DISSERTATION

DIETARY MODULATION OF CANINE METABOLISM FOR OBESITY MANAGEMENT AND CANCER RISK REDUCTION

Submitted by

Genevieve Michele Forster

Department of Clinical Sciences

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2015

Doctoral Committee:

Advisor: Elizabeth P. Ryan

Rodney L. Page
John E. Bauer
Anne C. Avery

ABSTRACT

DIETARY MODULATION OF CANINE METABOLISM FOR OBESITY MANAGEMENT AND CANCER RISK REDUCTION

Metabolic aberrancies associated with environmental exposures and excess adiposity can increase risk for multiple chronic diseases. Obesity is the primary nutritional disorder of companion dogs and incidence and prevalence rates continue to increase, yet little is known about underlying metabolic disruption in obese dogs or the role of environmental contaminants to which companion dogs are exposed. Cooked common beans (*Phaseolus vulgaris,* L.) are a rich source of macro-, micro-, and phytonutrients that have potential to support healthy weight management in dogs, modulate metabolism, and decrease risk for chronic disease, but have yet to be evaluated for safety and digestibility in dogs. Given the prevalence of obesity in companion dogs and the potential of bean consumption to improve health, the overarching hypothesis for this dissertation is that cooked beans are well tolerated and palatable in dog food and beneficially modulate underlying metabolic pathways associated with canine obesity and environmental exposures. The major objectives in this dissertation were to 1) determine environmental exposures and obesity associated metabolic aberrancies in companion dogs; 2) investigate the feasibility, safety and digestibility of incorporating cooked bean powders into nutritionally complete dog food formulations; and 3) determine the effects of weight loss and bean intake on metabolic parameters. To accomplish these objectives, 66 clinically healthy, adult companion dogs of various breeds and genders were recruited to participate in randomized, controlled bean-based dietary intervention

studies performed at Colorado State University Veterinary Teaching Hospital and Wellington Animal Hospital. The specific hypotheses tested were:

- Companion dogs are exposed to detectable levels of environmental pollutants and exhibit altered metabolomes associated with obesity.
- Dry dog foods formulated with 25 % weight/weight cooked bean powders are safe, digestible, and well tolerated compared to a nutrient matched control diet.
- Dogs consuming bean-based diets will have altered lipid and carbohydrate metabolism, reduced inflammation, increased expression of satiety gut hormones, and decreased insulin resistance.

We first determined the background exposures to pesticides as detected in urine from 21 normal weight dogs. Urine samples collected from the dogs were screened for a panel of 301 pesticides using an established ultra-performance liquid chromatography-mass spectrometry (UPLC-MS/MS) platform. Fifteen distinct pesticides were detected: the most frequently detected compounds in canine urine were atrazine, fuberidazole, imidacloprid, terbumeton, and clopyralid. We next evaluated 66 clinically healthy dogs that were normal weight, overweight, or obese for differences in serum biochemistry, microbiome, and metabolome. The proportion of overweight and obese dogs with hemolysis, creatinine kinase, and aspartate aminotransferase (AST) levels outside reference ranges was higher than normal weight dogs. Levels of AST, chloride, and gamma-glutamyl transpeptidase were lower in overweight or obese dogs. The fecal microbiomes were evaluated in a subset of 50 dogs using 16S Illumina based sequencing. The fecal microbiome comprised, in order of abundance, the phyla Firmicutes, Bateroidetes, Proteobacteria, and Actinobacteria. Significant variation existed between all dogs with no differences found at the level of phyla, class, order, family, or genus level. The fecal, plasma, and urine metabolome of 66 dogs were evaluated by liquid chromatography (LC) and gas chromatography coupled to mass spectrometry (MS), and 266 compounds were differentially expressed by weight phenotype.

Difference in plasma metabolites accounted for 44 % of the variation between normal weight, overweight, and obese dogs.

To determine the safety and digestibility of incorporating cooked bean powders into nutritionally complete dog foods, three clinical trials were carried out. Dogs consuming bean-based diets maintained indices of adequate nutritional intake as mandated by the Association of American Feed Control Officials (AAFCO) feeding trials. Bean-based diets were as digestible as the matched, standard ingredient, control (CON) dog foods. In overweight dogs undergoing weight loss, the black bean (BB) diet had higher total dry matter and crude protein digestibility and both the navy bean (NB) and black bean diets had higher carbohydrate digestibility than the CON diet. No increased flatulence or major change in fecal consistency was reported by any of the owners for any dogs. Normal weight dogs consuming beans had lower serum cholesterol levels than the CON dogs, and dogs undergoing weight loss on bean-based diets had decreases in serum HDL, LDL, and triglycerides. Metabolites associated with lipid, carbohydrate, and protein metabolism were altered in bean consuming dogs; however in dogs undergoing weight loss, the greatest shift in the metabolome was observed in response to weight loss, independent of diet.

This work highlights the utility of metabolomic platforms for evaluating the metabolism of dogs and determining intervention responsive metabolic pathways. These data provide a foundation for continued investigation into the role of beans for healthy weight management and obesity and cancer prevention in dogs.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Dr. Elizabeth Ryan for her guidance, support, and mentorship. Thank you for your compassion and patience while modeling the highest standards of scientific integrity and professionalism. I would like to thank my committee members, Drs. Rod Page, Anne Avery, and John Bauer for their guidance and encouragement. I have learned so much from each of you and have truly enjoyed your feedback and collaboration. To each of my lab mates, especially Erica Borresen, Dustin Brown, and Nora Jean Nealon, thank you for your time, assistance, and inspiring conversations! I have learned so much from each of you and know you will each do amazing things! To Dr. Adam Heuberger, thank you for the countless hours you spent teaching me how to generate and process data. To Dr. Ajay Kumar, thank you for challenging me to see the bigger picture, while I don't miss pouring plates at 2 am, I do miss our debates. To Dr. Andrew Goodyear, thank you for setting an example of excellence and professionalism. To Dr. Irfan Ghazi, thank you for allowing me to be a part of your research, your encouragement and friendship have meant so much to me. To Dr. Job Mapesa, thank you for reminding me of the responsibility scientists to have to the world. Your vision and passion are a continuing inspiration to make the world a better place.

I would like to acknowledge the mentorship and support I have received from countless individuals at Colorado State University. Dr. Steve Dow co-mentored me for my undergraduate research project and helped set the stage for this research. Members of the Dow lab answered my unending questions, helped me set up experiments, and taught me to love immunology: thank you, Drs. Amanda Guth, Scott Hafeman, Joe Sottnik and Leah Mitchell. I would also like to thank Dr. Mark Zable for giving me my first research lab experience and encouraging me to think outside the box. To Dr. Alan Schenkel and Pete Justice, thank you for always believing in me and being there

to answer my never-ending supply of questions. I would like to acknowledge the directors, administrators, students, and graduates of the combined DVM/PhD program at CSU for their support and inspiration. Drs. Terry Nett, Anne Avery, and Ed Hoover have worked tirelessly to set up and maintain excellent mentorship, support and encouragement through this program. Thank you. The journey down this path would not have started without the intervention of Dr. Steve Withrow, thank you for taking the time to see my potential and passion. And a special thank you to Lynda Reed for finding the best in everyone, and making Dr. Withrow come try the bean cake.

I would next like to thank my husband, Zach, for his unending support and patience. Thank you for always being there, believing in me, and making me laugh. Thank you for the sacrifices you make every day to let me follow my passion. To Manda Watkins, thank you for being a wonderful friend, always having my back, and living your truth. My sanity wouldn't be the same without you. To John and Lynda Leetham, thank you for teaching me the joy, wonder, and limitations of science, and the hugs and support, you have both been there for every milestone, and the in between times too. To Dr. Laurie Fonken, thank you for your compassion and creating space for me to explore, heal, and grow. To Noelle Noyes, thank you for being there, and reminding me of why we do this. To Brea Smith, thanks for sharing your passion for science and life. To Crystal Reid, thank you for teaching me to be a compassionate mentor and friend. To Jeff and Beth Erkhart, thank you for teaching me to question everything. To Lindsey Riggle, thank you for challenging me to try to new things. To Chris Taylor, thank you for inspiring me to understand and accept myself. To my family, thank you for your support, love, and laughter.

This research would not have been possible without the collaboration of researchers across disciplines, institutions, and professions. I would first like to thank Dr. Dale Hill, Gordon Gregory, and Jolene Hoke of ADM Alliance Nutrition and Edible Bean Specialties Inc. for providing the

diet resources and analyses necessary for completing this research. I would especially like to thank Drs. Sue Lana, Kristen Weishaar, and Jenna Burton along with the rest of the Flint Animal Cancer Center Clinical Trials Core team for their help with the clinical trials. I would also like to thank Drs. Tracey Jenson and Teva Stone for allowing us to conduct a clinical trial from the Wellington Veterinary Hospital and Cadie Tillotson for her technical assistance. I would like to thank Dr. Kelly Swanson and his graduate students, Drs. Katherine Kerr and Alison Beloshapka for their microbiome and fiber analyses at the University of Illinois at Urbana-Champaign and the Wallenstein Lab, especially Guy Beresford, for isolating and amplifying DNA at the CSU Natural Resource Ecology Laboratory. Thank you to Dr. John Bauer for lipid analyses at Texas A&M University. Thanks to Drs. Corey Broeckling and Adam Heuberger for their collaboration with the metabolomics analyses at the Proteomics and Metabolomics Facility at CSU, and Dr. Greg Dooley for the pesticide analysis at the CSU Center for Environmental Medicine. I would like to thank Dr. Kim Cox-York for her help with the multiplex assays food Science and Human Nutrition Laboratory at the Kendall Nutrition Center. Thank you to Drs. Ann Hess and Sangeeta Rao for their assistance with statistical analysis. I would like to express my gratitude to Morris Animal Foundation, the CSU College of Veterinary Medicine and Biomedical Sciences College Research Council, the Center for Companion Animal Studies, and the Veterinary Summer Scholars Research Program for funding. I would especially to like to thank Lindsey Riggle for her help editing this dissertation.

DEDICATION

Dedicated to the memory of Shannon and Deb,

Thank you for teaching to me to laugh, love, and believe in myself -

You are both forever in my heart.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

OBESITY PREVALENCE, HEALTH RISKS, AND MANGAEMENT IN COMPANION DOGS

The human obesity epidemic remains at an all-time high in the United States with recent estimates showing that 39 % of all adults and 17 % of children are obese.[1] While the prevalence of human obesity seems to have stabilized over the last 10 years[1], the incidence of companion dog obesity is still increasing[2]: up to 40 % of dogs are overweight or obese in the U.S.[3], and canine obesity prevalence is also high in the UK[4], Australia[5], and in China.[6]

Health Risks and Metabolic Dysfunction Associated with Obesity

Excess adipose tissue is associated with an increased incidence of disease in both humans and dogs. The economic impact of obesity related health problems underscores the significance of the increased risk. For example, on a per capita basis in the U.S., the average medical spending for obese humans is 42 % higher than for lean humans.[7] Similarly, overweight dogs tend to require medical intervention for chronic diseases 2-3 years earlier than lean dogs.[8] Obese humans and dogs are at higher risk for developing orthopedic disease, such as osteoarthritis, cardiorespiratory complications, diabetes mellitus, dyslipidemia, urinary tract disorders, hormonal and reproductive anomalies, and some types of cancer.[9; 10] Some of the most challenging consequences of obesity to address are the sub-clinical aberrancies that often precede the development of overt disease.[10] Figure 1 summarizes the relationship between metabolic dysfunction associated with obesity and the increased risk for the development of disease.

Obesity is associated with dysregulated metabolic pathways that are often summed together as "metabolic syndrome" to flag individuals at higher risk of developing type II diabetes

mellitus and cardiovascular disease. Metabolic syndrome is clinically diagnosed with a combination of abdominal adiposity, hyperlipidemia, inflammation, and insulin resistance and is associated with increased risk of heart disease and insulin independent diabetes.[11] In a study of 35 obese dogs, 20 % met the criteria for metabolic syndrome.[12] Some authors argue the merit of diagnosing metabolic syndrome in dogs on the basis that cardiovascular disease is less common in dogs than people and that the type of diabetes seen in dogs is closer to the human type I diabetes.[13] However, the characteristics of metabolic syndrome in dogs still demonstrate underlying adipose dysfunction that merit further investigation to determine the relationship between obesity, metabolic dysfunction, and the increased risk for chronic disease.

The Complexities of Weight Management

In its simplest form, the cause of obesity is an energy imbalance: more energy is consumed than is expended, leading to an expansion of adipose tissue.[10] However, the environment, feeding and begging behavior, and individual physiology may all play roles in determining energy requirements, expenditure, and consumption in dogs.[14; 15]

Emerging evidence supports the role of environmental exposures in obesity and increased risk for chronic disease (Figure 1). Organophosphorus pesticides can increase insulin resistance, alter glucose and lipid metabolism, and increase inflammation.[16] High exposures to some organic contaminants are associated with increased levels of circulating glucose, insulin, cholesterol, and triglycerides, as well as higher body mass index (BMI).[17] Adult mice orally exposed to an organophosphate experienced significant increases in body weight, *ad libitum* food consumption, serum cholesterol, and insulin resistance.[18] In obese rats exposed to an empirically derived, environmentally relevant mixture of contaminants, Mailloux et al. demonstrated alterations in hepatic lipid and energy metabolism that contribute to obesity associated co-morbidities.[19]

Further demonstrating the relationships between obesity, altered metabolism, and pesticide exposures, Arrebola et al. demonstrated that certain pesticide exposures increased risk for hypertension, but only in individuals with a BMI over 26.3 kg/m^2.[20] The role of environmental contaminate exposures in canine obesity merits investigation, however little is known about baseline exposures in dogs, much less the physiological effects.

Multiple strategies exist to address weight management, including pharmacological, behavioral, exercise, and diet interventions. The primary target for the majority of weight management strategies starts with a diet or nutritional intervention that helps improve satiety, maintain lean muscle mass, and a healthy nutritional status.[21-24] Given the continued rise in canine obesity, novel dietary strategies are warranted to prevent and manage obesity in companion dogs. Common beans merit investigation as an ingredient in dog food as they have the potential to improve weight management in companion dogs, as well as reduce the risk for multiple chronic diseases and obesity associated comorbidities. The following section will explore the nutritional components of beans and their potential used in dog diet formulations.

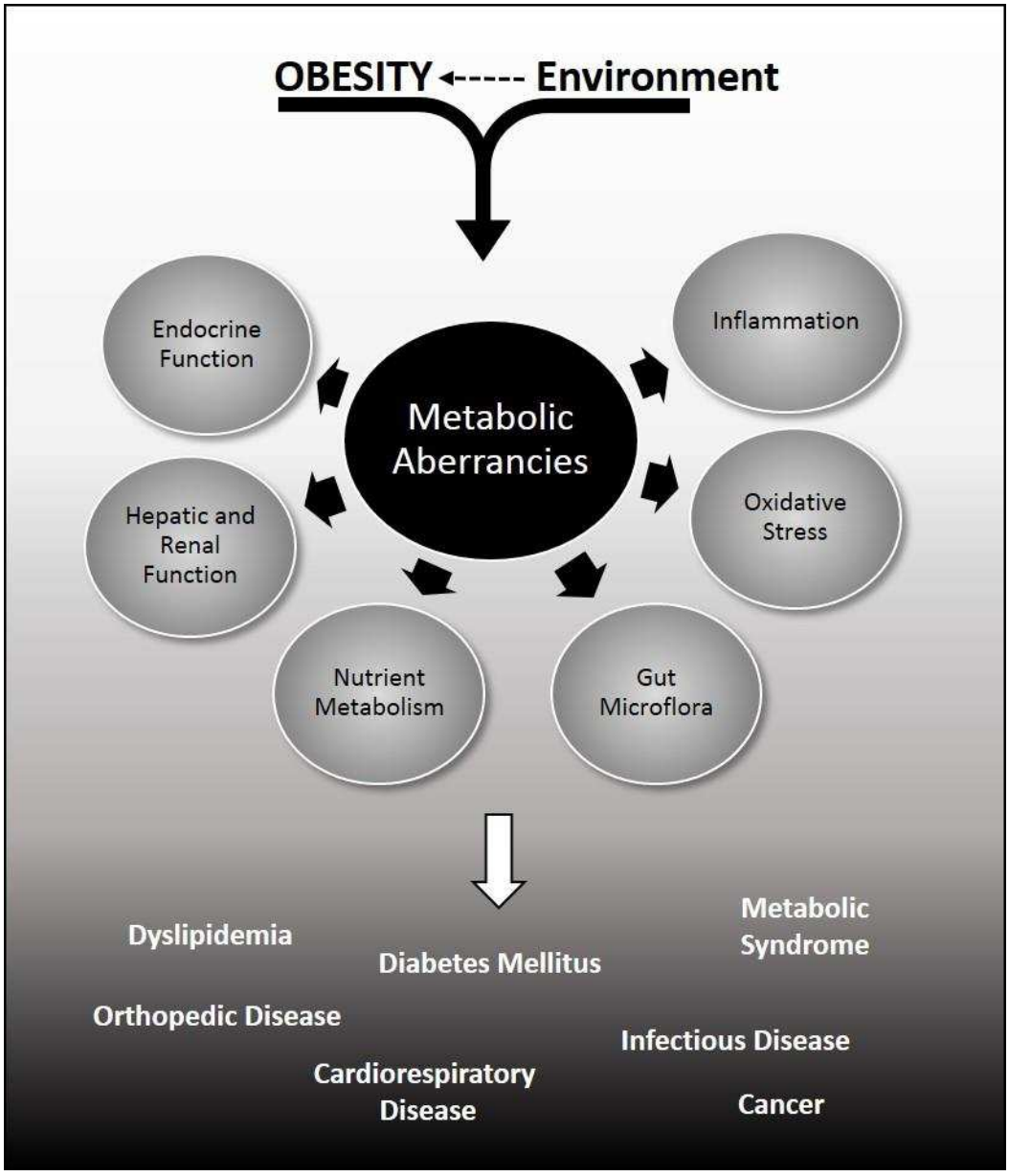

Figure 1: Metabolic dysfunction associated with obesity and environmental exposures. This figure summarizes data from human, dog, and murine studies demonstrating the effects of obesity and exposures to environmental pollutants that increase risk for developing disease.

BEANS ARE AN IMPORTANT FOOD FOR WEIGHT MANAGEMENT

Common beans (*Phaseolus vulgaris,* L.), such as black, pinto, and navy beans are edible, dry, non-oil seeds, or pulses, in the legume family.[25] They comprise over half of the consumed legumes worldwide[25], with over 18 million metric tons produced globally each year.[26] Of the bean classes grown in the U.S., the most common are pinto, followed by navy, and black.[27] Legumes represent an important crop for increasing environmentally sustainable agriculture practices because of their unique ability to fix nitrogen into soil[25] and potential to reduce reliance on resource intensive animal protein production[28], legumes can support the nutritional sustainability[29] of dog food formulations. In addition to providing potential environmental benefits, bean consumption is associated with improved health and weight management. For example, in epidemiological studies, bean intake is inversely associated with all-cause mortality[30] and strongly associated with longevity[31], higher intakes of fiber and minerals, better weight management, and lower blood pressure.[32] Beans contain unique macro and micronutrients when compared to other grains[33] and consumption has been shown to reverse dyslipidemia[34-36], reduce inflammation[37-39], improve gut hormone expression and insulin sensitivity[40-42], modulate apoptotic markers[43], and support a healthy microbial community.[44] Figure 2 summarizes the potential of common bean intake to correct metabolic aberrancies directly and through healthy weight management.

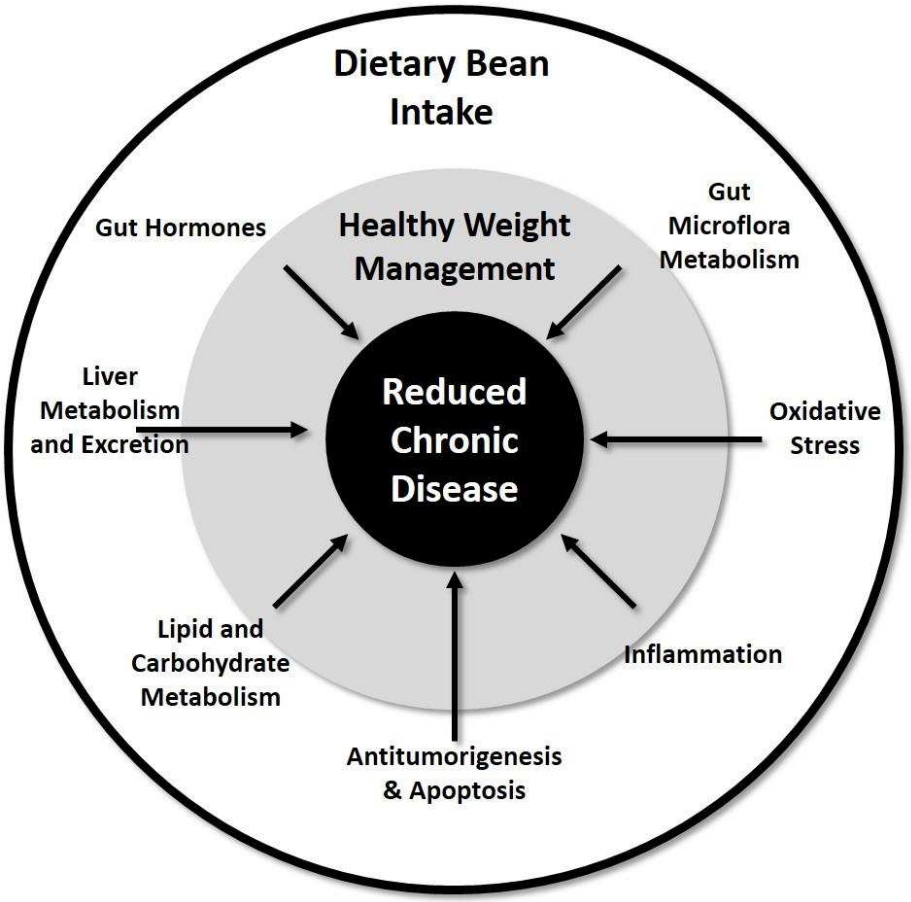

Figure 2: The role of bean intake to reduce risk for chronic disease directly and through healthy weight management. This figure represents the potential benefits of bean intake and weight management to ameliorate metabolic aberrancies associated with environmental exposures and obesity (Figure 1) to reduce the risk of chronic disease.

Cooked common beans are excellent sources of macro-, micro-, and phyto-nutrients that work together to support healthy weight management.[45] On average, cooked, dehydrated common beans contain about 70 % total carbohydrates, up to 30 % total dietary fiber, 15-30 % protein, less than 2 % crude fat, and supply about 386 Kcal/100 g dry matter.[46-48]

Table 1 shows the specific profiles of black and navy beans, the two cultivars evaluated in this dissertation for use in dog food formulations.

Table 1: Nutrient and energy composition of cooked black and navy beans by proximate analysis.

Per 100 g Dry Matter	Black Bean	Navy Bean
Carbohydrate, g	69.21	71.98
Total Dietary Fiber, g	25.39	29.01
Protein, g	25.86	22.74
Total lipid, g	1.58	1.71
Ash, g	3.36	3.59
Energy, kcal	385	387

Data from the USDA National Nutrient Database[48]

Carbohydrates

Bean carbohydrates can be categorized by the methods used to detect them and by their dietary interactions with a mammalian host. Cooked common beans contain 50 %- 70 % total carbohydrate, the majority of which is starch (up to 30 % of dry matter) and fiber (up to 30 % of dry matter).[49] The remaining carbohydrates are primarily made up of polysaccharides and oligosaccharides, but also contain some disaccharides and monosaccharides.[49; 50] Sucrose and fructose make up the majority of the monosaccharides, while stachyose, raffinose and verbascose make up the oligosaccharides.[51] About 85 % of the starch fraction can be digested by mammalian enzymes, the remaining 15 % is classified as resistant starch.[52] The digestible starch of cooked whole beans provides approximately 26 % rapidly digestible starch, 17 % slowly digestible starch, and 12 % very slowly digestible starch. Cooked whole beans have a starch digestibility index of 48[53], compared to wheat bread with an index of 100[54], resulting in a low glycemic response.[55]

Fiber

Fiber is defined as the non-digestible carbohydrates and lignin content.[56] A preponderance of evidence in human studies support that fiber intake is inversely associated with excess body weight and may improve weight loss by altering gut hormone expression, decreasing energy density of food, improving satiety, and altering nutrient metabolism and

absorption in the intestine.[57] In normal weight dogs, high fiber diets, especially those with fermentable fiber[58], have been shown to reduce caloric intake. When high fiber diets are combined with 30 % protein, satiety also improved.[59] In overweight colony beagles fed a weight management diet, a fiber supplement lowered caloric intake and increased weight loss.[60; 61] Companion dogs that consumed a high fiber and high protein diet lost, on average, 12 % more body weight with a 20 % greater loss of body fat mass than dogs consuming a high protein, moderate fiber diet.[62]

Fiber composes about half of the total carbohydrate fraction of cooked common beans and total dietary fiber ranges from 14 -30 % of the total dry matter.[49; 50] The fiber content of cooked beans is attributed with decreasing serum cholesterol levels, increasing satiety, improving weight management, and inhibiting the growth of cancer cells.[45; 50; 63-67]

Increasing bean intake in dogs, humans, and lab animals has had a minimal impact on the composition of a healthy, stable gut microbiota[44; 68; 69], but instead seems to alter the metabolic function of the bacteria in the gut by increasing short chain fatty acid (SCFA) production.[70] Propionate production increases both in *ex vivo* colonic fermentations[44] and in plasma samples of individuals consuming beans.[70] Butyric acid has been shown to increase with *in vitro* bean fermentations, and in rats fed a bean-based diet, is also associated with increasing lipid oxidation by altering carbohydrate metabolism in the liver.[71] Increased SCFA production may represent an important mechanism for the observed decreased lipemia, increased satiety, and improved weight loss associated with bean intake.[72; 73]

Protein and Amino Acids

Total dry matter of cooked, common beans comprises up to 30 % protein, about twice that of cereal grains.[49] The primary nutritional proteins in cooked beans are albumins and

globulins[74], the primary globulin is phaseolin, a storage protein with low levels of sulfur-containing amino acids. Thus the amino acid profiles of beans tend to be low in methionine, but high in lysine.[25] The albumin fraction contains lectins and enzyme inhibitors which are present in high amounts of raw beans and include alpha-amylase inhibitors and Bowman-Birk trypsin and chymotrypsin inhibitors – the presence of which are almost negligible after cooking.[75] Independently, lectins, alpha-amylase inhibitors and Bowman-Birk inhibitors demonstrate modulation of gut hormone secretion, interference with tumor cell growth and metabolism, influence on the gut microbiome, decreases in inflammation, and modulation of lipid metabolism.[75] Given the observation of these effects in clinical trials and epidemiological studies, cooked bean proteins may retain enough inhibitory activity to confer a health benefit, while minimizing any potential nutritional deficit.[75] Beans are also relatively high in arginine and glutamine, thermogenic amino acids that further modulate both carbohydrate and fat metabolism for improved glycemic control and weight loss.[71]

Micronutrients

Beans are exceptionally high in B vitamins, magnesium, and potassium. Beans are also rich in phosphorus, calcium, iron, and zinc, but low in sodium.[25] Linder, *et al*. evaluated the micronutrient concentration in a range of dog food diets when fed at caloric restriction levels suitable for weight loss. Magnesium, riboflavin, and niacin were among the micronutrients found to be deficient in commercial diets when at restricted levels.[24] Thus, beans have the potential to support micronutrient sufficiency during weight loss in dogs. Furthermore, the high potassium and low sodium content in beans have the potential to beneficially modulate blood pressure, further reducing the potential for obesity associated co-morbidities.[76]

Phytonutrients

A wide range of small, bioactive molecules in beans provide additional health and weight management benefits. Cooked beans have alpha-amylase inhibition activity that may be associated with slowing carbohydrate digestibility in the small intestine and reducing dietary energy extraction.[75] While bean derived alpha-amylase inhibitors have been used to improve human weight loss, the compounds isolated from raw bean extracts have had variable results in clinical trials.[77] Phytates also lower carbohydrate digestibility, and in high concentrations, can chelate calcium, iron, zinc, and magnesium.[25] Phytate is the primary storage molecule of phosphorus in plants[78] and while it is reduced with both soaking and cooking[79], bean seeds selected for low phytate concentration have been shown to improve iron absorption in women.[78] While this study did not report the protein content of the low phytate bean cultivars, other plants have shown a direct correlation between phytate content and protein content.[25] Furthermore, phytates demonstrate strong chemopreventive properties[80] and serum lipid lowering activity[45] indicating that in low concentrations, they may confer more of a health benefit than a nutritional liability in the context of a balanced diet formulation. Cooked common beans contain a wide range of polyphenols that include phenolic acids, flavonoids, and triterpenic acids. Total phenolics acids reported in cooked bean cultivars average around 1,600 ug/100 g dry matter gallic acid equivilents and some abundant acids include ferulic acid, o-coumaric and p coumaric acid, sinapic acid, and caffeic acid.[81] Beans with colored seed coats tend to have the highest concentration of flavonoids including catechin, genistein, epicatechin, kaempferol, and some of the polyphenol resveratrol.[81] In general, polyphenols have been shown to reduce inflammation, cardiometabolic disease, oxidative stress, and inhibit the cell cycle of neoplastic cells.[64; 65; 81-85] However, emerging evidence supports that these phenolic compounds are associated with

improved weight loss and dyslipidemia by altering fat metabolism and improving glucose homeostatsis.[86-90] Cooked beans also contain the phytosterols β-sitosterol, stigmasterol, and camesterol - plant-derived lipids with structural similarity to cholesterol[63]. Phytosterols have been shown to reduce hyperlipidemia by competitively inhibiting cholesterol incorporation into micelles at the intestinal lumen, upregulating reverse transporters in enterocytes that increase the rate at which lipids are excreted back into the lumen after initial absorption, and finally by again competitively inhibiting the incorporation of cholesterol into chylomicrons.[91]

DISSERTATION AIMS AND HYPOTHESES

Given the prevalence of obesity in companion dogs and the potential of bean consumption to improve health, our goal was to investigate the feasibility, safety, and digestibility of incorporating cooked bean powders into nutritionally complete dog food formulations. The overarching hypothesis for this dissertation research project is that cooked beans are well tolerated and palatable in dog food and beneficially modulate underlying metabolic pathways associated with canine obesity and environmental exposures. Our overall objectives were to 1) determine environmental exposures and obesity associated metabolic aberrancies in companion dogs; 2) investigate the feasibility, safety, and digestibility of incorporating cooked bean powders into nutritionally complete dog food formulations; and 3) determine the effects of weight loss and bean intake on metabolic parameters. Our goal was to formulate bean-based nutritionally complete dog diets with isocaloric and nutrient matched bean-free control diets and to investigate the effects of these diets on nutritional, clinical, metabolic, inflammatory, and endocrine function in normal weight and overweight companion dogs. Navy and black beans were chosen based on their availability in the U.S. and their differences in seed coat color from different phytochemical profiles. The nutritional composition of these two bean

cultivars are presented in Table 1. Sixty-six clinically healthy, adult, male and female dogs of diverse breeds participated in one of three clinical trials. The characteristics of individual dogs are presented in Appendix 1. The specific aims, experiments, and hypotheses are detailed below and summarized in Figure 3.

Specific Aim 1: Determine canine environmental and obesity related exposures.

Experiment 1A*:* Measure pesticide exposures in normal weight dogs. Exposure to environmental contaminants has been associated with increased risk for obesity, metabolic dysfunction, and chronic disease in humans and lab animals, and has been minimally investigated in companion dogs. Because natural exposures have not previously been reported in companion dogs, our objective was to determine baseline exposures to agrochemicals, believed to be important sources of potential metabolic and endocrine disruptors, which may increase risk for obesity and chronic disease. This experiment is presented in Chapter 2: *Multiresidue analysis of pesticides in urine of healthy adult companion dogs*.[92]

- Hypothesis: Companion dogs are exposed to low dose mixtures of pesticides that can be detected in urine using an agrochemical mass spectrometry based screening panel.

Experiment 1B: Determine differences in serum biochemistry, metabolome, and microbiome between normal weight, overweight, and obese dogs. Multiple studies have reported minor differences in clinical blood analytes and the microbiome of normal and overweight dogs; however differences in the metabolome have not yet been investigated. The objective of this experiment was to determine potential differences in this cohort of Northern Colorado dogs by comparing clinical blood analytes and metrics, the microbiome, and the metabolome that reflect obesity related exposures, and to correlate these differences with underlying subclinical disease.

This experiment is presented in Chapter 3: *Serum biochemistry microbiomes, and metabolomes of clinically healthy normal weight, overweight, and obese dogs.*

- Hypothesis: Overweight and obese dogs have higher levels of organ stress, altered gut microflora, and metabolic aberrancies than normal weight dogs.

Specific Aim 2: Determine safety and digestibility of bean-based dog foods

Experiment 2A*:* Evaluate the safety and digestibility of a navy bean-based diet in dogs for weight maintenance. Given the potential health benefits of bean consumption in companion dogs, the objective of this experiment was to determine the safety and digestibility of a 25 % weight/weight navy bean diet compared to a matched, placebo control diet for adult dog weight maintenance. This experiment is presented in Chapter 4: *Effects of cooked navy bean powder on apparent total tract digestibility and safety in healthy adult dogs.*[93]

- Hypothesis: A nutritionally complete, navy bean-based diet is equally digestible, palatable, tolerable, and supports weight maintenance and nutritional requirements of adult companion dogs, compared to a matched control diet.

Experiment 2B: Determine the safety and digestibility of navy and black bean-based diets in overweight dogs undergoing weight loss. Our objective was to determine if a bean-based diet is digestible and meets the nutritional requirements of adult dogs undergoing short- and long-term calorically restricted weight loss compared to an isocaloric, nutrient matched control diet. This experiment is presented in Chapter 5: *Navy and black bean-based dog foods are digestible during weight loss in overweight and obese adult companion dogs.*

- Hypothesis: Nutritionally complete black and navy bean-based diets support healthy weight loss, are digestible, and nutritionally adequate for short- and long-term calorically restricted companion dog weight loss compared to a control diet.

Specific Aim 3: Determine the effects of consuming a bean-based diet on the canine metabolome, serum biochemistry, gut hormones, and inflammation.

Experiment 3A: Determine the effects of consuming beans on the normal weight canine metabolome. The objective of this study was to evaluate the effects of beans on the small

molecule profiles of a nutritionally complete dog diet and the effects of consumption on serum lipids and metabolism in healthy, normal weight adult dogs. This experiment is presented in Chapter 6: *Consumption of cooked navy bean powders modulates the canine fecal and urine metabolome.*[94]

- Hypothesis: We hypothesized that the addition of navy bean powder results in distinct phytochemicals compared to the control diet and that consumption of the navy bean diet modulates the chemical composition of the canine fecal and urine metabolome.

Experiment 3B*:* Determine the effects of consuming beans during weight loss on canine biochemistry. The objective of this study was to measure changes in clinical, serum biochemistry of adult dogs undergoing weight loss on a bean-based or control diet. This experiment is presented in Chapter 7: *Nutritional weight loss therapy with cooked bean powders regulates serum lipids and biochemical analytes in overweight and obese dogs.*[95]

- Hypothesis: Overweight and obese dogs undergoing calorically restricted weight loss will have improved serum biochemical profiles and dogs consuming a bean-based diet will have improved serum lipid profiles compared to dogs consuming a matched, no-bean diet.

Experiment 3C*:* Determine the effects of consuming beans during weight loss on the canine metabolome, inflammasome, and gut hormones. The objective of this study was to evaluate the metabolic changes that occur in dogs during short term weight loss and the influence of bean consumption on metabolism. This experiment is presented in Chapter 8: *Effects of bean consumption on canine metabolism and inflammation during calorically restricted weight loss.*

- *Hypothesis:* Dogs consuming a bean-based diet during weight loss will have alterations in lipid and carbohydrate metabolism, reductions in inflammatory cytokines, less reduction in satiety hormones and increases in insulin sensitizing hormones.

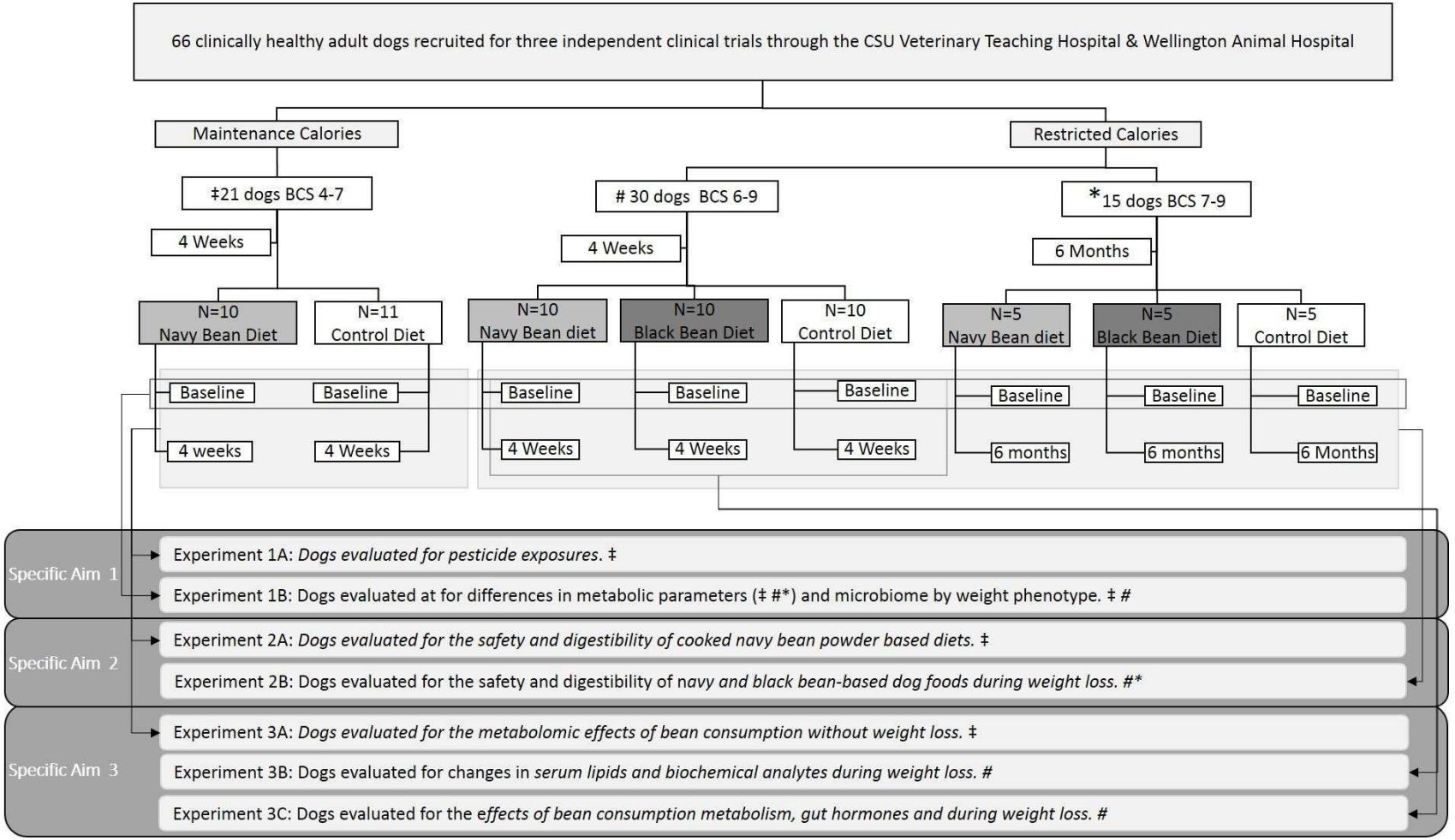

Figure 3: Overview of study design for investigating the role of bean-based diets for improving canine health. Dogs completed a double-blinded, randomized, controlled dietary intervention clinical trial to evaluate the safety, digestibility, and feasibility of incorporating 25 % weight/weight cooked bean powder into extruded dog foods.

CHAPTER 2: MULTIRESIDUE ANALYSIS OF PESTICIDES IN URINE OF HEALTHY ADULT COMPANION DOGS[1]

INTRODUCTION

Chronic diseases are the leading cause of death in both dogs and humans and prevalence is expected to increase.[96; 97] While there are multiple risk factors, the association between pesticide exposure and chronic disease risk has been of increasing public health concern. Environmental pesticide exposures in humans have been associated with heart disease, obesity, arthritis, cancer, respiratory disease, diabetes, and neurodegenerative disorders.[98] Emerging evidence supports that when exposures occur in combination, the aggregate, unintended consequences of pesticide toxicity increase[99-101], the effects cannot be simply accounted for by summing the effects from compounds in isolation[102], and therefore highlights the need to study relevant chemical mixtures. Studies investigating the effects of complex toxicant mixtures are difficult in that the exposure doses of the known pesticides can vary substantially[103], and the cumulative exposures during critical phases of growth and development are largely unknown. Thus it has been challenging to interpret results from studies that demonstrate a single target mechanism of action for a specific pesticide exposure and for determining a relationship to disease pathogenesis. Advancements are being made in overcoming the challenges associated with the study of complex mixtures.[104] For example, improved instrumentation methods have helped to establish lower limits of detection for many chemicals, permitting more accurate body-burden assessments with health and/or disease outcomes.

[1] This chapter was published in *Environmental Science and Technology* December 2014:48(24):14677-85. Doi: 10.1021/es503764s. Authors are Genevieve M. Forster, Dustin G. Brown, Greg P. Dooley, Rod L. Page, and Elizabeth P. Ryan

Companion dogs are relevant to study because they develop similar complex human diseases such as cancer.[105; 106] Shared lifestyles of dogs and humans support their use as biosentinels for monitoring environmental exposure[107], and was demonstrated with development of mesothelioma in dogs whose owners were regularly exposed to asbestos.[108] Elevated levels of flame-retardants and associated toxins found in dog serum[109], pesticides in urine after exposure to treated lawns[110; 111], and concentrations of organohalogenated compounds in dog serum were reported in 2011 to be consistent with reported levels in humans. [112] Studies that have demonstrated associations between exposure to environmental contaminants and canine disease risk in dogs include: 1) exposure to 2,4-dichlorophenoxyacetic acid and increased risk of transitional cell carcinoma[113]; 2) a diagnosis of lymphoma and herbicide exposure[114]; 3) topical insecticide use and increased risk for bladder cancer[115]; and 4) the presence of pyrethroids in adipose tissue adjacent to canine mammary tumor tissues and the aggressiveness of the tumor phenotype.[116] Monitoring environmental exposures in dogs has the potential to increase our knowledge of human exposures, alongside our understanding of mechanisms for disease progression.

Until recently, a background exposure profile in healthy individuals has been difficult to establish for a defined population given lifestyle differences, namely variation in diet, living environment, travel, illness, and geographical location.[103; 117; 118] Advances in mass spectrometry applications, including metabolomics, provides a unique opportunity for toxicologists to advance our knowledge of low dose chemical mixtures in humans and animals.[119] This study was designed to determine a chemical exposure mixture relevant to healthy companion dogs on controlled ingredient diets during a one-month period.[120] A panel that was validated for detecting fungicides, herbicides, and insecticides on food products was applied to canine diets and urine

samples from a cohort of healthy dogs to assess total exposure. The findings presented demonstrate the complexity of chemical mixtures of environmental organic compounds across diverse, companion dog breeds.

MATERIAL AND METHODS

Diets

Two diets were formulated and fed to pet dogs as previously described: "Control" and "Navy Bean".[120] Both diets were matched in nutrient and energy content and contained rice, corn, wheat, beet pulp, flaxseed, meat and bone meal, and poultry fat. Corn and wheat ingredients were adjusted in the navy bean diet to account for the inclusion of 25% weight/weight cooked navy bean powder.[120] Diets were formulated to meet the Association of American Feed Control Officials and Nutritional Research Council guidelines for adult dog maintenance.[121; 122] Both kibble diets were extracted and analyzed for presence of pesticides.

Canine Participants and Urine Collection

Twenty-one clinically healthy, adult, male and female companion dogs, of various breeds and normal weights were enrolled at the Flint Animal Cancer Center and Veterinary Teaching Hospital at Colorado State University as part of a dietary intervention trial to investigate the digestibility of dry beans as a major ingredient in dog food.[120] The study veterinarian determined the enrolled dogs were clinically healthy upon determination of a normal physical exam and blood chemistry results. Owners completed a health history questionnaire for each dog and were asked to provide information on any medication use including anthelmintics or supplements. Two dogs received heartworm prevention, C10 and C11. No owners reported the use of flea or

tick prevention. Dogs were recruited from the Fort Collins, CO area, which consisted of both rural and urban environments; water sources were primarily municipal, and specific data on drinking water sources was not collected. As previously described, dogs were randomized to one of two dietary groups: "Control" or "Navy Bean", n=11 and 10, respectively.[120] Owners were instructed to exclusively feed the study diets, and were provided with diet logs to record any additional food consumed by the dogs. Table 2 shows the month of each urine sample collection, the major animal and plant ingredient of each diet consumed before starting the study, and any common treats or table scraps fed as reported by owners.

Table 2: Urine collection dates and baseline dietary intake for 21 clinically healthy dogs evaluated for pesticide exposure.

	Collection Date (2010)		Baseline Diet Major	
Dog ID	**Baseline**	**4 Week**	**Ingredients**	**Treats and Snacks[1]**
NB1	August	August	Chicken and Wheat	Biscuits, CET chews, human food
NB2	August	August	Lamb and Rice	Milk bones
NB3	August	September	Chicken and Corn	Chewie's and Purina Dog Biscuits
NB4	August	September	Meat/Bone Meal and Corn	Biscuits and table scraps
NB5	August	September	Meat/Bone Meal and Corn	Human food, bones, and rawhides
NB6	August	September	Meat/Bone Meal and Corn	Human food, bones, and rawhides
NB7	September	October	Chicken and Corn	Dog biscuits
NB8	September	October	Meat/Bone Meal and Corn	Dog biscuits
NB9	September	October	Chicken and Rice	Peanut butter
NB10	September	October	Chicken and Corn	
C1	August	August	Chicken and Corn	
C2	August	August	Chicken and Pea	Hills dental and chicken jerky treats
C3	August	September	Chicken and Rice	Rawhide chews
C4	No Sample	September	Chicken and Rice	Rawhide chews
C5	August	September	Chicken and Rice	
C6	September	October	Chicken and Corn	Milk Bones
C7	September	October	Chicken and Corn	Bread
C8	September	October	Chicken and Corn	Science diet biscuits
C9	No Sample	October	Chicken and Corn	Science diet biscuits
C10	October	November	Chicken and Rice	
C11	October	November	Chicken and Rice	

[1]As reported by owners

Two urine samples were collected either by "free-catch" as urine was voided or by ultrasound-guided cystocentesis, before ("baseline") and after consuming a study diet for 4 weeks. Urine samples were stored at -80°C until the time of analysis. Two of the "Control" dogs had insufficient amounts of urine for pesticide detection at baseline and were excluded from statistical analysis, however all 4 week urine samples were screened and presented. Urinary pesticide detection data from both baseline and 4 week timepoints were obtained for a total of 19 dogs. All study protocols were approved by the Colorado State University Institutional Animal Care and Use Committee and dog owners provided informed consent for study participation.

Pesticide Extraction

Approximately 1 mL of each urine sample was adjusted to pH 5.2 with a 2M sodium acetate buffer and incubated with 10 µL of β-glucuronidase (Sigma, St Louis, MO) for 2 hr at 55˚C. Urine samples were diluted 1:10 into 9 mL of Milli-Q water and spiked with 20 µL of 10 µg/mL D5-atrazine (Sigma Aldrich) as an internal standard. A total of 5 g of each dog food diet was manually homogenized in 5 mL of Milli-Q water and also spiked with 20 µL of 10 µg/mL D5-atrazine. Diet and urine samples were extracted using Agilent SampliQ QuEChERS kits designed to follow AOAC Method 2007.01. Briefly, 15 mL of 1 % acetic acid in acetonitrile was added to each sample in a pre-cleaned 50 mL conical falcon tube. One pre-weighed packet containing 6 g of magnesium sulfate ($MgSO_4$) and 1.5 g sodium acetate (NaOAc) was then added and the sample shaken vigorously for 1 min. Solid material was separated by centrifugation at 2000 RCF. An 8 mL aliquot of supernatant was taken and added to a 15 mL vial containing 400 mg of primary and secondary amino sorbent and 1200 mg $MgSO_4$. Dispersive solid phase extraction was achieved by vortexing samples for 2 min and then centrifugation at 2000 RCF for 5 min. Six mL of supernatant were then removed, dried under

nitrogen gas to 500 µL, and diluted to 1 mL with 5mM ammonium formate/0.01 % formic acid for Ultra Performance Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (UPLC-MS/MS) analysis.

UPLC-MS/MS Qualitative Pesticide Analysis

This 301 pesticides screening method utilized UPLC-MS/MS with electrospray ionization in the positive mode. The instrument used in the analysis was an Agilent 1290 UPLC coupled to an Agilent 6460 triple quadrupole mass spectrometry, which was equipped with an ESI source using Agilent Jet Stream Technology (Agilent, Santa Clara, CA). Pesticides were separated on a Zorbax Eclipse Plus C18 column (2.1X 150mm, 1.8 µm particle size) (Agilent) at 60 °C. A volume of 15 µL from each diet and urine sample was injected and a binary mixture of 5mM ammonium formate/0.01 % formic acid (A) and methanol with 5mM ammonium formate/0.01 % formic acid (B) at a flow rate of 0.5 mL/min. The gradient used was 4 % B increasing to 98 % B at 15 min, and held for 5 min. The ionization source conditions used were as follows: nebulizer gas flow of 5 L/min at 325 °C and 30 psi; sheath gas flow of 11 L/min at 375 °C; and the capillary voltage 3500 V. The optimized fragmentor, collision energy, and two MS-MS transitions for each analyte were imported to the method from the Agilent Pesticide Screening Dynamic Multiple Reaction Monitoring (MRM) Database (Agilent # G1733AA). The dwell times for the transitions were maximized based on the number of concurrent MRMs. A test mix containing 136 pesticides was run prior to each sample set to calibrate retention time windows at 100 ng/mL for each analyte. The data collection and processing were performed by using Agilent MassHunter software (v. B.04.01). Samples were analyzed using Agilent Mass Hunter Quantitation software (v. B.04.01). For each sample, individual pesticides were inspected for the presence of two characteristic product ions at the correct retention time. Positive hits for

the pesticides were confirmed by retention time and the product ion ratio correlation between the sample peaks and corresponding standards (+/- 20 %). Table **3** lists the detected compounds, retention times, and precursor and product ions detected.

Table 3: Identification parameters for pesticides detected in canine urine and diet samples.

Compound	Retention Time (Min)	Precursor ion (m/z)	Product ions (m/z)	Classification	EPA Registered Use
Atrazine	9.46	216.1	174.1 132.0	Herbicide	corn, sorghum, sugarcane, and residential lawns [123]
Azinphos-ethyl	11.31	346.0	137.1 97.0	Insecticide	N/A
Bromacil	8.06	261.0	205.0 187.9	Herbicide	Citrus, pineapple, and non-agricultural weed control [124]
Bupirimate	11.90	317.2	272.0 166.1	Fungicide	N/A
Clopyralid	2.00	192.0	174.0 110.0	Herbicide	Sugarbeets, mint, and wheat [125]
Ethoxyquin	11.38	218.2	174.1 148.0	Insecticide	Pears [126]
Fuberidazole	6.91	185.1	157.1 156.0	Fungicide	N/A
Hydroxyatrazine	5.61	198.0	156.1 86.0	Herbicide	N/A
Imidacloprid	4.83	256.1	209.1 175.0	Insecticide	Corn, lettuce, broccoli, apples, potatoes [127]
Metobromuron	9.15	259.0	170.0 148.1	Herbicide	N/A
Nicotine	1.13	163.1	132.1 130.0	Insecticide	Ornamental plants (poinsettias)[128]
Propamocarb	2.95	189.2	102.1 144.1	Fungicide	Ornamental, non-crop grasses, shrubs, and vines [129]
Propham	9.26	180.1	138.1 120.0	Herbicide	Legumes, flax, lettuce, safflower, lettuce, and fallow land
Propoxur	8.22	210.1	168.1 111.0	Insecticide	Indoor applications [130]
Pymetrozine	3.57	218.1	105.0 79.0	Insecticide	Fruit and vegetables [131]
Spinosad A	13.20	732.5	142.1 98.0	Insecticide	Fruit, vegetables and grains [132]
Terbumeton	10.15	226.2	170.1 114.1	Herbicide	N/A
Thiabendazole	6.57	202.0	175.0 131.1	Fungicide	Fruit, vegetables and grains [133]
Triasulfuron	8.02	402.1	167.1 141.1	Herbicide	Wheat, barley, pastures, and rangeland [134]

N/A = not currently registered for use in the U.S.

LC-MS/MS Quantitative Atrazine Analysis

Urine samples were prepared for analysis by spiking 200 µL of urine with 25 µL of 1µg/ D5 atrazine, as an internal standard, followed by briefly vortexing. Two hundred µL of ice cold acetonitrile was added, samples vortexed again, and then centrifuged at 10,000 RCF for 5 minutes. Supernatants were transferred to autosampler vials for LC-MS/MS analysis. The instrument used in the analysis was an Agilent 1290 UPLC coupled to an Agilent 6460 triple quadrupole mass spectrometry, which was equipped with an ESI source using Agilent Jet Stream Technology (Agilent, Santa Clara, CA).

Atrazine was separated on a Zorbax Eclipse Plus C18 column (2.1X 100mm, 3.5µm particle size) (Agilent) at 40 °C. A sample volume of 10µL was injected and a binary mixture of 0.1 % formic acid (A) and acetonitrile/0.01 % formic acid (B) at a flow rate of 0.5 mL/min. The gradient used was 40 % B increasing to 100 % B at 5 min. The positive ionization source conditions used were as follows: nebulizer gas flow of 8 L/min at 325 °C and 45 psi; sheath gas flow of 11 L/min at 390 °C; and the capillary voltage 3500 V. The ion transitions monitored for atrazine were 216.1->132 /174.1 m/z and 221.1 ->179.1 for D5-atrazine. Atrazine identifications were confirmed by retention time (1.47 mins) and the product ion ratio (13.2) correlation between the sample peaks and corresponding standards (+/- 20 %). Samples were quantitated with linear regression using Agilent Mass Hunter Quantitation software (v. B.04.01). The lower limit of quantification for atrazine in dog urine for this study was 20 pg/mL or 20 parts per trillion.

Determination of Pesticide Bioactivity Potential

Pesticides with positive detections in canine urine were screened in the Pubchem BioAssay database for bioactivity. For each pesticide, all active bioactivity outcomes were

selected, confirmed, and recorded with the BioAssay Identification (AID) number. All results are reported in Table 4.

Table 4: Reported bioactivities of detected pesticides from PubChem BioAssay database.

Pesticide[1]	Bioactivity
Atrazine	Activates cAMP Responsive Element signaling (AID: 662) Activates insulin secretion (AID: 743287) Carcinogenic (AID: 1189, 1208, 1205) Inhibits the activation of platelets (AID: 1663)
Fuberidazole	Activates the AhR signaling pathway (AID:743085, 743122) Agonist of the ER-α signaling pathway (AID: 743079) Genotoxic (AID: 720516)
Imidacloprid	Inhibits cell surface uPA activity preventing formation of plasmin (AID: 540303) Inhibits tyrosyl-DNA phosphodiesterase I (AID: 686978)
Thiabendazole	Activates the AhR signaling pathway (AID: 743085) Increases SMN2 gene splice variant expression (AID: 1458) Activates 5'UTR Stem-Loop Driven Prion Protein mRNA translation (AID: 1999) Activates BRCA1 Expression (AID: 624202) Agonist of the ER-α signaling pathway (AID: 743079) Inhibits insulin promoter activity (AID: 1273) Inhibits tubulin formation (AID: 742594) Genotoxic (AID: 720516)
Clopyralid	Activates the AhR signaling pathway (AID: 743085)
Nicotine	Agonist of the nAchR (AID: 742462) Agonist of the AR signaling pathway (AID: 743036)
Bupirimate	Disruptor of mitochondrial membrane potential (AID: 720635) Antagonist of the GR signaling pathway (AID: 720725) Antagonist of the AR signaling pathway (AID: 743122, 743063, 743054) Antagonist of the PPAR-γ signaling pathway (AID: 743199) Agonist of the antioxidant response element signaling pathway (AID: 743219)
Propham	Agonist of the ER-α signaling pathway (AID: 743079)
Metobromuron	Activates the AhR signaling pathway (AID: 743122, 743085)
Ethoxyquin	Antagonist for Hypoxia Response Element Signaling Pathway (AID: 915) Inhibits Cytochrome p450 isoforms (AID: 894, 899, 883, 884, 1851) Activates Caspase-3/7 (AID: 588813) Inhibits hydroxyacyl-coenzyme-A dehydrogenase, Type II (AID: 886) Inhibits 15-human lipoxygenase 1 and 2 (AID: 881, 887) Inhibits hydroxysteroid (17-beta) dehydrogenase 4 (AID: 893) Inhibits Histone Lysine Methyltransferase G9a (AID: 504332) Inhibits vitamin D receptor (AID: 504847) Inhibits bromodomain adjacent to zinc finger domain, 2B (AID: 504333) Inhibits human tyrosyl-DNA phosphodiesterase 1 & 2 (AID: 686979, 720702) Activates heat shock response signaling pathway (AID: 743228) Agonist of the PPAR-γ signaling pathway (AID: 743094, 743140) Activates the AhR signaling pathway (AID: 743122) Antagonist of the thyroid receptor signaling pathway (AID: 743065) Agonist of the antioxidant response element signaling pathway (AID: 743202)

[1]https://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi. AhR: aryl hydrocarbon receptor; AR: androgen receptor ER-α: estrogen receptor-alpha; GR: glucocorticoid receptor, nAchR: neuronal acetylcholine receptor; PPAR-δ/γ: peroxisome proliferator-activated receptor delta/gamma; uPA: urokinase plasminogen activator

RESULTS AND DISCUSSION

Pesticide screening reveals mixed chemical exposures in dogs.

The purpose of this study was to assess pesticide exposure in a cohort of twenty-one, clinically healthy adult dogs by analyzing diets and urine using a targeted UPLC-MS/MS pesticide screening panel. Fifteen pesticides that have not been previously reported in dog urine were detected at either baseline or 4 week time points (Figure **4**, A and B) in 19 of the 21 dogs with both baseline and 4 week urine samples. Eight pesticides were detected at both time points. In order of detection frequency, indicated by the percent of positive dogs at baseline and 4 weeks, respectively, were atrazine (100 % and 100 %), fuberidazole (74 % and 53 %), imidacloprid (42 % and 26 %), terbumeton (21 % and 11 %), hydroxyatrazine (11 % and 5 %), triasulfuron (5 % and 21 %), clopyralid (5 % and 5 %), and nicotine (5 % and 16 %). At baseline thiabendazole (11 %), propham (5 %), and metobromuron (5 %), were detected; however these three pesticides were below detection limits in all dogs at four weeks. Bupirimate (5 %), spinosad A (5 %), and propamocarb (5 %) were detected at 4 weeks only. Individual exposure profiles are shown for each of the 21 dogs at baseline and 4 weeks (Figure 4C). In the first of the two dogs with unavailable baseline data, atrazine, fuberidazole, and triasulfuron were detected in the 4 week sample and in the second dog, atrazine and ethoxyquin were detected. The use of heartworm prevention was reported in dogs C10 and C11, however the owner did not report the specific medication administered.

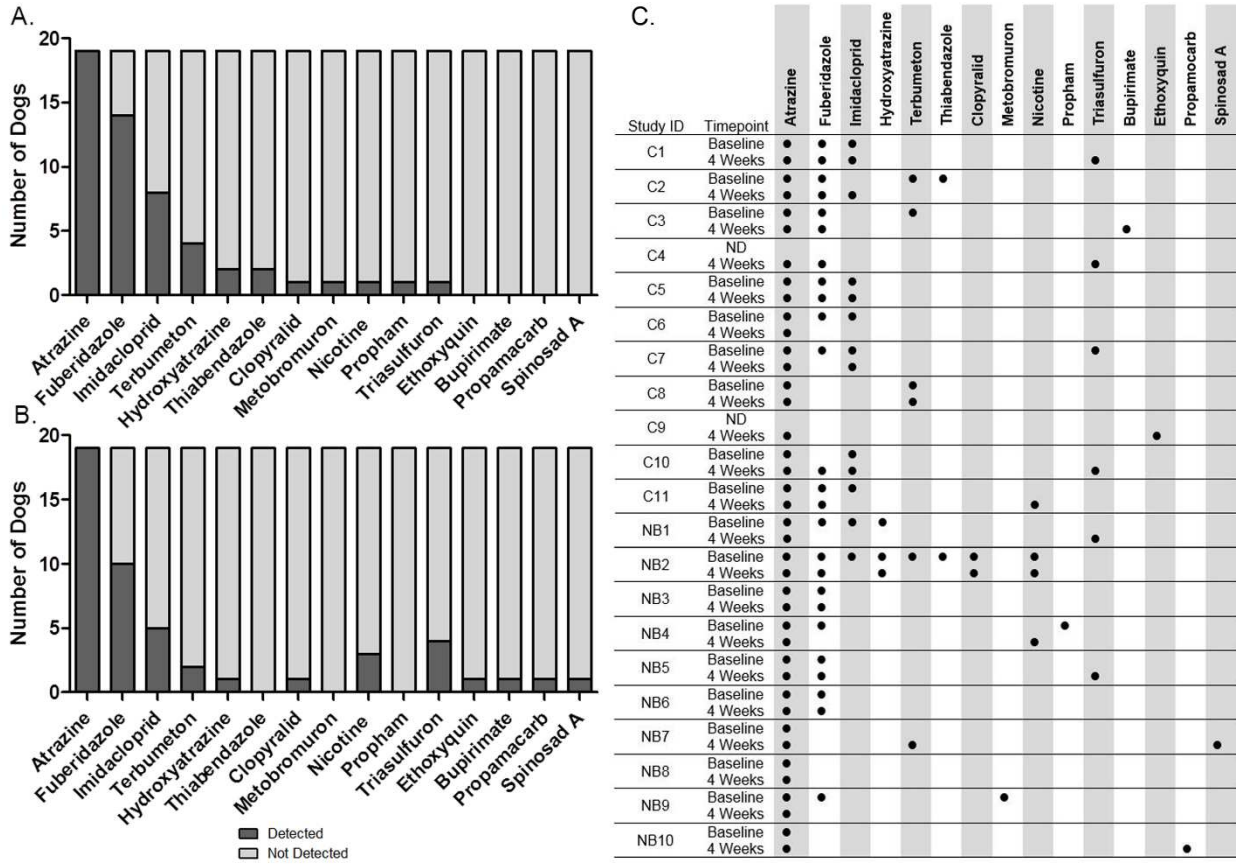

Figure 4. Detected pesticides in dog urine at baseline and 4 weeks. Detection frequency is shown in figures A and B. Each bar represents the total number of dogs screened for urinary excretion of pesticides by UPLC-MS/MS. The dark shaded portion of each bar represents the number of canine urine samples in which the pesticide was detected and the light shaded portion of the bar indicates the number of urine samples that the pesticide was not detected at (A) baseline and (B) after 4 weeks of controlled dietary intake. Profile exposures for each dog are shown at baseline and 4 weeks, where detected pesticides are illustrated by a dot (C). ND= No data.

To demonstrate the accuracy of the pesticide detection in urine, Figure 5 shows the positive detection of atrazine in a canine urine sample compared to control. The retention time of a 50 ng/mL atrazine standard was 1.47 min, which was identical to the peak detected in the urine sample. The ion ratio of the product ions 174 m/z and 132 m/z was 14.2 for the 50 ng/mL atrazine standard. The product ion ratio for the peak detected in the urine sample was 14.6,

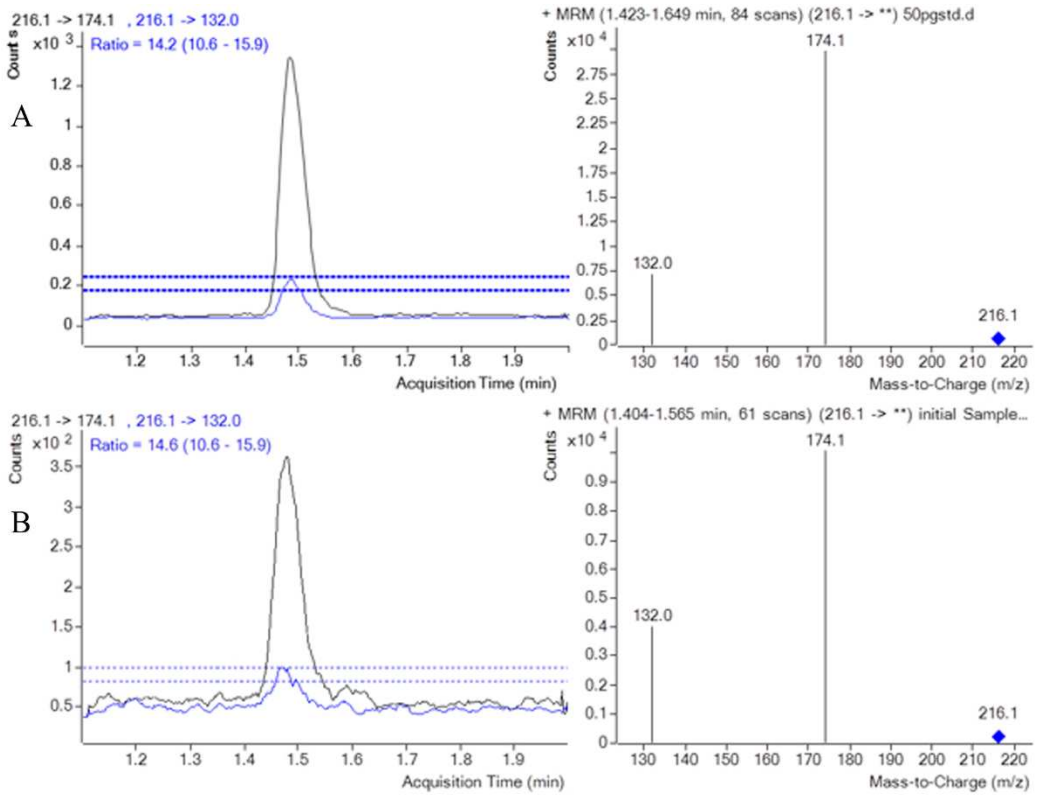

Figure 5. Validation of atrazine identification. Ion ratio correlation for atrazine's qualifier (216.1 > 132.0) and quantifier (216.1 > 174.1) ions in (A) 50ng/mL standard and (B) an individual dog urine sample at 4 weeks. Positive identifications were confirmed by retention time and the ion ratio (+/-20 %).

which was within 10 % of the atrazine standard. This positive detection methodology was applied to all pesticides detected in this study. β-glucuronidase was added to remove glucose moieties in urine, however detoxification of pesticides could include metabolites that undergo conjugation, hydrolysis, oxidation, methylation, and acetylation. Future detection panels for biomonitoring should account for biotransformation of parent pesticides and recent advancements in pesticide and metabolite identification as well as non-targeted metabolomics have improved biomonitoring capabilities.[119] The UPLC-MS/MS screening method utilized in this study was selected for the advantage of high-throughput sensitivity and specificity as this profile of pesticide residues have not been reported in dogs from natural exposures. The comparative quantification of these residues between urine samples using this method is imprecise due to potential differences in extraction efficiencies, ion suppression potential, and

urine concentration between biological replicates. Importantly, a relatively large number of parent pesticides were detected in canine urine, which supports the inclusion of parent compounds for biomonitoring and the utility of UPLC-MS/MS as a screening tool.

Atrazine levels in canine urine were below limits of quantification.

Given that atrazine was detected all in canine urine samples, we next quantified atrazine using LC-MS/MS quantification methods. Atrazine was only found in 3 samples above the limit of quantification equal to 20 pg/mL (20 parts per trillion). One dog with both baseline and 4 week timepoints had atrazine present in levels above the limit of quantification: 108.6 pg/mL at baseline and 86.9 pg/mL at 4 weeks. The third sample with quantifiable atrazine exposure levels was one of the dogs excluded from previous analysis due to the lack of a baseline urine sample. This dog's urine had 117.1 pg/mL at the 4 week time point. Blank samples spiked with D5-atrazine did not contain atrazine product ions indicating the atrazine peaks in urine were authentic and not the result of an impurity of the internal standard at low levels. Hydroxyatrazine was detected in 2 dogs and may have been the result of either atrazine metabolism or exposure to the hydroxylated metabolite of atrazine. The screening panel did include metabolites of atrazine but none were detected in urine samples.

Diet effects on urinary pesticide contents.

To determine the contribution of dietary intakes to canine pesticide exposure, we controlled the diets of all dogs for 4 weeks. The control diet was formulated using common pet food ingredients of corn, wheat, rice, poultry, and meat and bone meal. Wheat and corn were decreased and 25 % weight/weight replaced with a novel cooked navy bean powder ingredient.[120] Pesticide detection in the study diets consisted of the insecticides pymetrozine, azinophos-ethyl, and propoxur, the fungicide/anthelmintic fuberidazole, and the herbicide bromacil (Table

5). No commonly used crop pesticides included in our screening panel were detected in the study provided diets, yet some of the classes of chemicals were detected in urine, such as triazines, benzimidazoles, carbamates, and pyrimidines. The detected pesticides with elimination or absorption data available, the reported half-life is less than 48 hours: atrazine[135], triazines in general[136], clopyralid [137], imidacloprid[138], nicotine[139], terbumeton[140], and triasulfuron.[141]

Table 5: Detected pesticides in canine diets.

	Detection in Diet	
Pesticide	**Control**	**Navy Bean**
Pymetrozine	✓	✓
Fuberidazole	✓	✓
Bromacil	✓	✓
Propoxur	✓	✓
Azinophos-Ethyl	ND	✓

ND = not detected

These findings suggest that the acute dietary intake was not the primary route of pesticide exposure in this companion dog cohort study, however, given the wide variety of dog foods available and the potentially diverse sources of ingredients globally, future investigations should still consider dietary pesticide exposures, the relevant time frame for exposures to accumulate in the host, and other, non-dietary, sources of exposure, such as water, air, and exposures to medications.

Pesticide Bioactivities.

Atrazine, fuberidazole, imidacloprid, thiabendazole, clopyralid, nicotine, bupirimate, propham, metobromuron, and ethoxyquin have records of bioactivity as indexed in the PubChem BioAssay database (Table 4). These data suggest that this mixture of pesticides has potential to impact xenobiotic transformation by activating the aryl hydrocarbon receptor (AhR) and modulating cytochrome p450 expression (fuberdiazole, thiabendazole, clopyralid, metobromuron, and ethoxyquin).[142-147] Energy metabolism may also be affected by atrazine, thiabendazole, bupirimate, and ethoxyquin by modulating insulin secretion and activity, disrupting oxidative phosphorylation by disrupting membrane potential, and via preventing

reduction of nicotinamide adenine dinucleotide during β-oxidation.[148-151] Cell cycle regulation, proliferation, and DNA integrity may also be influenced by imidacloprid, thiabendazole, bupirimate, and ethoxyquin by DNA phosphodiesterase inhibition, inducing BRCA1 expression, promoting oxidative stress, and activating caspases.[152-156] Modulation of endocrine function by atrazine, thiabendazole, nicotine, bupirimate, propham, and ethoxyquin may occur via activating multiple pathways including the cAMP responsive element signaling upstream of phosphodiesterase IV; as well the estrogen receptor alpha (ER-α), androgen receptor (AR), and glucocorticoid receptor.[90; 157-162] Fluctuations in lipid metabolism and inflammation may occur from bupirimate and ethoxyquin as antagonists to the peroxisome proliferator-activated receptor (PPAR) signaling pathway and inhibitors of lipoxygenases.[163-166] Protein expression modifications both directly and epigenetically were shown by thiabendazole and ethoxyquin via induction of gene splice variations, activation of translation, and inhibition of histone methlytransferases.[167-169] Increased prevalence of chronic diseases in dogs and humans in conjunction with emerging evidence supporting that pesticide exposure increases risk for chronic diseases provide the rational for more rigorous pesticide mixture biomonitoring.[98] Moreover, exposure effects of pesticides as single compounds may be exacerbated by exposures to complex mixtures of chemicals.[170] This study confirmed the presence of a complex mixture of pesticides in a cohort of healthy dogs and demonstrates low-dose, environmentally relevant contents. Further research is warranted to determine the exposure duration, possible synergies, and dose dependent effects of combined pesticide exposures on chronic disease risk. Results from broad-spectrum screening methods, as applied in this study, demonstrating a wide range of low-level environmental toxicant exposures, provide rationale for an exposome study. Links between

complex exposure combinations and a mechanistic understanding of how environmental toxicants may impact health merit continued analysis throughout the lifespan.[171]

Companion dogs as sentinels for human environmental exposures and models for disease.

Dogs and humans share the same living environments and thus harbor a similar variety of exposures from materials, air, water, and food. Dogs experience a shorter lifespan than humans, allowing for cost effective and timely prospective studies. By utilizing companion dogs as sentinels for environmental exposures in humans and increasing our capability to screen for multiple chemicals, we can determine relevant mixtures for future studies that will help to better understand how pesticide mixtures target multiple cell pathways important for health and affecting disease risk.[107] The findings reported herein suggest that more advanced biomonitoring studies will be needed to investigate synergistic and dose dependent effects of relevant pesticide exposures as chemical mixtures as was recently accomplished for parabens.[103] This study highlights a significant knowledge gap with respect to environmental toxicant burden in healthy, adult, companion dogs and demonstrates the feasibility for continued investigations of the canine exposome during the lifespan, and the relationships to health and disease.[2]

[2] We would like to thank Amy Keller and Cadie Tillotson for their technical assistance. EPR and DGB are members of the Halifax Project (www.gettingtoknowcancer.org) which was established to collate information on relevant, low-dose mixtures of chemical exposures and has provided a conceptual framework for this publication.

CHAPTER 3: SERUM BIOCHEMISTRY, MICROBIOMES, AND METABOLOMES OF CLINICALLY HEALTHY NORMAL WEIGHT, OVERWEIGHT, AND OBESE DOGS

INTRODUCTION

Obesity is a serious multi-factorial nutritional disorder impacting over half of all dogs in developed countries. Overweight and obese dogs are at greater risk for multiple diseases including osteoarthritis, dyslipidemia, and cardiovascular disease, and some types of cancer, and suffer from decreased quality of life and shorter life expectancy.[10; 172] Advancements are being made in the nutritional management of obesity and emerging evidence supports the role of the owner's awareness of obesity related issues, socioeconomic status, activity level, and veterinary supervision.[21] Recent studies have demonstrated alterations in serum biochemistry [173], intestinal microflora composition [174], inflammatory biomarkers [175], and gut hormones [176] between obese and normal weight dogs, however the impact of these parameters on disease outcome are largely speculative.

Metabolomics is the study of global metabolite profiles present in a sample at a snapshot in time and may provide a deeper layer of metabolic analysis to determine subclinical metabolic alterations that are associated with disease outcomes.[177] Metabolomic platforms have been used in multiple species to demonstrate previously unknown alterations in amino acid, lipid, and carbohydrate metabolism with underlying links to inflammation, oxidative stress and perturbations in the gut microflora[177-190], however, this approach has been minimally applied to understanding obesity in dogs.

The objective of this study was to determine difference metabolic differences in normal weight (NW), overweight (OW) and obese (OB) dogs. We utilized clinical hemograms, serum

biochemistry and metabolic panels, evaluated the fecal gut microflora, and assessed the fecal, plasma and urine metabolome of 66, clinically healthy, adult, companion dogs of various ages, breeds, genders, and levels of excess adiposity. We hypothesized that OW and OB dogs will have higher levels of serum lipids, altered microflora composition, and shifts in the metabolome associated with altered lipid, amino acid, and carbohydrate metabolism when compared to NW dogs.

METHODS AND MATERIALS

Canine Participants and Sample Collection

Informed, written consent was obtained from all owners before dogs began participation in the study. Dogs were enrolled at the Colorado State University Veterinary Teaching Hospital (Fort Collins, CO) and the Wellington Veterinary Hospital (Wellington, CO).

Clinically healthy adult dogs with no known medical problems or dietary allergies were recruited to participate in one of three previously reported clinical trials (Chapters 4 & 5, Table 6).[95; 120] A physical exam was performed by a study veterinarian and a complete serum biochemistry panel and whole blood cell differential performed to confirm that each dog was healthy prior to enrollment. A Body Condition Score (**BCS**) was assigned to each dog on a 9-point scale [191] where a score of 5 is considered "ideal" and every unit increase in BCS over 5 corresponds to a 10-15 % increase in bodyweight, such that a BCS of 8/9 corresponds to approximately an 30 % excess bodyweight. Dogs with a BCS of 4-5/9 were considered "normal weight" (n=17), dogs with a BCS of 6-7/9 were considered "overweight" (n=27), and dogs with a BCS 8-9/9 were considered "obese" (n=22)[192]. Signalment data were also collected from owners and medical records and included breed, age, and sex. A wide range of breeds were represented in this study: Labrador Retrievers and Lab mixes, n=8 and n= 6, respectively; Australian Cattle Dogs,

n=5; Terriers (American Pit Bull and Boston), n=5; Border Collie and mixes, n=1 and n=4, respectively; Golden Retriever, N=4; Australian Shepherd and mixes, n=3 and 1, respectively; Hounds (Daschund and Basset), n=3; Dalmatian, n=2; Saint Bernard, n=2; Spaniels, n=2; Boxer, n=1; Corgie, n=1; Karelian Bear Dog mix, n=1, Keeshond, n=1, Rottweiler, n=1; Shiz Shu, n=1; Standard Poodle, n=1; Wire-hair Pointer, n=1; and otherwise unknown mixed breeds, n=12.

Table 6: Characteristics of 66 normal weight, overweight, or obese clinically healthy dogs analyzed to determine metabolic aberrancies between weight classes.

	Normal Weight	Overweight	Obese	p-value[2]
Age[3], yr	3.0[a] (2.5-4.5)	5.0 (3.0-6.0)	5.5[b] (3.0-7.0)	0.037
Weight, kg	22.5[a] (19.4-30.8)	30.6 (22.4-37.3)	37.7[b] (24.6-39.8)	0.031
Sex[4]				0.905
Female	9	16	12	
Male	8	11	10	

[1]Weight class was determined by Body Condition Score (BCS) using a 9 point scale (Laflamme, 1997): Normal Weight, BCS 4-5/9, n=17; Overweight, BCS 6-7/9, n=27; Obese, BCS 8-9/9, n=22. [2]Continuous variables (age and weight) were evaluated for differences across groups using a Kruskal-Wallis test and categorical variables (sex) were evaluated using a Chi-square test. $P < 0.05$ was considered significant. Groups with significant differences are indicated with different letter superscripts. [3]Age as reported by owner. [4]All dogs were neutered with the exception of one male in the normal weight group, and one female in each of the overweight and obese groups.

Serum samples for biochemical analysis were collected via venipuncture into a tube without anticoagulant, allowed to coagulate for 15 minutes and centrifuged for 15 minutes at 1,500 x g. Plasma samples for metabolomic analysis and complete blood cell counts were collected into tubes containing EDTA, immediately placed on ice, and centrifuged within 20 minutes of collection at 4 °C for 15 minutes at 1,500 x g. The plasma was removed and stored at -80 °C until analysis. Urine was collected by free catch or ultrasound guided cystocentesis at the discretion of the veterinarian, immediately placed on ice or stored at 4 °C, then transferred to -80 °C in 1ml aliquots. Owners were instructed to collect fecal samples within 5 hours of being voided by the

dog. Fecal samples were immediately frozen after collection and stored at -20 °C, lyophilized until dry, and stored at -80 °C until extracted for microbial and metabolomic analysis.

Serum Biochemistry and Hemograms

All clinical analyses were performed at the Clinical Pathology Laboratory at Colorado State University as previously described.[193] Briefly, serum biochemical analysis was performed under standard conditions on a clinical chemistry analyzer (Hitachi 917, Roche Diagnostics; Indianapolis, IN) and the complete blood count was performed on an automated analyzer (Advia 120, Bayer; Tarrytown, NY).

Microbiome Analysis

In brief, DNA was extracted from 150 mg of lyophilized stool using the MO BIO PowerFecal DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) per the manufacturer's instructions. The targeted 16S small-subunit ribosomal genes were amplified using universal 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R GGACTACVSGGGTATCTAAT primers (Caporaso et al. 2011) via polymerase chain reaction (PCR) using a 12bp unique Golay barcoded primer for each sample (Lauber et al. 2008). The sample amplicons were subsequently cleaned using MO BIO UltraClean-htp 96 Well PCR Clean-Up Kits (MO BIO Laboratories, Carlsbad, CA, USA), quantified using the Quant-iT PicoGreen dsDNA Reagent and Kits (Life Technologies Corporation, Grand Island, NY, USA) and pooled for sequencing. DNA extraction, amplification and cleaning were performed at the Natural Resource Ecology Laboratory at CSU.

Sequencing was performed on a MiSeq platform (Illumina, San Diego, CA, USA) at the RTSF genomic core facility at Michigan State University. Sequences reads were normalized to total number of reads. Sequencing data were processed using the Quantitative Insights Into Microbial Ecology (QIIME 1.7.0) pipeline (Caporaso et al. 2010) for determining operational

taxonomic unit, taxonomy assignment, and UniFrac analyses followed with Principle Coordinates Analysis (Lozupone and Knight 2005, Lauber et al. 2009).

Metabolomic Analysis

Fecal, plasma, urine, and diet samples were extracted and derivatized as previously described.[94] Samples were analyzed by GC and liquid chromatography (LC) coupled to mass spectrometry (MS) under previously described conditions.[94; 194] Individual features, described by mass, charge and retention time were generated in XCMS. The average abundance of duplicate injections were normalized to total ion current and clustered together into individual metabolites.[195] The relative abundance of each cluster was calculated based on the weighted sum of all features within the cluster and scaled to a median of one. Spectra were screened against in-house and external libraries including NIST v12 (www.nist.gov), Massbank, Metlin, and Golm (http://gmd.mpimp-golm.mpg.de) metabolite databases for annotation.

Statistical Analysis

Baseline characteristics of the dogs were evaluated using a Kruskal-Wallis test for the continuous variables of age, and weight followed by Dunn's Multiple Comparison test to determine the groups with significant differences. A Chi-square test was used to evaluate groups for differences in the categorical variable of sex and presence of abnormal clinical blood values for parameters where all expected values were greater than 1 and at least 20 % of the expected values were greater than 5. Differences in proportional data were considered significant when $p < 0.05$, and trends were considered when $p < 0.10$. Clinical blood parameters were analyzed with an ANCOVA where both weight class and age were evaluated. Pair-wise comparisons were Tukey-Kramer adjusted and parameters without normal distributions were log transformed for statistical evaluation. The raw values, not the transformed values are reported for all parameters. The

microbiome and metabolome data were evaluated with a one-way ANOVA in R.[196] An FDR correction was applied to all data with multiple comparisons.[197] Significance was considered at a corrected p value < 0.1 and trends were considered when p <0.2. For non-corrected p values, differences were considered significant when $p < 0.05$, and trends were considered when $p < 0.10$.

RESULTS

Baseline Characteristics of Canine Participants

Sixty-six adult NW, OW, and OB dogs were enrolled to evaluate differences in serum analytes, microbiome, and metabolome across weight phenotypes. The baseline characteristics of dogs in each group are presented as medians and interquartile ranges in Table 6. The median age in years, as reported by owners in the NW dogs was 3.0 (2.5-4.5), 5.0 (3.0-6.0) in OW, and 5.5 (3.0-7.0) in the OB group. There was a significant difference in age across the three groups ($p = 0.04$) with the median age of the NW dogs significantly younger than the OB dogs ($p < 0.05$). The median weight of the NW dogs was 22.5 kg (19.4-30.8 kg), 30.6 kg (22.4-37.3 kg) for the OW dogs, and 37.7 (24.6 – 39.8 kg) for the OB dogs. As expected, dogs in the OB group were significantly heavier than dogs in the NW group ($p < 0.05$). Ideal weights were calculated for the OW and OB dogs based on the assumption of a 10 % increase in total body weight for every unit increase in BCS over 5[192] and no differences were seen in calculated ideal weights across groups (data not shown, $p = 0.79$). Within the NW group, there were 9 neutered female dogs, 7 neutered male dogs, and one intact male dog. In the OW group, there were 15 neutered female dogs, 1 intact female dog, and 11 neutered male dogs. In the OB group, there were 11 neutered female dogs, 1 intact female dog, and 10 neutered male dogs. For the microbiome and urine metabolome analysis, fecal samples from a subset of 51 dogs were processed and analyzed. All dogs in the NW remained the same, the OW group was reduced by 5 (n=22) and the OB group was reduced by 10 (n=12).

There were no differences in weight, age, or sex distribution of the 66 dogs compared to the subset of 51 dogs ($p < 0.05$, data not shown).

Serum Biochemistry and Hemograms of Normal Weight, Overweight and Obese Dogs

Thirty-nine clinical serum and whole blood parameters were measured or calculated for all 66 dogs. Parameters evaluated included differential cell counts, cell morphology, and serum biochemical analytes (Figure 6). All dogs were considered healthy by the evaluating study veterinarian, however 63 of the 66 dogs had at least one value outside of normal ranges in 31/39 parameters. The most commonly observed parameter outside of normal ranges was the albumin:globulin (A/G) ratio which was elevated in 38/66 dogs. The distribution of dogs with elevated A/G ratios was not different by weight class ($p = 0.962$). Fifty-five percent of dogs with elevated A/G ratios (21/38) also had increased levels of albumin or decreased levels of globulin (data not shown), the distribution of which, was also not different by weight class. Hemolysis of blood samples was over 60.0 mg/dL in 25/66 dogs: the distribution across weight class was different across weight classes ($p = 0.014$) with OW dogs having the highest proportion of hemolytic samples (15/27), followed by OB dogs (8/22), and then NW dogs (2/17). Creatinine kinase (CK) was elevated in 9/66 dogs, all of which were OW or OB: this distribution was different across weight classes ($p = 0.038$), OW dogs had the highest proportion (7/27), followed by OB dogs (2/22). Aspartate aminotransferase (AST) was elevated in 5/66 dogs and slightly below 16 IU/L in 1 NW and OW dog. All 5 dogs with elevated AST levels were OW (5/27), thus the proportion of abnormal values by weight class was different by weight class ($p = 0.020$). Cholesterol was elevated in 5/66 dogs and decreased in one OB dog, the proportion of dogs with abnormal values tended to be different by weight class ($p = 0.080$): NW (2/17), OW (0/27), and OB (4/22). The fourth most frequently parameter outside of the normal range was platelet count

which were decreased in 14/66 dogs, 13 of which had clumped platelets (data not shown). The proportion of dogs with low platelets was equally distributed across weight classes: 5/17 NW, 5/27 OW, and 4/22 OB ($p = 0.631$). The proportion of dogs with low levels of potassium, globulin, or lymphocytes; high levels of packed cell volume (PCV) hemoglobin, alkaline phosphatase (ALP), lipemia, eosinophils, red blood cell counts (RBC), or calculated osmolality; or mixed levels of monocytes was not different by weight class ($p > 0.05$). A chi square statistic could not be calculated for red blood cells (RBC), mean corpuscular volume (MCV), red blood cell distribution width (RDW), mean platelet volume (MPV), glucose, sodium, bicarbonate, anion gap, segmented neutrophils, magnesium, alanine amino transferase (ALT), nucleated cell counts, mean corpuscular hemoglobin concentration (MCHC), or blood urea nitrogen (BUN) as these analytes were outside of normal ranges in less than 4 dogs and the distributions did not meet the assumptions for calculating a Chi-square statistic (Figure 6). There were no values outside of reference ranges for plasma protein, creatinine, phosphorus, calcium, total protein, total bilirubin, gamma-glutamyl transpeptidase (GGT), or chloride.

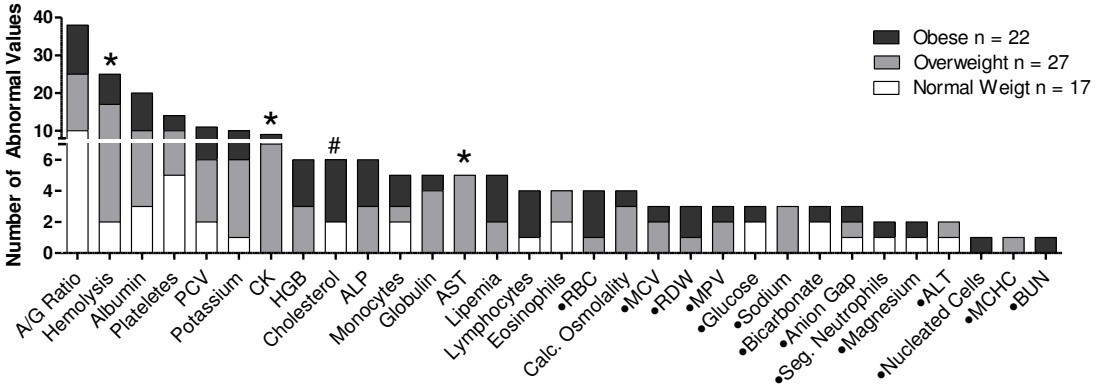

Figure 6: Number of serum, plasma, and whole blood parameters with values outside of reference ranges in normal weight, overweight, and obese dogs. Bars indicate the total number of values outside of reference ranges for each parameter by weight class: NW = white portion, OW = gray portion, OB= black portion. Distribution across was weight classes for each parameter was evaluated for differences using a Chi-square. Asterisks (*) indicate parameters with distribution differences by weight class ($p < 0.05$), pound signs (#) indicate a trend ($p < 0.10$), and dots (•) indicate parameters from Chi-square analysis. Abbreviations: RBC, red blood cell; HGB, hemoglobin; PCV, packed cell volume; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; RDW, red blood cell distribution width; MPV, mean platelet volume; BUN, blood urea nitrogen; A/G, albumin:globulin; CK, creatinine kinase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

All measured serum, plasma, and whole blood parameters and reported as medians and range (min-max, Table 7). Initially, a non-parametric one-way ANOVA was used to determine differences across groups and found that compared to NW dogs, OW or OB dogs had higher levels of segmented neutrophils, plasma protein, higher anion gap, CK, and tended to have higher levels of albumin; OW and OB dogs also had lower magnesium, lower levels of GGT, sodium, calculated osmolality, and AST (data not shown). However, age was found to co-vary with lymphocytes, monocytes, phosphorus, globulin, A/G ratios, cholesterol, CK, ALP, ALT and AST. An ANCOVA statistical approach was used to control for the effects of age and AST, GGT, and chloride were significantly different when age was accounted for. AST was decreased by an average of 4 IU/L in OB dogs compared to NW; chloride was decreased on average by 2 mEQ/L; and GGT was decreased in both OW and OB dogs by 3 IU/L and 2.5 IU/L, respectively. No differences were seen across groups in nucleated cells, lymphocytes, monocytes, eosinophils, plasma protein, RBC, HGB, PCV, MCV, MCHC, RDW, platelets, MPV, glucose, BUN, phosphorus, calcium, magnesium, total protein, albumin, globulin, A:G ratio, cholesterol, total bilirubin, ALP, ALT, sodium, potassium, bicarbonate, anion gap, lipemia, or hemolysis (Table 7).

Table 7: Serum biochemistry and whole blood counts of normal weight, overweight, and obese dogs.

Analyte[1]	Normal Range	Normal Weight *N=17*	Overweight *N=27*	Obese *N=22*	p-value[2]
Nucleated Cells	4.5-15.0 x10^9/L	7.9 (5.8-12.1)	9.3 (5.6-14.7)	10.05 (5.8-15.4)	0.260
Seg. Neutrophils	2.6-11.0 x10^9/L	4.9 (2.5-9.3)	6.3 (3.2-10.2)	7.25 (4.3-11.4)	0.109
Lymphocytes	1.0-4.8 x10^9/L	2.3 (0.9-4.4)	1.8 (1-3.8)	1.6 (0.6-3.0)	0.641
Monocytes	0.2-1.0 x10^9/L	0.5 (0.1-1.0)	0.5 (0.1-0.9)	0.6 (0.1-1.3)	0.978
Eosinophils[3]	0.1-1.2 x10^9/L	0.35 (0.2-2.1)	0.4 (0.1-1.8)	0.4 (0.1-1.1)	0.677
Plasma Protein	- g/dL	6.5 (6.1-7)	6.8 (6.3-7.5)	7 (6.4-7.8)	0.206
RBC	5.5-8.5 x10^{12}/L	7.3 (6.7-8.5)	7.3 (6.5-9.2)	7.6 (6.7-9.03)	0.510
HGB	13.0-20.0 g/L	17.2 (15.7-19.6)	18 (16-21)	18.25 (15.8-21)	0.309
PCV	40-55 %	50 (46-56)	51 (46-60)	53 (44-60)	0.542
MCV	62.0-73.0x10^{-15}L	68 (64-73)	70 (65-78)	68.5 (37-73)	0.331
MCHC	33.0-36.0 x10^9/L	34 (34-36)	35 (34-37)	35 (33-36)	0.221
RDW	12.0-15.0 %	12.5 (12.1-13.3)	12.9 (11.9-14.5)	13.2 (11.4-13.9)	0.206
Platelets	200.0-500.0x10^9/L	239 (142-324)	264 (106-453)	290 (123-444)	0.283
MPV	7.5-14.6 x10^{-15}L	11.1 (9.6-14.2)	10.4 (8.5-15.3)	11.2 (8.2-16.7)	0.589
Glucose	75-130 mg/dL	97 (71-120)	94 (80-123)	98.5 (81-139)	0.641
BUN	7-32 mg/dL	18 (12-31)	15 (11-32)	16 (9-39)	0.700
Creatinine	0.4-1.5 mg/dL	1.1 (0.8-1.4)	1.1 (0.8-1.5)	0.9 (0.7-1.3)	0.206
Phosphorus	2.1-6.0 mg/dL	3.8 (2.6-5)	3.5 (2.4-4.8)	3.3 (2.4-5.2)	0.827
Calcium	9.2-11.7 mg/dL	10.7 (10-11.5)	10.7 (10-11.7)	10.7 (10-11.5)	0.987
Magnesium	1.9-2.7 mg/dL	2.1 (1.8-2.4)	2.05 (1.9-2.7)	1.9 (1.7-2.2)	0.136
Total Protein	5.3-7.2 mg/dL	6.1 (5.6-6.8)	6.3 (5.4-7)	6.4 (5.8-7)	0.860
Albumin	2.5-4.0 mg/dL	3.8 (3.5-4.2)	3.9 (3.4-4.7)	4 (3.6-4.5)	0.125
Globulin	2.0-3.8 mg/dL	2.4 (2-3.2)	2.4 (1.6-3.3)	2.35 (1.7-2.8)	0.641
A/G Ratio	0.8-1.6	1.7 (1.1-2)	1.7 (1-2.4)	1.7 (1.3-2.4)	0.331
Cholesterol	130-300 mg/dL	229 (175-339)	232 (168-293)	241 (124-341)	0.542
Total Bilirubin	0.0-0.3 mg/dl	0.1 (0-0.2)	0.1 (0.1-0.3)	0.1 (0-0.2)	0.221
CK[3]	50.0-275.0 IU/L	90 (52-212)	146 (62-2179)	150.5 (73-350)	0.109
ALP[3]	20.0-142.0 IU/L	40 (10-138)	45 (16-830)	47 (21-477)	0.987
ALT[3]	10.0-110.0 IU/L	31 (27-164)	41 (19-118)	35 (21-109)	0.743
AST[3]	16.0-50.0 IU/L	29 (15-47)[a]	29 (14-93)[a]	25 (16-39)[b]	**0.043**
GGT	0.0-9.0 IU/L	3.0 (0.0-5.0)[a]	0.0 (0.0-5.0)[b]	0.5 (0.0-3.0)[b]	**0.040**
Sodium	142.0-152.0mEQ/L	147 (144-151)	146 (140-157)	147 (142-150)	0.199
Potassium	4.0-5.0 mEQ/L	4.4 (3.9-4.7)	4.3 (3.7-4.9)	4.3 (3.5-4.7)	0.677
Chloride	108.0-120.0mEQ/L	113 (110-118)[a]	112 (109-119)[a]	111 (108-115)[b]	**0.040**
Bicarbonate	16.0-25.0mEQ/L	21.5 (18.8-28.4)	20.2 (17.6-23.9)	20.15 (16.8-25.3)	0.330
Anion Gap	13.0-22.0mEQ/L	16 (11-21)	17 (12-22)	19 (14-23)	0.125
Calc. Osmolality	284.0-304.0 mOsm/Kg	293 (287-303)	290 (282-314)	291 (284-306)	0.237
Lipemia[3]	0.0-40.0 mg/dL	16 (0-39)	9 (0-770)	8.5 (4-122)	0.987
Hemolysis[3]	0.0-60.0 mg/dL	35 (7-82)	72 (13-403)	46 (3-220)	0.283

[1]Abbreviations: RBC, red blood cell; HGB, hemoglobin; PCV, packed cell volume; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; RDW, red blood cell distribution width; MPV, mean platelet volume; BUN, blood urea nitrogen; A/G, albumin:globulin; CK, creatinine kinase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase. [2]Data are shown as median and range (min-max) and were evaluated for differences across groups using an ANCOVCA with the variables of age and body weight classification and FDR corrected. Differences between groups were determined with pairwise comparisons and a Tukey-Kramer correction. $P < 0.1$ was considered significant. Groups with significantly different medians are indicated with different letters. [3]Parmeteres not normally distributed were log transformed for statistical analysis however the actual, untransformed values are reported.

Fecal Microbiome of Normal Weight, Overweight, and Obese Dogs

The fecal microbiome was assessed in 17 NW dogs, 21 OW dogs, and 12 obese dogs. Significant variation existed within dogs that was not consistent by weight class (Figure 7). Firmicutes was the most abundant phylum and comprised 56 % - 73 % of the canine fecal microbiome. Bateroidetes comprised 24 %-37 %, Proteobacteria 2 % - 3 %, and Actinobacteria 0.02 %. The most abundant genus was *Blautia,* followed by *Bacteroides,* unclassified members of the Lachnospiraceae family, and [*Rumminococcus*]. No differences between weight classes were seen at the level of phyla, class, order, family or genus (data not shown)

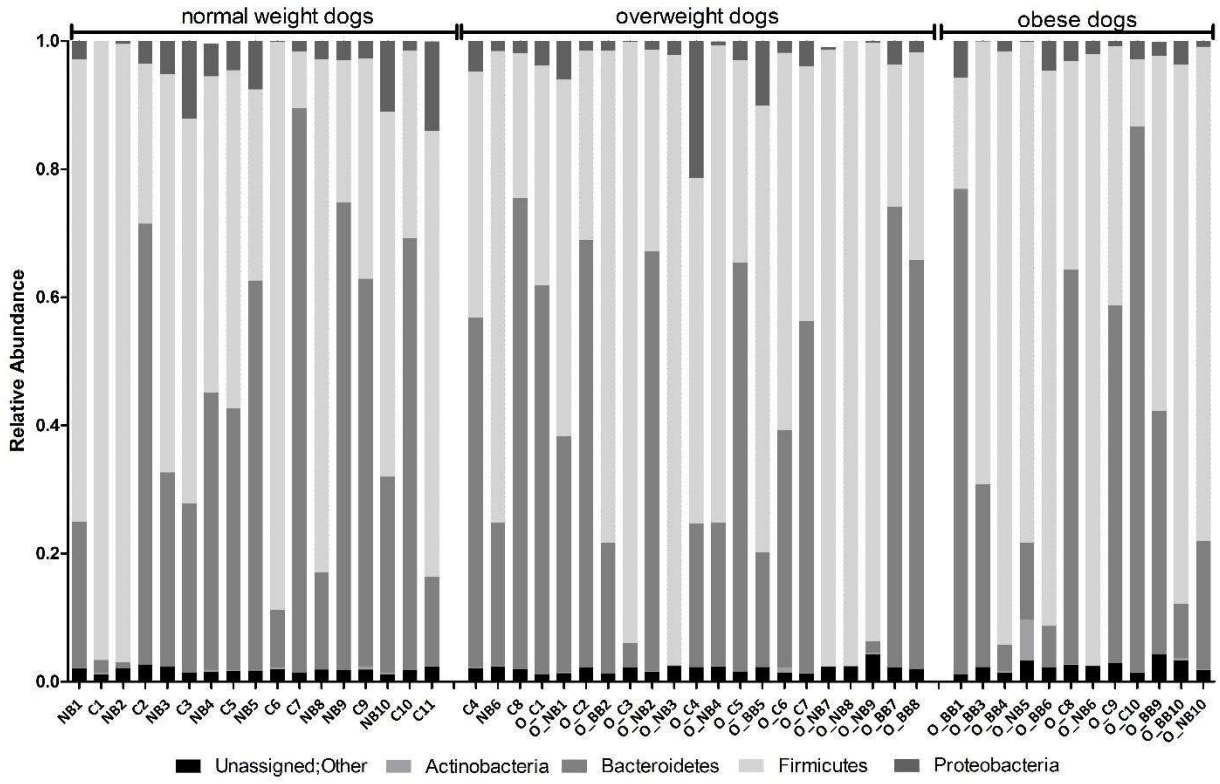

Figure 7: Phyla level variation in the fecal microflora of normal weight, overweight, and obese companion dogs. Each bar represents the total relative abundance and is colored by phyla. Dogs are separated by weight class based on BCS.

Fecal, Plasma, and Urine Metabolome of Normal Weight, Overweight, and Obese Dogs

To determine potential metabolic differences between NW, OW, and OB dogs, fecal, plasma and urine samples were analyzed via GC- and LC-MS (Table 8). None of the fecal metabolites detected by GC were significantly different between the weight groups, 37 fecal metabolites detected by LC were different: 36 were higher in the NW dogs relative to the OW and OB dogs while 1 was lower. Plasma contained the greatest number of metabolites that were different between dog weight classes: 30 were detected by GC, half of which were higher in the NW dogs; 155 were detected by LC, 57 were higher in NW dogs while 98 were higher in OW and OB dogs. In urine, only one compound was different in the GC data, however it was a very small peak with a low number of features and was therefore not counted in this dataset. By LC, 44 compounds were different by weight class, 23 were elevated in NW dogs and 21 were elevated in OW and OB dogs.

Table 8: Canine metabolites detected in fecal, plasma, and urine samples that differentiate normal, overweight, and obese dogs.

	GC	*LC*
Fecal	**1,112**	**795**
Significant by weight class	0	37
Elevated in NW dogs	0	36
Elevated in OW and OB dogs	0	1
Plasma	**554**	**348**
Significant by weight class	30	155
Elevated in NW dogs	15	57
Elevated in OW and OB dogs	15	98
Urine	**1,139**	**1,318**
Significant by weight class	0	44
Elevated in NW dogs	0	23
Elevated in OW and OB dogs	0	21
Total Significant	**30**	**236**

Total number of detected metabolites in each matrix by platform are bolded. Significance was determined by a one-way ANOVA and FDR corrected p values < 0.10 were accepted as significant. NW= normal weight, OW=overweight, OB=obese.

To determine differences in the global metabolite variation between the NW, OW, and OB dogs, we evaluated each matrix and platform with PCAs and found significant variation in fecal, plasma, and urine samples (**Figure 8**). The greatest variation between weight phenotype was captured in the plasma metabolome: compounds detected by LC (**Figure 8**A) and GC (**Figure 8**B) explained 45 % and 14 % of the variation, respectively. Urine metabolome explained 5 % of the variation (**Figure 8**C) and variation in the fecal metabolome was 3.9 % (**Figure 8**D).

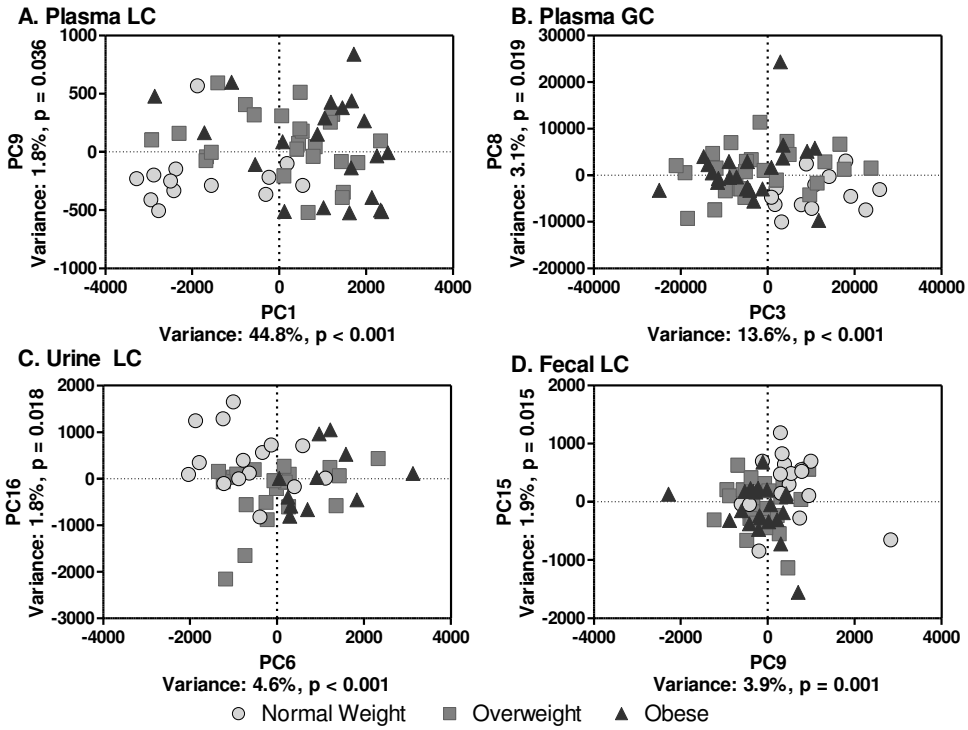

Figure 8: Variation in the normal weight, overweight, and obese canine metabolome. Significant variation in A) plasma metabolites detected by LC-MS; B) plasma metabolites detected by GC-MS; C) urine metabolites detected by LC-MS; and D) fecal metabolites detected by LC-MS. Dogs classified as normal weight are indicated by circles, overweight dogs by squares, and obese dogs by triangles.

DISCUSSION

The objective of this study was to determine the subtle, obesity-related changes in the physiology of OW and OB dogs compared to NW dogs to provide insight into metabolic aberrations that could lead to the onset of overt, clinical disease. Clinical blood and serum, and fecal and plasma metabolomic analyses were performed on 66 clinically healthy adult, companion dogs with a wide range of BCSs, breed, age, and living environments. Fecal microbiome and urine metabolomics were performed on a subset of these dogs as well.

Overweight and Obese Dogs had More Clinical Blood Parameters Outside of Reference Ranges than Normal Weight Dogs.

The proportion of OW and OB dogs with clinical blood values outside of reference ranges was higher than NW dog for hemolysis, CK, and AST, and tended to be higher for

cholesterol (Figure 6). However, of these parameters, only AST levels were significantly different between groups and were lower in the OB dogs compared to NW. In addition to AST, GGT and chloride were also reduced in OW and OB dogs (Table 7). In this cohort of dogs, age was significantly correlated to analytes related to liver and kidney function, white blood cell populations, and phosphorus. Previous studies have reported that compared to lean dogs, overweight dogs have higher levels of cholesterol[176; 198-200], triglycerides[176; 198; 199; 201], and lipoproteins[199], while other studies have found no differences in one or more lipid parameters.[198; 199; 201] Age associations have been reported for lipids, CK, and creatinine[201; 202], however a relationship between hemolysis and obesity has not, to the best of our knowledge, previously been reported in dogs. In this cohort of dogs, the level of hemolysis was not associated with obesity, but overweight and obese dogs were more likely to have a hemolytic blood draw than NW dogs. While this could be a finding specific to this study, it could also represent a true phenomenon where dogs with excess adiposity may have more traumatic blood draws, and thus a higher probability of high RBC lysis. In these dogs, the level of hemolysis was associated with segmented neutrophil counts, plasma protein, CK, and AST. While not controlled for in this analysis, future studies involving venipuncture for blood collection may need to account for obesity associated hemolysis during collection.

Overall, very few differences were seen across clinical blood parameters in NW vs. OW and OB dogs and conflicting results have been published in different cohorts of dogs. These findings support the need for sub-clinical metabolic assessments in OW dogs as the clinical biochemical analyses inconsistently detected differences in otherwise healthy dogs and may not be sensitive enough to detect subtle physiological alterations in the absence of overt disease.

The Canine Fecal Metabolome Comprised Significant Individual Variation and Did Not Correspond to Weight Classification.

The fecal microbiome of 50 dogs was analyzed to determine differences in the microbiota composition that could contribute to metabolic alterations in NW and OW or OB dogs. Consistent with previous reports, the phyla Firmicutes was the most abundantly represented.[69; 174] Contrary to previous reports, no changes were seen between any of the weight classes (NW, OW, OB) while increases in Actinobacteria[174] and Proteobacteria[200] have been reported in lean and obese companion dogs and colony dogs, respectively. While sample size is comparable between this study and reported results, the individual variations was much higher and could reflect the diet, age, and environment of the dogs, however there could also be differences in sample collection. Other studies have reported immediate collection and subsequent freezing of samples after spontaneous defecation, however in this study, owners were instructed to collect each fecal samples within 5 hours of defecation and then samples were frozen. Additional bias may also have been introduced via the normalization approach: total sum scaling was used to normalize each read to the total number of reads, however this has been found to skew results in samples with zero or low reads of a particular sequence. To overcome this potential bias, a weighted, cumulative sum scaling has been proposed and seems to overcome some of this bias.[203] While confirmatory studies with larger samples sizes are needed to establish the relationship between obesity and the fecal microbiota of dogs, future studies should also incorporate an approach to assess the function of the microbiome, not just the composition.

Overweight and Obese Dogs Have Significant Variations in the Metabolome Compared to Normal Weight Dogs

Two hundred, sixty-six compounds were found to differ significantly across weight classes. The majority of different compounds were found in the plasma, detected by LC, which explained

45 % of the variation between NW, OW, and OB dogs (**Figure 8**A). Previous metabolomic studies in humans have found significant variation in plasma amino acids, glycerides, and phosphatidylcholines between obese and lean individuals[187; 188] which may also be revealed in this canine data set. To the best of our knowledge, this is the first metabolomic report in obese dogs and may reveal important associations between metabolic aberrancies and risk for the development of overt chronic disease.

CONCLUSION

In this cohort of 66 NW, OW, and OB companion dogs, obesity was associated with minor changes in clinical serum analytes, however OW and OB dogs were more likely to have altered serum analytes in relation to the reference range. We also found that OB dogs were more likely to have hemolytic blood draws which could further limit the sensitivity of clinical metabolic blood panels for detecting subtle alterations in metabolism. The fecal microbiome of these dogs exhibited significant variation between individual dogs and differences across weight class could not be determined. In spite of the significant variation between dogs, the metabolome provided robust differences between NW, OW, and OB dogs. Identification of these metabolites could reveal important indicators of aberrant metabolism that could be used to identify dogs at risk for developing obesity associated co-morbidities. This study has demonstrated that OW and OB dogs have significant metabolic differences from NW dogs and has highlighted the potential of metabolomics to further our understanding of obesity in companion dog.[3]

[3] We would like to thank Guy Beresford for processing the fecal samples for microbiome analysis, Ann Hess for her assistance with the statistical analysis, Husen Zhang for the microbiome analysis, Kelly Santangelo for her assistance with interpreting the clinical blood panel, and Cadie Tillotson for her assistance with the collection of samples. We would also like to acknowledge the Clinical Trials Core at the CSU Flint Animal Cancer Center and the Wellington Veterinary Hospital for study oversight.

CHAPTER 4: EFFECTS OF COOKED NAVY BEAN POWDER ON APPARENT TOTAL TRACT DIGESTIBILITY AND SAFETY IN HEALTHY ADULT DOGS[4]

INTRODUCTION

Staple foods are important determinants of health in humans and companion animals.[204; 205] Developing nutritional guidelines to improve health and prevent disease in dogs has contributed to the production of numerous commercial dog foods with novel carbohydrate[206], fiber[26; 207], and protein sources.[208; 209] Despite the success of plant-based nutrients in dog food, dry bean (*Phaseolus vulgaris* L., Fabaceae) is one staple food crop of global agricultural and nutritional importance that has been overlooked in commercial pet food formulations. Evidence supports that in addition to providing excellent sources of protein, fiber, minerals, essential vitamins, and bioactive compounds in higher concentrations than cereal grains, such as wheat and corn[33], beans have chronic disease fighting properties, which slow or prevent disease progression.[210]

Navy beans were selected for this study because of their reported health benefits[211; 212] and availability in a cooked powder form. Navy bean consumption has not previously been examined in colony or companion canines. Digestibilities of the starch and fiber fractions of uncooked legumes were evaluated for canines *in vitro* and showed lower digestibility compared to other carbohydrate sources.[207] However, cooked beans have been successfully incorporated

[4] This chapter was published in *Journal of Animal Science* August 2012:90(8):2631-38. DOI: 10.2527/jas.2011-4324. Authors are Genevieve M. Forster, Dale Hill, Gordon Gregory, Kristen M. Weishaar, Sue Lana, John E. Bauer, and Elizabeth P. Ryan(*120*). Forster, G. M., D. Hill, G. Gregory, K. M. Weishaar, S. Lana, J. E. Bauer, and E. P. Ryan. **2012**. Effects of cooked navy bean powder on apparent total tract nutrient digestibility and safety in healthy adult dogs. *Journal of animal science* 90: 2631-2638.

into homemade canine diets (R. L. Remillard, Veterinary Nutritional Consultations, Inc., Hollister, NC, personal communications) and soybeans, another legume, are digestible by dogs.[213]

The major objective of this study was to examine safety and digestibility of cooked navy bean powder in healthy dogs compared to a placebo control, commercial canine diet formulation. We hypothesized that cooked navy bean powder as a major ingredient in an adult canine diet formulation, is palatable, safe, and digestible.

MATERIALS AND METHODS

The Colorado State University Institutional Animal Care and Use Committee approved all clinical trial operations, animal care procedures, and collection of biological samples for safety and digestibility of experimental research diets before beginning the study.

Study Design

Twenty-one healthy, adult, free-living dogs of different breeds were recruited to participate in a randomized, double-blinded, and placebo controlled canine dietary intervention study at the Veterinary Teaching Hospital's Animal Cancer Center at Colorado State University. Dogs were randomized in a 1:1 manner for equal allocation to study groups and the study clinician determined a BCS during the baseline physical exam. Each dog received a study code number and both the owner and clinician were blinded to the assigned study arm. Dogs were transitioned on to the study diets over a 4 d period. Blood, urine, and feces were collected at baseline, d 14, and d 28 of the study. A 96 h pooled fecal sample was collected on d 15-19 of the study for digestibility analysis. On d 28, dogs were transitioned over a 4 d period back to their original diet.

Inclusion/Exclusion Criteria for Canine Participation

Male and female dogs between the ages of 2 and 7 yr, had BCS between 4 and 7 on a 9 point scale [214], and weighed at least 10 kg were eligible to participate. Of the 21 dogs recruited, 10 different known breeds and several mixed breeds were represented in the study and randomly distributed across treatment groups (Table 9). Dogs were excluded if they had any reported dietary allergies, intestinal sensitivities/discomforts, or prior history of cancer. Dogs must not have taken antibiotics or analgesics for at least 1 mo before starting the study. Heartworm prevention was allowed. Dog owners provided informed consent before their pet's enrollment at the Colorado State University Animal Cancer Center. Participants were required to be present for baseline, d 14, and d 28 study visits, provide a 96 h fecal sample collection after the dog(s) had consumed the study diets for 10 d, and record daily food intake and daily fecal scores for 28 d. Compliance to study protocol was determined by clinical trials coordinator with weekly phone calls to dog owners and during each study visit. Palatability and tolerance of the diet were determined using a pet health history survey that was completed by the owners on d 14 and d 28. Owners were asked about any incidence of vomiting, nausea, diarrhea, flatulence, and changes in physical activity, appetite and water intake as well as to report any apparent changes in behavior.

Table 9: Breeds of twenty-one canine dietary intervention study participants by diet group.

	Number of Dogs	
Breed[1]	**Control**	**Navy Bean**
Australian Cattle Dog	2	2
Dalmatian	-	1
Hound Mix	1	-
Mixed (unknown)	2	2
Pitbull Mix	2	-
Pointer	-	1
Retriever (Golden/Lab)	3	1
St. Bernard	1	-
Standard Poodle	-	1
Terrier/Terrier Mix	-	2
Total	**11**	**10**

[1]Breeds as reported by owners.

Canine Diet Formulations

Two canine diet formulations were used in this study that meet nutritional recommendations according to published feeding guidelines.[215] A formula similar to an AAFCO approved, commercially available adult canine diet formulation containing 27 % protein and 12 % crude fat was used for the 0 % bean, placebo control. The control diet was mixed and manufactured under the same conditions and locations as the experimental bean diet (ADM Alliance Nutrition Feed Research Pilot Plant, Quincy, IL & Applied Food Biotechnology Plant in St. Charles, MO). The bean diet was formulated to match the control diet in macronutrient and caloric content, except for the inclusion of 25 % cooked navy bean powder (ADM Edible Bean Specialties, Decatur, IL). Adjustment of major food ingredients, such as wheat and corn, were made to account for differences in the contribution of cooked navy bean powders to macro and micronutrients and total caloric

Table 10: Ingredient and chemical composition of cooked navy bean powder and control diets fed to twenty-one healthy, adult dogs.

	Control	Navy bean
Ingredient, % (as-fed)		
Navy bean powder	-	25.00
Meat and bone meal	14.83	13.86
Brewer's rice	12.50	12.50
Corn	11.25	11.25
Corn gluten meal	14.24	9.35
Wheat middlings	14.50	8.27
Poultry fat	7.77	7.75
Poultry by product meal	6.50	6.50
Wheat grain	14.50	1.67
Beet pulp	1.00	1.00
Ground flaxseed	0.75	0.75
Salt	0.50	0.50
Brewer's yeast	0.50	0.50
Vitamin Premix[1]	0.50	0.50
MCaP	0.08	0.39
KCl	0.30	0.10
L-Lys•HCl	0.22	-
Met	-	0.07
ChCl	0.05	0.05
Analyzed composition (as-fed)		
DM, %	95.01	94.28
n-6 Fatty acids, %	2.26	2.02
n-6/n-3 fatty acid ratio	10.91	10.06
LA/ALA ratio	10.54	9.79
(LA + ARA)/(ALA + EPA + DHA)	10.7	9.93
AAFCO ME, kcal/kg	3,380	3,416
Analyzed composition (%, dry matter)		
Organic matter	91.37	91.83
Ash	8.63	8.17
Crude protein	31.15	29.91
Acid hydrolyzed fat	14.00	13.58
Crude fiber	2.95	3.18
Gross energy, kcal/kg	4,968	4,958

[1]ADM Alliance Nutrition, Quincy, IL. Provided per kilogram of navy bean diet: vitamin A, 7,500 IU; vitamin D, 750 IU; vitamin E, 93.75 IU; thiamine, 3.75 mg; riboflavin, 30 mg; pantothenic acid, 12 mg; niacin, 15 mg; pyridoxine, 1.875 mg; folic acid, 0.26 mg; vitamin B_{12}, 37.5 μg; choline, 1,700 mg; iron from ferrous sulfate, 282 mg; copper from copper sulfate, 15 mg; manganese from manganous oxide, 31 mg; zinc from zinc oxide, 187 mg; iodine from calcium iodate, 2 mg; selenium from sodium selenite, 0.7 mg. Provided per kilogram of control diet: vitamin A, 7,500 IU; vitamin D, 750 IU; vitamin E, 93.75 IU; thiamine, 3.75 mg; riboflavin, 30 mg; pantothenic acid, 12 mg; niacin, 15 mg; pyridoxine, 1.875 mg; folic acid, 0.26 mg; vitamin B_{12}, 37.5 μg; choline, 1,711 mg; iron from ferrous sulfate, 303 mg; copper from copper sulfate, 16 mg; manganese from manganous oxide, 39 mg; zinc from zinc oxide, 196 mg; iodine from calcium iodate, 2 mg; and selenium from sodium selenite, 0.7 mg. LA = linoleic acid, ALA = alpha linoleic acid, ARA = arachidonic acid,

contents. The fatty acid content of both diets was matched as well. Marine type long chain n-3 fatty acids were not present in either diet. The percentage of ingredients and chemical components are listed for each study diet in Table 10.

Canine Diet Intervention

Dog owners were instructed to feed only the research diet provided by study clinical coordinator for the entire study duration and to measure out a prescribed amount of food for canine consumption each day. The prescribed daily caloric consumption was determined by body weight and according to the dog's normal feeding habits (1 or 2 feedings daily). Water was provided *ad libitum*. The total required daily caloric intake for maintenance of each dog was calculated at the baseline study visit using the following formula: daily ME requirement (kcal) = 110 x BW $^{0.75}$, where BW is measured in kg.[216] This formula was used to maintain a stable weight in dogs for the study duration. An inappropriate weight change was defined by a change in BW of 2 % per wk or 4 % change from each visit (AAFCO, 2010). Owners measured and recorded the volume of food offered and refused. The total amount consumed was calculated by subtracting the weight of the refused food from offered food. The owner completed a daily intake record for 28 d and a space was provided to record any intake aside from research diet that may impact study results.

Blood and Urine Sample Collection

Non-fasted blood samples were collected via jugular puncture at baseline, d 14, and d 28 of the study. At each visit approximately 18 mL of blood was collected; 1 mL of whole blood was collected into an evacuated red top tube without anticoagulant for biochemistry panel analysis. Another 1 mL of blood was collected into an evacuated lavender top tube containing EDTA for complete blood counts (**CBC**), hemoglobin (**HGB**), and hematocrit determination.

The additional blood was used to isolate peripheral blood mononuclear cells and serum for future analyses. Urine samples were usually collected by the owner at home using provided specimen containers. In some cases when the owner was unable to obtain a urine sample, ultrasound guided cystocentesis was used.

Blood and Urine Analysis

The Clinical Pathology Laboratory at Colorado State University performed all blood and urine analyses. The biochemistry panel was analyzed using a clinical chemistry analyzer (Hitachi 917; Roche Diagnostics, Indianapolis, IN) and CBC was detected using an analyzer (Advia 120; Bayer, Tarrytown, NY). Urinalysis was measured using standardized clinical laboratory procedures. Color and clarity were assessed visually, specific gravity was determined using a refractometer with water as a reference. A chemstrip (Cobas Chemstrip 10 MD; Roche Diagnostics, Indianapolis, IN) was used to determine pH, protein, glucose, ketones, bilirubin, and blood concentrations. Any samples positive for protein were further analyzed with the sulfosalicylic acid turbidometric test (Exton's Method). Microscopic analysis of the urine sediment was used to determine and quantify cellular components, crystals, casts, and bacteria. All methods used have been previously described.[217]

Fecal Scores and Sample Collection

Owners reported daily fecal scores using a 3-point scale with 1 = well formed, 2 = soft, and 3 = runny. A comment space was provided on the score sheet to obtain any observational changes per owner's discretion. A 4 d (96 h) total fecal collection was performed for the measurement of apparent total tract macronutrient digestibility after 10 d of consuming 100 % of the investigational (placebo control or bean containing) diets. Samples were collected daily and

stored at -20 °C. At the end of the collection period, the samples were weighed, pooled, and stored at -20 °C and then freeze-dried prior to proximate analysis.

Proximate Analyses for Assessing Apparent Total Tract Nutrient Digestibility

Proximate analysis of both the research diets and the 96 h pooled fecal samples were performed accordingly: Method 935.29 for DM, 920.39, for fat, 942.05 for ash, and 990.03 for CP.[218] Organic matter was calculated by subtracting ash from DM or 100 %. Gross energy was measured using an oxygen bomb calorimeter (Model 1261; Parr Instruments, Moline, IL). Crude fiber content was determined (Crude Fiber Method; ANKOM Technology, Macedon, NY) and study samples were coded and blinded for proximate analyses ADM Alliance Nutrition Laboratory, Quincy, IL). The lipid profile of the navy bean and control diets was determined according to previously reported protocols.[219] Digestibility of protein, fat, OM, and DM were calculated by the following formula where nutrients were measured in grams on a DM basis: Nutrient digestibility (%) = [(nutrient intake – nutrient in feces)/ nutrient intake] × 100. Metabolizable energy was calculated by the following formula: ME (kcal/kg of food) = {GE of food consumed – GE of feces collected – [(g of protein consumed – g protein in feces) × correction factor for energy lost in urine]}/grams of food consumed × 1,000. [215] Feed and fecal values on a DM basis were used in all calculations and the correction factor for energy lost in urine was 1.25 kcal/g.[215]

Statistical Analysis

Data are presented as means and SEM. Non-paired *t*-test probabilities were used to determine differences in digestibility, nutrient intake, ME, fecal output, age, and weight means between the 2 diet groups. Blood results were analyzed in both diet groups and across time points using repeated measures ANOVA. Within each time point, outliers were detected by a

Grubbs test. Fisher's Exact Test was applied for assessing differences in BCS and sex between diet groups. A probability of $P < 0.05$ was accepted as statistically significant. Statistical analyses were performed using a software package (GraphPad Prism version 5.03 for Windows; GraphPad Software, San Diego, CA).

RESULTS

Canine Participant Characteristics

A diverse set of breed participants were recruited for this study to provide broad representation of the canine population. Table 11 shows the mean and SEM of age in years, weight in kilograms, median BCS, and sex by study arm of the 21 dogs who participated in the study. No differences were determined at baseline across treatments.

Table 11: Comparison of age, weight, body condition scores, and sex of twenty-one canine dietary intervention participants by diet.

Characteristic	Navy bean[1]	Control[2]	*P*-value
Age, yr	4.1 ± 0.53	3.2 ± 0.35	0.17
Weight, kg	23.4 ± 1.47	28.2 ± 3.30	0.21
BCS[3], number of dogs			1.0
4 and 5	8	9	
6 and 7	2	2	
Sex[4], number of dogs			0.67
Female	6	5	
Male	4	6	

Data are reported as mean ± SEM. [1]n = 10; [2] n = 11. [3]Purina 9-point scale where BCS 4 and 5 are ideal and 6 and 7 are slightly overweight (Laflamme, 1997). [4]All female participants were spayed and all but 1 male in the control group was castrated. For age and weight, *P*-values were determined using a non-paired *t*-test. For count data, *P*-values were determined using a Fisher's Exact test. Differences were considered significant when $P < 0.05$.

Peripheral Blood Outcome Measures

We evaluated the safety of 25 % (wt/wt) navy bean powder dietary intake compared to a 0 % bean, placebo control canine diet. No changes in blood diagnostic test results including HGB, packed cell volume (**PCV**), serum albumin concentrations, and serum alkaline phosphate activities (**ALP**) were detected across treatment groups. Table 12 shows the average serum levels of these characteristics for both groups at baseline, d 14, and d 28 with the laboratory reported normal ranges. Average values for the navy bean and control diet groups on d 28, respectively,

were 17.3 and 18.3 for HGB; 49 and 53 for PCV; 3.8 and 4.0 for serum albumin; and 37 and 44 for ALP. In addition to these blood characteristic measurements required for assessing the nutritional adequacy of diets [122], complete blood cell count and biochemistry profiles were conducted to determine safety. Measured characteristics included: glucose, blood urea nitrogen, creatinine, phosphorus, calcium, magnesium, total protein, globulin, albumin to globulin ratio, cholesterol, total bilirubin, creatinine kinase, alanine aminotransferase, aspartate transaminase, gamma-glutamyl transpeptidase, Na, K, Cl, lipemia, and hemolysis.

No adverse changes were detected in any of the laboratory values examined between the treatment groups. The characteristics of the CBC and biochemistry panel were determined to be within normal ranges (data not shown).

Table 12: Assessment of blood characteristics of twenty-one clinically healthy adult dogs consuming a control or navy-bean based diet for 28 days.

Analyte (Reference Range)	Control	Navy Bean	p- value
Hemoglobin (13 – 20 g/dL)			0.15
Baseline	17.8 ± 0.34	17.4 ± 0.42	
Day 14	18.0 ± 0.35	17.3 ± 0.67	
Day 28	18.3 ± 0.42	17.3 ± 0.32	
Packed cell volume (40 – 55 %)			0.13
Baseline	51 ± 0.81	51 ± 1.12	
Day 14	52 ± 1.02	50 ± 1.00	
Day 28	53 ± 1.28	49 ± 0.82	
Albumin (2.5 – 4.0 mg/dL)			0.16
Baseline	3.9 ± 0.08	3.8 ± 0.08	
Day 14	4.0 ± 0.08	3.8 ± 0.07	
Day 28	4.0 ± 0.07	3.8 ± 0.06	
Serum alkaline phosphatase (20 – 142 IU/L)			0.39
Baseline	55 ± 11.7	43 ± 6.30	
Day 14	46 ± 8.63	37 ± 5.00	
Day 28	44 ± 8.63	37 ± 5.27	

Data are reported as mean ± SEM. Control, n = 10; Navy Bean, n = 11. Reference ranges used at the Diagnostic Medicine Center, Colorado State University.

Furthermore, no laboratory values were changed or outside of the normal range in any of the dogs from baseline. In one control group participant, ALP levels were chronically elevated above the normal range; however, there was no significant change over baseline and the absence of any other abnormalities or clinical signs supported continued study participation. Inclusion of this subject elevated the mean ALP value of the control group; this result was determined to be an outlier and was, therefore, excluded from the analysis.

Urinalysis Results

A urinalysis, as an additional safety measure, was applied herein given the potential for beans at 25 % (wt/wt) of diet to modulate overall metabolic status as well as liver and kidney metabolism. Urinalysis was conducted at baseline, d 14, and d 28 post intervention in all participants. All laboratory values assessed, including specific gravity, protein, bilirubin, ketones, blood, and crystal formation were determined to be within normal ranges and no differences were observed between groups (data not shown). An intriguing trend was observed for urine pH such that the bean diet group had an average pH at baseline of 6.5 that decreased after d 14 to 5.5, and then normalized to an average baseline value of 6.5 at d 28. The control group demonstrated an opposite trend whereby the average urine pH was 6 at baseline, increased to a pH of 7 at d 14, and then returned to urine pH of 6 at d 28. There was no observed clinical or statistical significance for this transient response in urine pH between the bean diet and control diet groups because both groups normalize at d 28 and there were no clinical changes in urate lithogenesis. These findings were reported herein as the experimental relevance of the acidic, yet transient urinary response to bean consumption may be important for future clinical dietary bean investigations.

Nutrient Intake, Body Weights, Digestibility, Metabolizable Energy, and Fecal Characteristics

No differences in macronutrient intakes, apparent total tract digestibility, fecal characteristics, or ME were observed between the bean and control diet groups, as shown in Table 13. Protein, fat, and OM intakes are reported on a DM basis. Average DMI: 325.70 g/d and 336 g/d; CP intake: 97.42 g/d and 104.7 g/d; fat intake: 44.23 g/d and 45.83 g/d; and OM intake: 299.10 g/d and 307.2 g/d, for the navy bean and control diets, respectively. Average bean powder intake per kg of BW was 3.7 g/d. Total tract apparent digestibilities were 68.58 % &

68.89 % DM, 78.22 % & 79.49 % CP, 77.57 % & 74.91 % OM, 94.49 % & 93.85 % acid hydrolyzed fat, and 3,313 kcal/kg & 3,195 kcal/kg ME, for the navy bean diet and control diet groups, respectively. No differences were observed between the groups. The total amount of fecal matter and fecal quality scores did not change between the 2 groups. Fecal output average was 96.07 g/d and 103.7 g/d for the bean diet and control diets, respectively. Owners most frequently reported well-formed stools. Two dogs, 1 from each group increased weight by 4 % on d 14 and the prescribed amount of food was reduced by 37.5 g/d for the dog consuming the bean diet and 32.6 g/d for the dog consuming control diet. All other dogs maintained weight throughout the study duration. Owners reported a minimal incidence of food intake outside of the provided diet; however these occurrences were equally distributed across groups. Isolated incidences of vomiting and diarrhea were reported in both groups as unrelated to diet by owner. None of the owners reported increased incidence of flatulence and both diets were reported as equally palatable to all study participants based on no differences detected in reported dietary intake or eating preferences as determined and reported by dog owners.

Table 13: Nutrient intakes, apparent total tract digestibility, metabolizable energy, and fecal characteristics of 21 dogs fed either a 25 % navy bean or isocaloric, nutrient matched control diet.

Nutrient/Characteristic	Control	Navy Bean	*P*-value
Daily Nutrient Intake (dry matter basis)			
Dry matter, g	336.3 ± 30.60	325.7 ± 19.10	0.78
Crude protein, g	104.7 ± 9.53	97.42 ± 5.71	0.53
Acid hydrolyzed fat, g	45.83 ± 4.21	44.23 ± 2.59	0.76
Organic matter, g	307.2 ± 27.96	299.1 ± 17.54	0.81
Apparent Total Tract Digestibility, %			
Dry matter	68.89 ± 5.08	68.58 ± 5.60	0.96
Crude protein	79.49 ± 3.52	78.22 ± 3.90	0.81
Acid hydrolyzed fat	93.85 ± 1.17	94.49 ± 1.05	0.69
Organic matter	74.91 ± 3.30	77.57 ± 3.81	0.60
Metabolizable energy, kcal/kg	3,195 ± 150	3,313 ± 164	0.60
Fecal characteristics			
Fecal output, g/d	103.7 ± 15.75	96.07 ± 14.38	0.72
Fecal score	1	1	

Data are shown as mean ± SEM. Control, n=11, Navy Bean, n=10. Fecal samples were scored according to the following system: 1 = well formed, 2 = soft, and 3 = runny.

DISCUSSION

The results reported herein demonstrate that cooked navy bean powder incorporated at 25 % (wt/wt) in an extruded dog diet is safe, palatable, and a digestible source of carbohydrates, protein, and fat for healthy, adult canines. Digestibility of selected commercial dry canine diets have been reported between 73.2 % and 84.5 % for DM, 77.2 % and 87.8 % for CP, 88.1 % and 97.1 % for fat, and 72.5 % for OM in both dogs and other model systems. [220; 221] Navy bean diet apparent total tract digestibility met these values. Compelling findings of no changes in any of the blood and urine characteristics tested further substantiates safety in systemic markers of canine wellness after consumption. Of particular note in the urinalysis results was the lack of clinically relevant changes in crystal formation in any of the individual dogs consuming the navy bean diet, in spite of the transient change in urine pH, as legume consumption is contraindicated for dogs at risk for urolithiasis (Hand, 2000). These results in healthy canines provide a strong basis for future nutritional investigations of cooked bean powder intake in companion animals with chronic diseases, as beans are gaining widespread popularity for dietary disease prevention strategies in humans.[32; 67; 210; 222; 223]

In addition to the novelty of including beans as a staple food ingredient for dogs, it is possible that beans may serve as a higher quality source of protein and fiber, provide a low glycemic index food, and deliver unique bioactive compounds for enhanced canine nutrition. Navy beans contain essential amino acids and may have improved bioavailability when compared to other plant protein sources. They may also provide less fat when compared to animal protein sources.[223] Bean fiber was shown to be readily metabolized by human intestinal flora into short chain fatty acids in an *ex vivo* incubation model with fecal samples.[224] Canine intestinal microflorae were also modifiable by dietary fiber to change short chain fatty acid

profiles. [225; 226] Diets high in fiber and protein have been shown to improve satiety [213; 227] and low glycemic index starch has been shown to alter lipid profiles [228] in dogs during weight loss.

Rodent and human studies have shown that increased dietary bean intake inhibits tumor formation [229], controls and manages diabetic outcomes [230; 231], improves blood lipids for reduced risk of cardiovascular disease [232], and enhances intestinal health.[233] Epidemiological studies suggest that legume consumption may be an important dietary predictor of longevity in humans and model organisms.[234-236] These chronic disease-fighting properties for increasing bean consumption reported from studies in humans and rodents merit investigation in companion canines.

Dry beans may not have been incorporated into commercial canine diet formulations because of perceptions that increased cooked bean intake would result in increased flatulence, intestinal distress, or be unpalatable to dogs. The lack of change in fecal characteristics or flatulence between the study groups was a compelling finding from this trial given the relatively high concentration of navy beans at 25 % (wt/wt). The lack of change in flatulence from canine bean consumption supports the experimental strengths and advantages of conducting double blinded, placebo controlled nutrition intervention studies in free-living animals.

The antinutrient bean components may be another reason why beans have not been utilized in commercial canine diet formulations. Bean antinutrients are primarily found in raw, uncooked beans.[237] Cooking significantly reduces antinutrient levels (e.g. amylase inhibitors, trypsin inhibitors, and the lectin protein phytohemagglutinin), and utilization of cooked bean powders further reduces potential for deleterious effects.[238; 239] Utilizing a commercial source of cooked navy bean powder and incorporation into a dry expanded product reduces the potential for canine antinutrient ingestion.

In support of the safety and digestibility of beans reported herein, the effects of beans for canine chronic disease control and prevention merits further exploration. We judge that the incorporation of 25 % (wt/wt) bean powder is a practical starting dose for achieving results from functional food attributes and that further digestibility testing may be required to safely increase the quantity of beans in canine diets. It should be noted that for dogs with a high risk for urolithiasis, inclusion of legumes may be inappropriate as legumes are relatively high in purines, protein, calcium, and magnesium and may therefore aggravate symptoms.[205] In conclusion, canine diets containing cooked navy bean powder are safe, digestible, palatable, and demonstrate promise to become a novel food ingredient for dogs. This study sets the foundation for future canine nutritional intervention studies with cooked bean powders and for a variety of disease conditions.

CHAPTER 5: NAVY AND BLACK BEAN-BASED DOG FOODS ARE DIGESTIBLE DURING WEIGHT LOSS IN OVERWEIGHT AND OBESE ADULT COMPANION DOGS[5]

INTRODUCTION

Obesity is the primary nutritional disorder in companion dogs.[240] Recent surveys estimate that 34-59 % of pet dogs in the United States, Europe and China are overweight or obese.[5; 6; 241] Overweight dogs may have shorter, reduced-quality of life[8; 240; 241] and increased risk for developing chronic diseases such as diabetes, cardiovascular and respiratory disease, urinary tract infections, pancreatitis, osteoarthritis, and some types of cancer.[3; 242]

For clinically healthy dogs, the primary treatment for obesity is nutritional therapy.[243] Because excess adiposity is directly related to a positive energy balance, the most practical dietary approach for weight loss is caloric restriction. An adequate weight loss diet has a nutrient composition that supports lean mass retention, induces fat mass reduction, and increases satiety.[241] Diets high in protein and fibre have been shown in both humans and dogs to promote weight loss and maintain lean muscle mass[62; 244], as well as reduce voluntary food intake in dogs.[245] Emerging research supports that in addition to macronutrients, there are specific foods and dietary patterns that may promote weight loss as a function of bioactive components and phytochemicals.[246-248] For example, in people, consumption of non-soy legumes such as common beans (*Phaseolus vulgaris,* L.), split peas, lentils, and chickpeas, is associated with decreased risk for obesity[32], reduced adiposity without caloric restriction[249], voluntary reduction of caloric

[5] This chapter has been accepted for publication to *Journal of Applied Animal Nutrition.* The authors are Genevieve M. Forster, Nora Jean Nealon, Dale Hill, Tracey D. Jensen, Teva L. Stone, John E. Bauer, and Elizabeth P. Ryan. G.M.F and N.J.N. contributed equally to this manuscript. British spelling is used per journal requirements.

intake[250], increased satiety, and in some cases, resulted in higher levels of weight loss with 30 % caloric restriction compared to an isocaloric, low legume or legume-free diet intervention.[251]

Common beans, such as navy, black and pinto, are excellent candidates for a weight loss-promoting food because they contain high quality proteins, have a carbohydrate profile with a low glycemic index, are abundant in dietary fibre, and are rich sources of iron, zinc, folate and magnesium.[252] The high protein content and amino acid profiles of beans have been associated with increased energy expenditure during weight loss and the arginine and glutamine content in particular was associated with improved carbohydrate and fat oxidation.[76] The fibre fraction from beans is abundant in resistant starch, which can augment weight loss via slower carbohydrate digestion and increased microbial fermentation.[251; 253] Common beans also contain a wide range of bioactive phytochemicals such as alpha-amylase inhibitors, phenolic compounds, and phytosterols which may modulate excess nutrient absorption, reduce dietary energy availability, promote satiety, and improve lipid metabolism.[251; 254-256] Due to the fact that dry bean consumption promoted weight loss in humans and rodents, the potential of beans to promote weight loss in dogs merits investigation because dogs have similar digestive physiology, obesity related co-morbidities, and environmental exposures to people.

Common beans are safe and digestible in normal, healthy weight dogs.[257] Bean-based diet formulations support short-term apparent weight loss, and were reported effective at reducing low density lipoprotein (LDL), high density lipoprotein (HDL), and triglycerides (TG) when compared to a control, no bean diet.[258] Therefore, the objectives of this current study were to: 1) evaluate the apparent total tract digestibility (ATTD) of nutritionally complete, navy (NB) and black (BB) bean diets in overweight or obese dogs undergoing calorically restricted weight loss and 2) determine the nutritional adequacy and utilisation bean-based diets compared to a no-

bean, nutrient-matched control (CON) diet using the outcome measurements defined by the Association of American Feed Control Officials (AAFCO)[122] compared to an isocaloric, nutrient matched, standard ingredient, control diet. We hypothesise that cooked bean powders added at 25 % weight/weight (w/w) into a nutritionally complete extruded dog food formulation will be digestible, support weight loss, and maintain indices of nutritional adequacy as compared to no-bean control diet.

MATERIALS AND METHODS

Study Design

The four-week short-term and twenty-six-week long-term studies were prospective, randomised, double-blinded, controlled, three-arm, dietary intervention clinical trials for calorically-restricted weight loss. The three study groups were CON, NB, or BB. The short-term weight loss study was conducted at the Colorado State University Veterinary Teaching Hospital (Fort Collins, CO) and the long-term study was conducted at the Wellington Veterinary Hospital (Wellington, CO). Owners signed an informed consent form and provided a medical history before dogs were enrolled in the study. All dogs were transitioned to the study-provided diet (CON, NB, BB) over a 4-day period by increasing the proportion of the study-provided food mixed into the dog's regular food as previously described.[257] At the end of the study period, all dogs were transitioned back to their regular food. Owners were instructed to exclusively feed the study kibble in the amounts prescribed. For the short-term study, owners were given pre-measured daily packets of food, and in the long-term study owners were given measuring cups with lines marked to indicate the appropriate amount of kibble to feed daily. All owners were

instructed to feed only the prescribed dog food for the duration of the study. Water was provided *ad libitum* and no treats were allowed.

Body weights were assessed bi-weekly and caloric intake was adjusted as needed to achieve a target weight loss of 0.5 % - 2 % body weight per week. For the short-term study (n=30), a 96-hour faecal collection was performed at 2 weeks, after the dogs had been exclusively consuming the study food for 10 days. For the long-term study (n=15) a 96-hour faecal collection was performed at 12 weeks. Owners were instructed to collect the canine faecal samples within 5 hours of being voided. Samples were frozen and stored at -20° C until analysis. Compliance to the study protocol was determined by owner surveys, diet logs (short-term study only), number of faecal samples collected, and apparent weight loss.

Eligibility Criteria for Companion Dog Participants

The Colorado State University Institutional Animal Care and Use Committee approved all clinical trial operations, animal care procedures, and collection of biological samples for analysis before beginning the study (IACUC 13-4316A).

Adult, male and female dogs between the ages of 2-7 years, with a body condition score (BCS) of at least 6 on a 9-point scale [191] and body weight of at least 10 kg, with no known health concerns were recruited for study participation. All dog owners provided written informed consent for participation. After enrollment, all dogs were evaluated by the study veterinarian, assessed for hematologic and biochemical anomalies, assigned a BCS, and screened for hypothyroidism with a total thyroxine (T4) test as previously described.[258] Dogs were excluded from participation for hypothyroidism, abnormal blood results (unless determined by the veterinarian to be within normal limits for a specific dog), or a history or diagnosis of cancer, inflammatory disease, or current infection. Dogs were also excluded if they had been

administered antibiotics or analgesics within 1 month of starting the study. The preventive use of anthelmintics was allowed. Dogs could be removed from the study at the discretion of the study veterinarian or request of owner. All dogs were monitored throughout the study for adverse changes in clinical blood, serum, or plasma analytes. At the end of each study period, hemoglobin, packed cell volume (PCV), albumin, and alkaline phosphatase (ALP) were compared to the AAFCO reference ranges for nutritionally adequacy.[122] One dog (short-term study, CON) had chronically-elevated ALP (830 g/dl at baseline that decreased to 320 g/dl at 4 weeks) and participated at the discretion of the attending veterinarian. Hemoglobin and PCV values were not obtained from one dog at the end of study (long-term study, BB) due to a clotted blood sample post-collection.

Fifty-six dogs were screened for participation in the short term or long term weight loss study and 49 were enrolled. Seven dogs failed the pre-screen exam for either renal or hepatic abnormalities (n=2), detection of previously undiagnosed cancer (n=2), hypothyroidism (n=1), urinary tract infection (n=1), or aggression and difficult handling (n=1). Thirty-three dogs were enrolled in the short-term study and randomised based on BCS to CON, BB, or NB study groups. Three dogs were withdrawn due to physical injury (n=1), owner unable to keep study-related appointments (n=1), and not consuming the study provided dog food (n=1).[258] Sixteen dogs were enrolled in the long-term study and randomised based on BCS to the CON, BB, or NB study groups. One dog was withdrawn from the long-term study after diagnosis with tapeworm and the owner's non-compliance to study protocol by feeding dog treats. Individual characteristics of each dog are presented in Appendix 1and summaries of the baseline characteristics are shown in Table 14. Breeds included dogs from retriever, terrier, herding, and working lineages, and spanned both purebred and mixed breeds. Dogs were equally distributed

between study diet groups for age, weight, sex, and BCS. There was one dog in each of the short-term and long-term studies that was not neutered. One 8-year-old dog was included in the short-term study and one 10-year-old dog was included in the long-term study at the discretion of the study veterinarian.

Table 14: Baseline characteristics of dogs completing cooked bean powder-based calorically restricted weight loss study interventions.

	Control Group		**Black Bean Group**		**Navy Bean Group**		
Characteristic	*Median (IQR)*		*Median (IQR)*		*Median (IQR)*		***p-value[4]***
Age[1], yr							0.16
Short-term	6.0 (4.7-7.0)		5.0 (2.8-5.3)		4.5 (3.5-6.0)		
Long-term	3.0 (2.0-8.5)		3.0 (3.0-4.5)		6.0 (4.5-7.0)		
Body weight, kg							0.25
Short-term	34.5 (20.7-39.6)		28.8 (16.5-34.1)		29.25 (20.4-38.1)		
Long-term	36.5 (29.2-40.4)		37.7 (26.7-45.7)		39.5 (27.3-56.1)		
			Number of Dogs				
Sex[2]	*Female*	*Male*	*Female*	*Male*	*Female*	*Male*	0.61
Short-term	7	3	6	4	4	6	
Long-term	3	2	4	1	2	3	
BCS[3]	*BCS 6-7*	*BCS 8-9*	*BCS 6-7*	*BCS 8-9*	*BCS 6-7*	*BCS 8-9*	0.33
Short-term	7	3	4	6	7	3	
Long-term	2	3	2	3	1	4	

Thirty dogs completed the short-term, 4 week study: Control Diet, N=10; Black Bean Diet, N=10; Navy Bean Diet, N=10. Fifteen dogs completed the long-term, 6 month study: Control Diet, N=5; Black Bean Diet, N=5; Navy Bean Diet, N=5.[1]Age as reported by owner.[2]All dogs were neutered with the exception of one female in the short-term study control group and 1 female in the long-term study black bean group. [3]Body Condition Score (BCS) was determined using a 9 point scale (Laflamme, 1997).[4]Continuous variables (age and weight) were evaluated for differences across groups using a Kruskal-Wallis test and categorical variables (sex and BCS) were evaluated using a Chi-square test. $P < 0.05$ was considered significant.

Dietary Formulations

CON, BB, and NB dietary study groups were provided a dry, extruded, pelleted dog food that was formulated to meet nutritional recommendations for adult dog maintenance [122; 243] and adjusted to consist of 27 % protein and 8 % fat. The CON, BB, and NB diets were mixed and manufactured under the same conditions and location (ADM Alliance Nutrition Feed Research Pilot Plant, Quincy, IL; Applied Food Biotechnology Plant, St. Charles, MO) and formulated to be isocaloric and nutrient matched. The CON diet ingredients consisted of poultry meal, wheat, corn, brewer's rice, pork and bone meal, flaxseed, fishmeal, brewer's yeast, and added vitamins

and minerals (Table 15). The BB and NB diets contained identical ingredients as the CON diet with the inclusion of cooked BB or NB bean powder (ADM Bean Specialties, Decatur, IL) added at 25 % w/w to the BB and NB diets. To account for the inclusion of the cooked bean powders, the wheat and corn ingredients were reduced to achieve iso-nutrient formulations to the CON diet. The metabolisable energy (ME) of the diets was calculated using modified Atwater Factors and estimated at 3,314 kcal/kg.[243]

Table 15: Diet ingredients and chemical composition of control, black, and navy bean-based dog foods fed to overweight and obese dogs during calorically restricted weight loss.

Ingredient, % (as-fed)	Control Diet	Black Bean Diet	Navy Bean Diet
black bean (cooked powder)	-	25.00	-
cooked navy bean powder	-	-	25.00
poultry meal	19.53	19.00	19.61
wheat grain	19.00	2.66	3.62
wheat middlings	19.00	11.61	9.42
corn grain	16.11	17.67	19.00
brewer's rice	10.00	10.00	10.17
pork and bone meal	7.32	3.95	2.56
poultry fat	3.00	3.00	3.00
flaxseed	1.00	1.00	1.00
fish meal	1.00	1.00	1.00
brewer's yeast	1.00	1.00	1.00
digest	1.00	1.00	1.00
calcium carbonate	0.80	1.28	1.47
salt	0.50	0.50	0.50
vitamin-trace mineral premix [a, b, c, d]	0.50	0.50	0.50
potassium chloride	0.14	0.05	0.05
choline chloride	0.10	0.10	0.10
monocalcium phosphate	-	0.68	1.00
Analysed Composition, % (as-fed)			
dry matter	95.02	95.59	94.96
moisture	4.98	4.41	5.04
crude protein	26.60	26.90	26.30
nitrogen free extract	47.82	47.99	48.96
acid hydrolyzed fat	8.40	8.10	8.00
crude fibre	3.90	4.30	3.70
total dietary fibre	16.98	17.96	18.65
soluble fibre	4.05	3.36	5.25
insoluble fibre	12.93	14.60	13.40
ash	8.30	8.30	8.00
Gross energy, kcal/kg	4,505	4,371	4,375
Est. metabolisable energy, kcal/kg	3,314	3,314	3,314

a. Provided per kilogram of control, black bean, and navy bean diets: vitamin A, 7,500 IU; vitamin D, 750 IU; vitamin E, 93.75 IU; thiamine 3.75 mg; riboflavin, 30 mg; pantothenic acid, 12 mg; niacin, 15 mg; pyridoxine, 1.88 mg; folic acid, 0.26 mg; vitamin B12, 37.5 µg; choline, 534.4 mg; Fe from ferrous sulfate, 282 mg; Cu from copper sulfate, 15 mg; I from calcium iodate, 2.025 mg. b. Manganese from manganous oxide provided per kilogram: 10.125 mg (control, black bean), 32.01 mg (navy bean). c. Zinc from zinc oxide provided per kilogram: 213.068 mg (control), 150 mg (black bean), 198.02 mg (navy bean). d. Selenium from sodium selenite provided per kilogram: 0.6463 mg (control), 0.2250 mg (black bean, navy bean).

Calculations for Energy Requirements and Caloric Restriction

Body condition scoring (BCS) was used to estimate ideal bodyweight (BW), and determined using a 9-point scale.[191] A score of less than 4 was considered underweight, a score of either 4 or 5 was considered ideal BW, a score of 6 or 7 was overweight, and a score of 8 or 9 was considered obese.[258] For each point over 5, a dog was considered to be 10 % above his or her ideal body weight in kilograms.[192] Using ideal weights determined by BCS, daily ME requirements for weight maintenance were calculated for each dog using the following formula: ME (kcal/day) = 110 x (ideal BW, kg)$^{0.75}$.[243; 257] Dogs were calorically restricted to approximately 60 % of their maintenance energy requirement. Apparent BW (kg) was measured at least every two weeks throughout each study.

Proximate Analysis, Apparent Total Tract Digestibility (ATTD), and Bomb Calorimetry

Proximate analysis was used to determine the crude nutrient profiles of the food and faecal samples as previously reported.[257] Soluble and insoluble fibre fractions were determined as previously described.[259] ATTD were evaluated at two weeks for the short-term study, and at twelve weeks for the long-term study and were calculated for total dry matter (TDM), crude protein (CP), crude fat (CF), and nitrogen free extract (NFE). The following formula was used to determine NFE: NFE % = TDM % – CP % – CF % - crude fibre % – ash %. For each nutrient component, the ATTD was calculated on a dry matter (DM) basis using the following formula [122]: % ATTD = [(g of nutrient consumed – g of nutrient excreted) / (g of nutrient consumed)] × 100.

Total gross energy (GE) content was measured by bomb calorimetry for each diet, and in each faecal sample at two weeks for the short-term study and at twelve weeks for the long-term study. ME was determined at two weeks during the short-term study, and at twelve weeks during

the long-term study and reported in kcal/kg using the following formula[122]: ME (kcal/kg of food) = {GE of food consumed – GE of faeces collected – [(g of protein consumed – g protein in faeces) × 1.25]}/ grams of food consumed × 1,000, where GE was in kcal/g, 1.25 kcal/g was the correction factor for energy lost in urine, and both diet and faecal values were on a DM basis.

Dogs were excluded from the ATTD and ME analysis if owners reported dietary indiscretion during the faecal collection period, were unable to differentiate between samples from different dogs, or collected faecal samples for less than 3 days. CON group exclusions: short-term, n=4 and long-term, n=1; BB group exclusions: short-term, n=1 and long-term, n=1; and NB group exclusions: short-term, n=3 and long-term, n=0.

Statistical Analysis

Non-parametric analyses were performed on all measures. For percent apparent weight loss, a two-way ANOVA (repeated measures) was performed within each study. For ATTD, ME, and food intake/kg BW, a two-way ANOVA (non-repeated measures) was performed. Bonferroni post-hoc tests were applied to correct for multiple comparisons. Statistical analyses for all measurements were performed using GraphPad Prism, Version 5.03 (San Diego, CA). Significance was reported at a p-value of $p < 0.05$.

RESULTS AND DISCUSSION

Nutrient Profiles of Bean-Based Dog Foods

The primary objective of this study was to determine the digestibility of nutritionally complete, NB and BB based dog food when compared to a nutrient matched CON dog food in overweight or obese dogs undergoing calorically restricted weight loss. Proximate analysis and bomb calorimetry results confirmed that the CON, NB and BB dog food formulations were

nutrient matched and isocaloric (Table 15). On an as-fed basis, for all diets, the estimated ME content of the diets was 3,314 kcal/kg, CP content was approximately 26 %, and CF was 8 %. Crude fibre was similar between the CON, BB, and NB diets (~4 %), while TDF was ~1 % higher in NB and BB diets when compared to CON. Compared to the CON diet, insoluble fibre was slightly increased in the BB diet (~1.5 %), while soluble fibre was slightly increased in the NB diet (~1 %).

Companion Dog Characteristics, Dietary Intake, and Apparent Weight Loss

Forty-five clinically healthy, adult, companion dogs, BCS classified as overweight or obese completed a short or long term calorically restricted weight loss trial and were assigned to either the NB, BB, or CON study groups. There was no difference in sex, median age, or BCS between dietary treatment groups or studies (Table 14). In both the short and long-term studies, percent apparent weight loss increased over time ($p < 0.0001$) and was similar between dietary treatments in each study; in the short-term study the median weight loss was 4.05 % in CON, 5.98 % in BB, and 6.14 % in NB; for the long-term study, the median weight loss was 17.90 % in CON, 14.0 % in BB, and 12.21 % in NB (Figure 9).

Daily nutrient intake (g/kg ideal BW) was not different between groups (Table 16) except for soluble fibre, which was significantly higher in NB group (~0.4 g/kg ideal BW) compared to BB (~0.25 g/kg ideal BW), but not different from CON (~0.3 g/kg ideal BW). All dogs consumed approximately 2.5 g CP per kg ideal BW (medians ranged from 2.1 g – 2.7 g/kg ideal BW), and dogs within the BB and NB group consumed, on average, 2 g cooked bean powder per kg ideal body weight (Table 16).

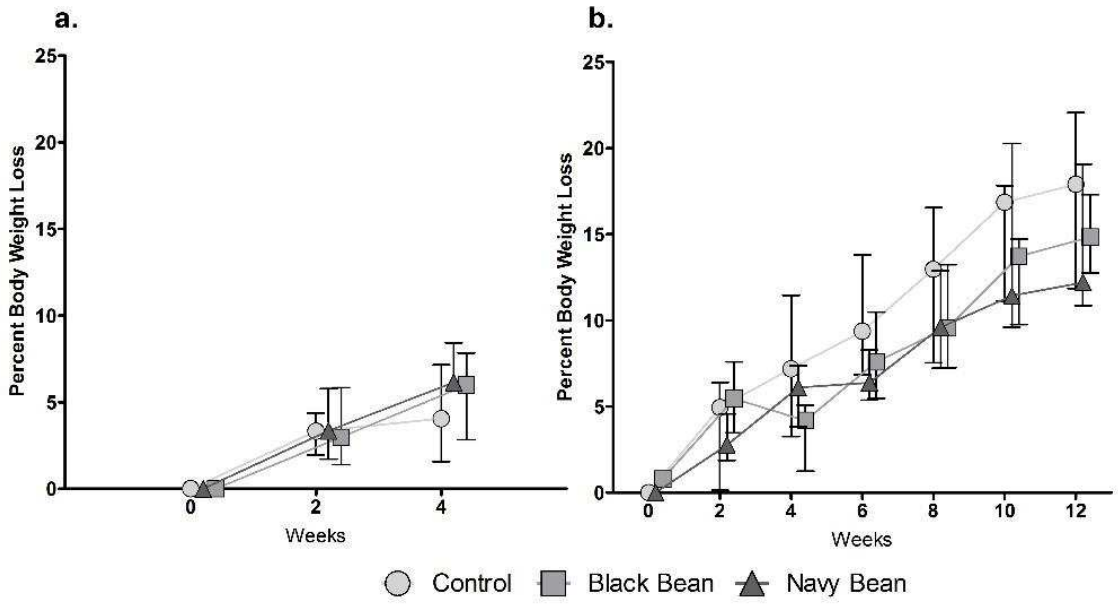

Figure 9: Percent apparent weight loss in dogs consuming a bean-based or control diet over (a) 4-weeks (short-term study, n =30) and (b) 12-weeks (long-term study, n = 15). In both (a) and (b) percent apparent weight loss increased over time ($p < 0.05$), but not between dietary treatments at any time point. Data are shown as median and IQR.

Table 16: Daily nutrient intake of forty-five overweight or obese adult, companion dogs undergoing calorically restricted weight loss on nutritionally complete bean-based or nutrient-matched control diets.

Daily Intake/kg ideal BW	Control Diet *Median (IQR)*	Black Bean Diet *Median (IQR)*	Navy Bean Diet *Median (IQR)*	***p-value***
Total Dietary Intake, g (*As-fed basis*)				
Short-Term	9.0 (8.5-9.7)	9.5 (8.8-10.2)	8.9 (8.6-10.3)	0.939
Long-Term	8.8 (8.2-10.1)	8.8 (8.5-9.0)	8.7 (8.6-9.1)	
ME (Estimated) Intake, Kcal (*DM Basis*)				
Short-Term	1.4 (1.4-1.7)	1.6 (1.5-2.0)	1.5 (1.5-1.8)	0.510
Long-Term	1.3 (1.3-1.6)	1.3 (1.2-1.5)	1.5 (1.3-1.6)	
Total DM Intake g/kg ideal BW				
Short-Term	8.7 (8.4-10.0)	9.5 (8.5-12.0)	8.5 (8.2-10.0)	0.900
Long-Term	8.0 (7.8-10.0)	7.4 (7.1-8.9)	8.3 (7.3-9.0)	
Crude Protein, g (*DM Basis*)				
Short-Term	2.4 (2.4-2.9)	2.7 (2.4-3.3)	2.4 (2.3-2.8)	0.808
Long-Term	2.2 (2.2-2.8)	2.1 (2-2.5)	2.3 (2.0-2.5)	
Crude Fat, g (*DM Basis*)				
Short-Term	0.8 (0.7-0.9)	0.8 (0.7-1.0)	0.7 (0.7-0.8)	0.729
Long-Term	0.7 (0.7-0.9)	0.6 (0.6-0.8)	0.7 (0.6-0.8)	
NFE, g (*DM Basis*)				
Short-Term	4.4 (4.2-5.2)	4.8 (4.3-5.9)	4.4 (4.2-5.2)	0.967
Long-Term	4.0 (3.9-5.1)	3.7 (3.6-4.5)	4.3 (3.8-4.7)	
Crude Fibre, g (*DM Basis*)				
Short-Term	0.4 (0.3-0.4)	0.4 (0.4-0.5)	0.3 (0.3-0.4)	0.087
Long-Term	0.3 (0.3-0.4)	0.3 (0.3-0.4)	0.3 (0.3-0.4)	
TDF, g (*DM Basis*)				
Short-Term	1.4 (1.4-1.7)	1.6 (1.5-2.0)	1.5 (1.5-1.8)	0.510
Long-Term	1.3 (1.3-1.6)	1.3 (1.2-1.5)	1.5 (1.3-1.6)	
Soluble Fibre, g (*DM Basis*)				
Short-Term	0.3 (0.3-0.4)^{ab}	0.3 (0.3-0.4)^{a}	0.4 (0.4-0.5)^{b}	< 0.001
Long-Term	0.3 (0.3-0.4)^{ab}	0.2 (0.2-0.3)^{a}	0.4 (0.4-0.4)^{b}	
Insoluble Fibre, g (*DM Basis*)				
Short-Term	1.1 (1.0-1.3)	1.3 (1.2-1.6)	1.1 (1.0-1.3)	0.133
Long-Term	1.0 (1.0-1.2)	1.0 (1.0-1.2)	1.1 (1.0-1.1)	
Cooked Bean Powder, g (*DM Basis*)				
Short-Term	0 (0-0)^{a}	2.4 (2.1-2.9)^{b}	2.1 (2.1-2.5)^{b}	< 0.001
Long-Term	0 (0-0)^{a}	1.8 (1.8-2.2)^{b}	2.1 (1.8-2.3)^{b}	

To determine differences in daily intake between diets and studies, each nutrient was evaluated with 2-way ANOVA. There were no differences between studies or interactions terms. A Bonferroni post-test was used to determine the groups with significant differences. Groups not sharing the same letter superscript are significantly different from each other.

Apparent Total Tract Digestibility and Metabolisable Energy of Black and Navy Bean-Based Dog Diets during Weight Loss

In the CON, BB, and NB diets, nutrient ATTD was consistent with expected ranges for standard ingredient and bean-based extruded dog diets.[257] TDM, CP, CF, and NFE ATTD are presented as median and range (min-max, Table 17). There were no differences in ATTD between each study group of the short and long-term study. In the short-term study, median TDM ATTD was higher ($p < 0.05$) for BB (83.0 %) than CON (74.6 %), while NB was similar to both (80.2 %); in the long-term study, TDM ATTD was similar for all three diets: CON (73.7 %), BB (79.6 %), and NB (77.5 %). For the NB diet, these results were consistent with previous studies demonstrating equal TDM ATTD compared to a nutrient matched CON diet.[257] To our knowledge, this is the first report of a BB based canine diet ATTD. To verify that TDM ATTD was higher for the BB diet compared to CON, we performed a pooled analysis on the results from both trials. The differences in TDM ATTD between CON and BB remained significant (data not shown), further supporting that that the BB TDM was indeed more digestible than CON and may have not been significant in the short-term study due to the sample size.

In the short-term study, CP ATTD was higher in BB (85.7 %) compared to CON (78.6 %), and NB was similar to both (83.5 %); in the long-term study, CP ATTD was similar between the CON (80.9 %), BB (82.5 %), and NB (79.4 %). Again, the difference in CP ATTD between CON and BB remained significant when data from both the long and short-term studies were pooled (data not shown). The CP digestibility of these cooked bean powder diets supports the use of common beans as a major ingredient in weight loss dog food formulas, particularly because dietary protein intake is an important contributing factor towards lean mass retention.[260] Results from this study support that BB may be particularly effective at contributing bioavailable proteins with higher digestibility than standard animal source ingredients, and future studies are

needed to determine if BB based diets differentially preserve lean muscle mass during weight loss.

CF was equally digestible between all diets in the short-term study: CON (89.8 %), BB (95.2 %), and NB (92.9 %); and the long-term study: CON (93.2 %), BB (94.1 %), and NB (89.4 %).

Carbohydrate ATTD, as measured by NFE, was higher in both the BB (89.3 %) and NB (87.6 %) diets compared to CON (83.2 %) in the short-term study, and similar between all diets in the long-term study: CON (82.1 %), BB (86.5 %), and NB (85.1 %). The NFE ATTD remained significantly higher in both bean groups when data from both the short and long-term studies were pooled (data not shown) supporting that the carbohydrates derived from the BB and NB were more digestible than those derived from corn and wheat based diets. Recent metabolomic studies support that metabolism of carbohydrates may be modulated in normal weight dogs consuming bean-based diets [94] even though carbohydrate digestibility was the same as the a control diet. [257] Therefore, the effects of bean intake on carbohydrate metabolism in dogs undergoing weight loss may be greater than for dogs maintaining weight. These data support the further investigation of carbohydrate metabolism in overweight and obese dogs consuming bean-based diets during weight loss.

ME was calculated for each group to determine the amount of utilisable energy provided by the CON, BB, and NB foods. Measured ME was similar between both the short and long-term studies and across all diets. Results are presented as a median (min-max, Table 17). The median ME for the CON diet was 3,446 kcal/kg for the short-term and 3,519 kcal/kg for the long-term study. In the BB diet, ME was 3,632 kcal/kg for the short-term and 3,507 kcal/kg for long-term study. In the NB diet, ME was 3,571 kcal/kg for the short-term and 3,434 kcal/kg for the long-

term study. ME was highest in the BB short-term study (3,632 kcal/kg) and this was the only measured ME that was higher than the estimated ME of 3,314 kcal/kg ($p = 0.004$). These data support that the energy utilisation from bean based dog food was equivalent to standard ingredient dog food formulations in dogs undergoing calorically restricted weight loss.

Table 17: Nutrient digestibility, metabolisable energy, and energy extraction efficiency of three nutritionally complete diets fed to overweight or obese adult companion dogs undergoing calorically restricted weight loss.

	Control Diet	Black Bean Diet	Navy Bean Diet	
Digestibility, %	*Median (IQR)*	*Median (IQR)*	*Median (IQR)*	***p-value***
Total dry matter %				
Short-term	74.6 (67.0-80.7)[a]	83.0 (75.8-89.0)[b]	80.2 (66.8-83.5)[ab]	0.015
Long-term	73.7 (69.9-79.1)	79.6 (76.9-83.7)	77.5 (71.8-88.1)	
Crude protein				
Short-term	78.6 (73.1-84.6)[a]	85.7 (80.4-91.4)[b]	83.5 (78.7-87.4)[ab]	0.040
Long-term	80.9 (78.5-84.3)	82.5 (81.8-85.9)	79.4 (77.0-91.4)	
Crude fat				
Short-term	89.8 (88.7-92.9)	95.2 (88.5-97.7)	92.9 (77. 3-96.4)	0.120
Long-term	93.2 (90.7-93.5)	94.1 (90.5-96.1)	89.4 (87.1-95.9)	
Nitrogen free extract				
Short-term	83.2 (76.9-86.8)[a]	89.3 (82.3-91.9)[b]	87.6 (84.1-89.8)[b]	0.002
Long-term	82.1 (77.7-85.4)	86.5 (83.9-89.5)	85.1 (83.5-91.6)	
Metabolisable energy, kcal/kg				
Short-term	3,446 (3,188-3,674)	3,632 (3,348-3,804)	3,571 (3,235-3,689)	0.617
Long-term	3,519 (3,271-3,670)	3,507 (3,367-3.618)	3,434 (3,328-3,844)	

Digestibility was calculated on a DM basis. To determine differences in digestibilities between diets and studies, each nutrient was evaluated with 2-way ANOVA. There were no differences between studies or interactions terms (data not shown). A Bonferroni post-test was used to determine the groups with significant differences. Groups not sharing the same letter superscript are significantly different from each other.

Whole Blood Analyses and Serum Biochemistry Reveal Safety of Bean Diets During Companion Dog Weight Loss.

To determine if dogs consuming the bean-based foods maintained indices of nutritional adequacy, comprehensive blood cell counts and serum biochemical analyses were performed for every dog at baseline and throughout the study as previously described.[257; 258] No negative physiological effects were observed in any analyte measured (data not shown). To demonstrate the nutritional adequacy of the dog food formulations, each dog's results were compared to

AAFCO reference limits or hemoglobin, PCV, albumin, and ALP at the end of the study period. Results are presented as a median (min-max) along with the AAFCO limits for each analyte (Table 18). The median albumin for the CON group was 3.9 g/dL and 3.9 g/dL; 3.9 g/dL and 4.0 g/dL for the BB group: and 3.9 g/dL and 4.1 g/dL for the NB group, for the short and long-term studies respectively. The median ALP for the CON group was 40.5 IU/L and 41.0 IU/L; 51.0 IU/L and 31.0 IU/L in the BB group; and 27.5 IU/L and 47.0 IU/L in the NB group for the short and long-term studies, respectively. The median PCV for the CON group was 51.0 % and 51.0 %; 49.5 % and 56.0 % in the BB group; and 51.0 % and 51.0 % in the NB group for the short and long-term studies, respectively. The median hemoglobin for the CON group was 17.7 g/dL and 18.0 g/dL; for BB was 17.7 g/dL and 20.0 g/dL; and for NB was 17.8 g/dL and 17.9 g/dL for the short and long-term studies, respectively. Serum analytes values fell within AAFCO established reference limits, supporting that the experimental NB and BB dog foods provided adequate nutrition and were safe to consume during short and long-term weight loss. This study applied AAFCO values for adult dog weight maintenance because there are no established values for dogs undergoing calorically restricted weight loss. Given that differences in canine serum analytes have been reported for overweight and obese dogs compared to normal weight dogs, and also that changes occur during weight loss [258; 261], future studies will need to determine if AAFCO reference values should adjusted for diets targeting weight management.

Table 18: Plasma and serum biochemical analysis of three diets fed to overweight or obese adult companion dogs undergoing calorically restricted weight loss.

Analyte	Control Group *Median (Min-Max)*	Black Bean Group *Median (Min-Max)*	Navy Bean Group *Median (Min-Max)*	Reference Values *Group (Individual)*
Hemoglobin, g/dL				
Short-term	17.7 (16.2-18.4)	17.7 (16.7-19.4)	17.75 (16.0-19.2)	≥ 14.0 g/dL
Long-term	18.0 (16.7-19.0)	20.0 (17.1-20.4)	17.9 (15.1-19.2)	(≥ 12.0)
Packed Cell Volume, %				
Short-term	51.0 (46.0-54.0)	49.5 (48.0-55.0)	51.0 (47.0-55.0)	≥ 42%
Long-term	51.0 (47.0-51.0)	56.0 (50.0-59.0)	51.0 (42.0-53.0)	≥ (36%)
Albumin, g/dL				
Short-term	3.9 (3.6-4.2)	3.9 (3.6-4.3)	3.9 (3.3-4.4)	≥ 2.8 g/dL
Long-term	3.9 (3.7-4.1)	4.0 (3.9-4.1)	4.1 (3.9-4.3)	(≥ 2.4)
Alkaline Phosphatase, IU/L				
Short-term	40.5 (27.0-320.0)	51.0 (26.0-152.0)	27.5 (16.0-76.0)	≤ 150 IU/L
Long-term	41.0 (23.0- 75.0)	31.0 (28.0-85.0)	47.0 (12.0-74.0)	(≤ 300)

Values for blood and serum analytes were determined at four weeks (short-term), and twenty-six weeks (long-term). Reference values were taken from AAFCO guidelines [122] for group means and individual dogs.

Conducting weight loss and digestibility studies with companion dogs, as opposed to colony dogs, presents new challenges because owner compliance in feeding and sample collection must be accounted for, as well as lapses in dietary discretion when feeding and collecting samples in a multiple dog household. Although this study was successful in achieving weight loss, many dogs did not achieve ideal weight during or following completion of the long-term study. This investigative team appreciates that lapses in study compliance may complicate interpretation of the results. However, these challenges emphasise the need for effective communication and perhaps an accelerated translation of canine weight loss study findings to real-clinic settings for body weight management planning.

CONCLUSIONS

In this study, we showed that nutritionally complete dog foods containing cooked bean powders were digestible by overweight or obese, adult, companion dogs undergoing short or long-term calorically restricted weight loss. The dog foods supported apparent weight loss and

provided utilisable energy, and the dogs maintained indices of nutritional adequacy when compared to a bean-free control dog food. Our findings of higher NFE ATTD in both the BB and NB diets compared to CON suggests that bean based dog foods may differentially impact canine carbohydrate metabolism. This study concludes that cooked common beans are safe and digestible as a major food ingredient during canine weight loss, when fed in a nutritionally complete, extruded kibble, and provides rationale for the continued investigation of the potential for cooked beans to improve protein, lipid, and carbohydrate metabolism, which are important for overall canine health. [6]

[6] We would like to thank ADM Alliance Nutrition for providing the dog foods and Gordon Gregory at ADM Edible Bean Specialties for providing the cooked bean powder. We would also like to thank the Wellington Veterinary Hospital and the Flint Animal Cancer Center Clinical Trials Core for providing companion dogs for participation in this study and clinical oversight. Additionally, we would like to thank Cadie Tillotson for technical assistance with study coordination. This research received no specific grant from any funding agency, commercial or not-for profit sectors.

CHAPTER 6: CONSUMPTION OF COOKED NAVY BEAN POWDERS MODULATES THE CANINE FECAL AND URINE METABOLOME[7]

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. [262] 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 (Chapter 4).[193]

In addition to providing a unique source of nutrients that may improve nutritional status [263], dry beans also contain a rich array of phytochemicals with health promoting properties, such as tocopherols, flavonoids, phytosterols, and enzyme inhibitors.[264-266] 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.[30; 265; 267] Dry bean intake has also been associated with improving longevity, promoting gastrointestinal health, facilitating healthy weight maintenance, and supporting hepatic function [44; 77; 234; 268; 269]. 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).[267; 270-272] The combination of dry bean

[7] This chapter was published in *Current Metabolomics* 2015*:*3(2):90-101. DOI: 10.2174/2213235X03666150519234354*.* Authors are Genevieve M. Forster, Adam L. Heuberger, Corey D. Broeckling, John E. Bauer, and Elizabeth P. Ryan.

macronutrients, micronutrients, and phytochemicals work together to promote health and reduce disease risk [262] 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.[273-275] 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.[276; 277]

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 biochemistry 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.[193] 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

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).

Canine 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.[193] A body condition score (BCS) was assigned to each dog by the study clinician using a 9-point scale.[191] Dogs were evenly distributed and no differences were detected between diet groups with respect to BCS, age, weight, and sex (Table 19).

Table 19: Baseline characteristics of 21 canine study participants evaluated for metabolomic changes in response to consumption of bean-based diets.

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]			
Female	5	6	0.670
Male	6	4	
BCS[4]			
BCS 4 & 5	9	8	1.00
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.[191]

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.[193]

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.[122; 278] 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.[193] Table 10 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.[193] 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)$^{0.75}$.[278]

Targeted Analysis of Dietary Nutrient Profiles

The macronutrient composition, on a dry matter basis, for the CON and NB diets respectively, was 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.[193] To determine the lipid profiles of the diets, total fat was

extracted and separated using thin layer chromatography (TLC) and analyzed via GC under previously reported conditions.[219] 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.[259]

Canine Diet Preparation for Non-Targeted 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.[279] 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 °C 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 °C 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[280; 281]. Specific run conditions are shown in Table 20.

Table 20: LC and GC run conditions for metabolite separation and detection.

Conditions	LC	GC
System	Acquity UPLC coupled to Q-TOF Micro (Waters)	Trace GC Ultra coupled to a Thermo DSQ II (Thermo Scientific)
Sample Injection	1 µl, in discrete random blocks (n=2)	1µl, 1:10 split ratio in discrete random blocks (n=2)
Column	Acquity UPLC T3 column (Waters, 1.8 µM, 1.0 x 100 mm)	30 m TG-5MS column (Thermo Scientific, 0.25 mm i.d., 0.25 µm film thickness)
Condition A	100% water, 0.1% formic acid	80°C
Condition B	95% methanol, 5% water, 0.1% formic acid	330 °C
Hold Time (Condition A)	1 min	0.5 min
Gradient Endpoint	100% B	330 °C
Rate from A to B	6.25% B/min (16 min)	15 ºC/min (16.67 min)
Hold Time (Condition B)	3 min	8 min
Time to A	0.1 min	-
Reequilibration Time	5.9 min	-
Total Run Time	26 min	30 min
Flow Rate	140 ul/min	1.2 mL/min (helium gas)
Column Temp	40°C	-
Sample Temp	5°C	-
Ion mode	Positive	Electron impact
Scan Range	50-1200 m/z	50-650 m/z
Scan Rate	1 scan/sec 0.1 sec delay	5 scans/sec
Calibration	5ppm (sodium formate)	-
Capillary Voltage	2200 V	-
Source Temp	130°C	-
Desolvation Temp	300°C	-
Flow Rate	400 L/hr (nitrogen gas)	1.2 mL/min (helium gas)
Collision Energy	7 V	-

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.[282] 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.[195] 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 Appendix 2 with its corresponding "cluster" number (C), identification confidence level [283], PubChem Compound Identifier (CID), and Simplified Molecular Input Line Entry Specification (SMILES).

Statistical Analysis

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 [284] in R.[196] 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. [197] 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 21). A detailed ingredient list has been previously reported in Table 10.[193]

Table 21: Major diet ingredients fed to 21 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 [193].

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, 34.39 % and 33.5 %; linoleic acid, 24.49 % and 25.71 %; palmitic acid, 22.44 % and 21.40 %; stearic acid, 6.25 % 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 (Figure 10A). 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, n-3, n-6, and n-9 fatty acids comprised 2.50 %, 25.15 %, and 34.76 % of total fat, respectively. For the CON diet, n-3, n-6, and n-9 fatty acids comprised 2.44 %, 26.61 %, and 34.00 % of total fat, respectively.

Crude protein levels were similar between the diets [193], and total amino acid profiles were likewise similar (Figure 10B). 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 and amines 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.[193]

Crude fiber was similar between diets.[193] 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 (Figure 10C).

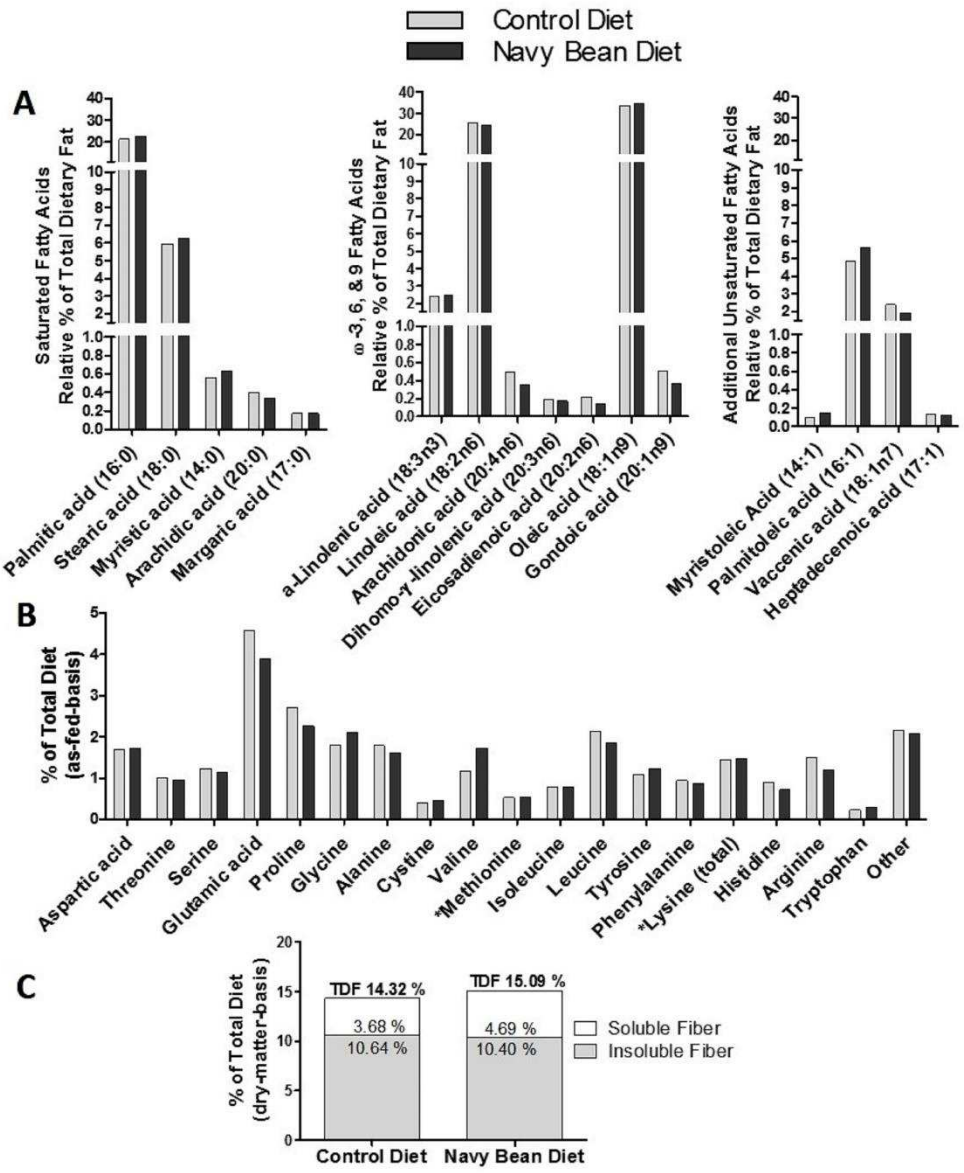

Figure 10: Macronutrient diet components in 25 % navy bean powder and control diets. A) Detected saturated, n -3, 6, 9, 5, & 7 fatty acids in navy bean and control diets as a relative percent of total dietary fat. B) Dietary amino acid profiles, as a percentage of total diet. Amino acids supplemented in the diets are indicated with an asterisk (*). C) Total dietary fiber (TDF) with soluble and insoluble fiber.

Non-Targeted Metabolomics 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 (Figure 11A**).** 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) (Figure 11B).

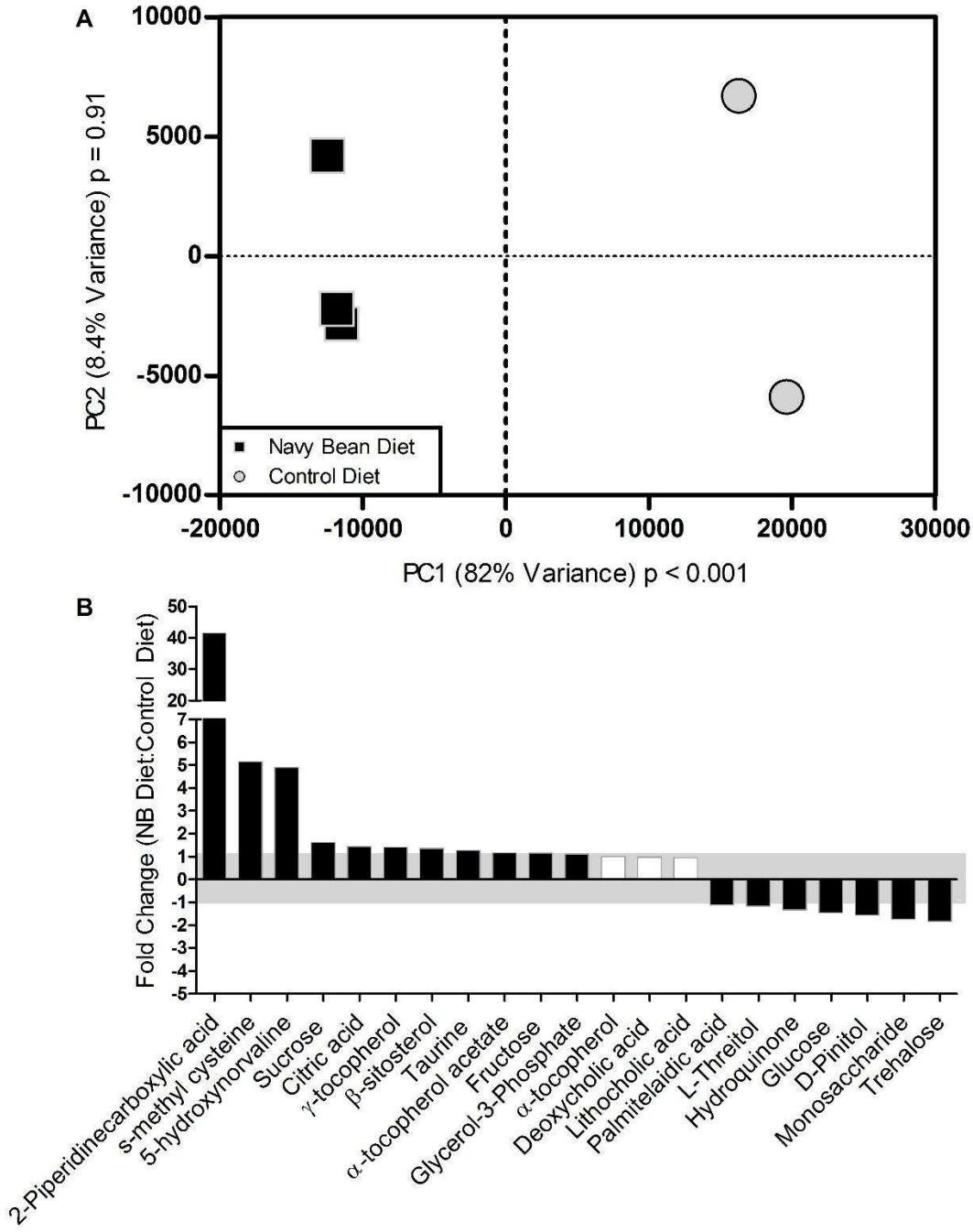

Figure 11: Metabolite profiles of a nutritionally complete, standard ingredient diet compared to an isocaloric, nutrient matched diet containing 25 % w/w cooked navy bean powder. A) Metabolite variance between 25 % navy bean powder and control diets. The Navy Bean diet is represented by squares and the 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 (Figure 12). 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.

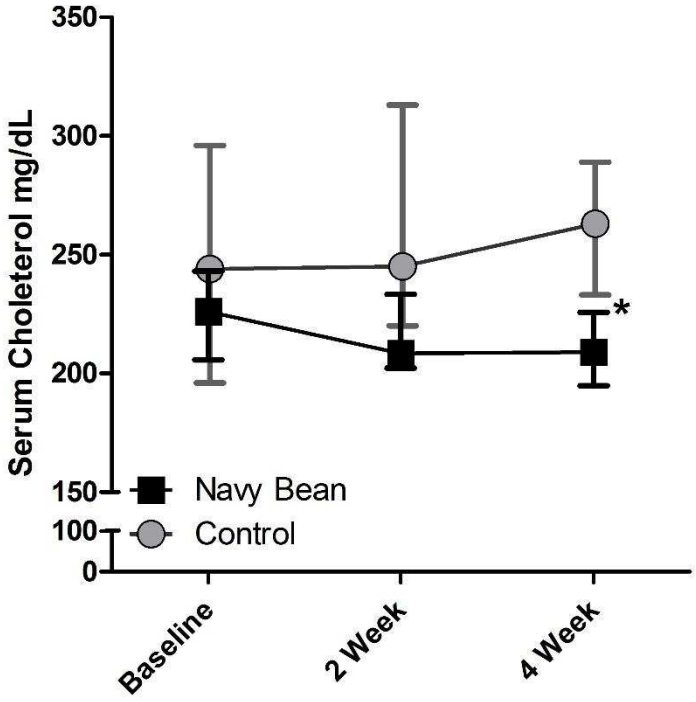

Figure 12: Serum cholesterol levels of 21 clinically healthy adult dogs during a 4 week dietary intervention trial establishing the safety and digestibility of 25 % w/w navy bean powder consumption compared to an isonutrient control diet. Dogs consuming the navy bean diet (n=10) had significantly lower 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.

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 principal components analysis (PCA, Figure 13A). 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 (Figure 13B). 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 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 (Figure 13C).

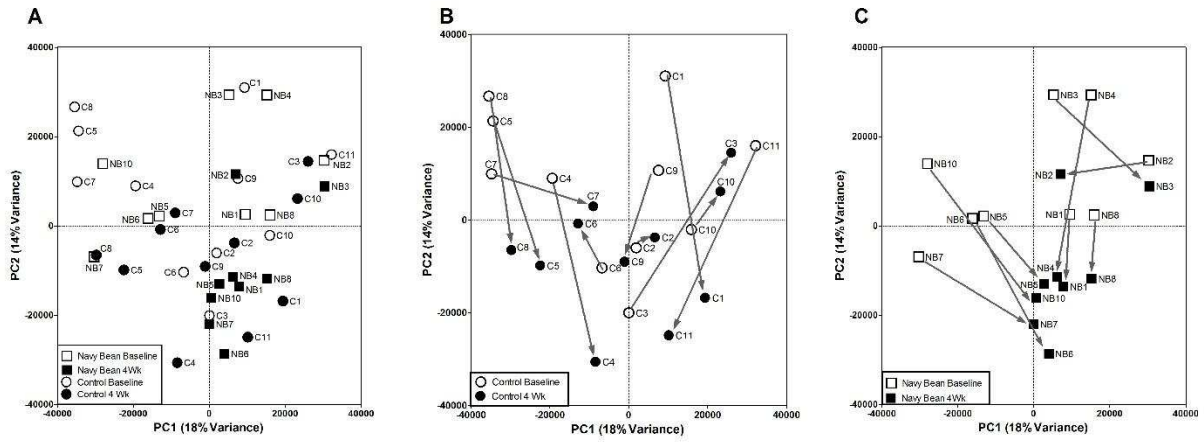

Figure 13: Fecal metabolite variance between both diet groups, control and navy bean, at baseline and after 4 weeks of consuming either a 25 % w/w navy bean powder or control diet (A). Dogs consuming the Navy Bean diet (n=9) are represented by squares and dogs consuming the control diet (n=11) are represented 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.

Fifteen fecal metabolites were identified that showed significant changes over time within each group, or were different between groups at 4 weeks (Table 22). 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 polycyclic 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 22**.**

Table 22: 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.

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 (Figure 14).

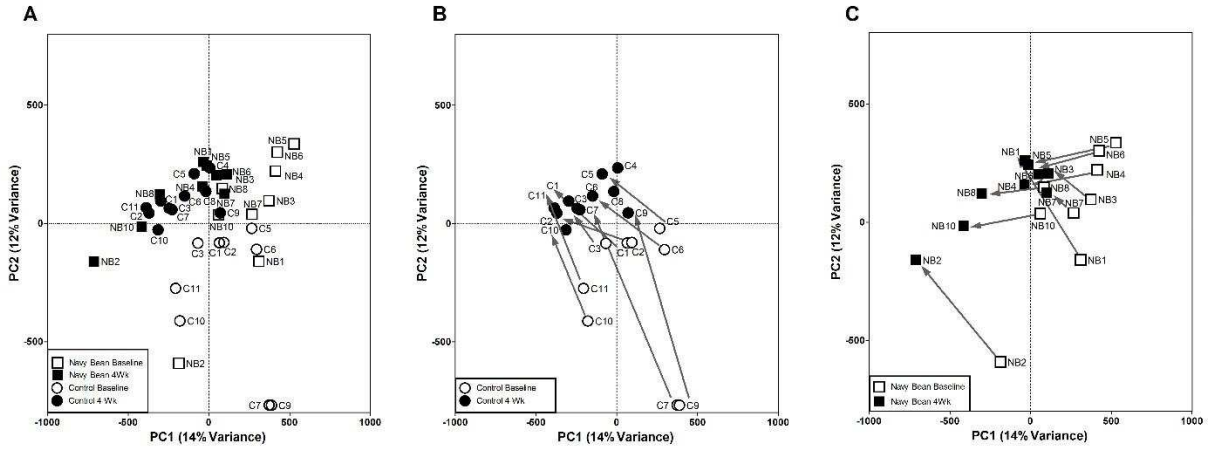

Figure 14: Urine metabolite variance between both diet groups, control and navy bean at baseline and after 4 weeks of consuming either a 25 % w/w navy bean powder or control diet (A). Dogs consuming the navy bean diet (n=9) are represented by squares and dogs consuming the control diet (n=9) are represented 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.

Fifteen metabolites were identified that were significantly different by diet or time (Table 23). 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).

Table 23: Diet responsive canine urine metabolites.

	Metabolite (Library Cluster)	Control Diet Group Abundance Day 0	Control Diet Group Abundance Day 28	Control Diet Group Fold Change Day 28:Day 0	Navy Bean Diet Group Abundance Day 0	Navy Bean Diet Group Abundance Day 28	Navy Bean Diet Group Fold Change Day 28:Day 0	P values Diet	P values Time	P values 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.

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 (Figure 10**)**. 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. (Figure 11A**)**. Increases in specific food metabolites such as 2-piperdinecarboxylic acid and s-methyl cysteine (Figure 11B) have been reported in multiple common bean cultivars [285] and validated in human and mouse studies as a predictive biomarker of dry bean intake in plasma and fecal samples [286; 287]. 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 to CON, while α-tocopherol content was similar across diets (Figure 11B). This finding is consistent with previous reports for legume seeds showing that the most abundant vitamin E isoform is γ-tocopherol.[288] β-sitosterol is the most abundant phytosterol in beans [289] 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.[264]

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.[290] 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.[291] Oligosaccharide content of the diets was not determined in this study, however extrusion of bean powders has been shown to reduce oligosaccharide content.[292] 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.[193]

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, variations within dog and triglyceride levels were not significantly different, supporting that the reported difference in 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.[44; 267] 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.[293] 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[264; 294; 295], prevent reuptake of bile acids via enterohepatic recirculation[296; 297], or increase biliary bile acid and cholesterol or related sterol secretion.[298-300] Contrary to expectations, given the increased cholesterol and bile acid content in bile associated with bean intake, fecal cholesterol excretion does not increase [297] and fecal bile acid excretion, especially secondary bile acids, actually decrease.[296; 298; 301] 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 22). Coprostanol, a microbial byproduct of cholesterol degradation [302], has been reported to increase with bean consumption.[296] However, it is not present in canine feces possibly because the canine gut microbiome lacks the organisms responsible for this conversion.[303] 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 22) 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 (Table 23). Interestingly, trigonelline, in humans, is more strongly associated with coffee intake [146], but was recently described as a candidate biomarker of bean intake in human plasma.[286] 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.

CONCLUSIONS

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.[8]

[8] This work was supported in part by the Flint Animal Cancer Center at Colorado State University, Archer Daniels Midland Alliance Nutrition, and Edible Bean Specialties. The authors wish to thank Cadie Tilliotson and Dr. Kristen Weishaar for technical assistance with sample collection from canine participants, Dr. Kelly Swanson for fiber analysis, and Dr. Ann Hess for statistical consultation.

CHAPTER 7: NUTRITIONAL WEIGHT LOSS THERAPY WITH COOKED BEAN POWDERS REGULATES SERUM LIPIDS AND BIOCHEMICAL ANALYTES IN OVERWEIGHT AND OBESE DOGS[9]

INTRODUCTION

Over 35 % of American adults are obese[304] with the consequence that obesity has now surpassed smoking as the leading cause of preventable disease in the U.S..[305] Companion animal obesity is estimated at 30 – 40 % [227] and mirrors the obesity epidemic in humans. Similarities between dogs and humans with obesity associated co-morbidities also exist for osteoarthritis, diabetes, nephropathy, cancer, and dyslipidemia.[306-309] Dogs represent an advanced translational model for cancer treatment [310; 311] and other disease management therapies[312-314] and have been used extensively to evaluate surgical methods such as endoscopic pyloric suturing[315], reversible gastric restriction implants[316], and gastric bypass techniques.[317] Additionally, dogs have been shown to be successful transitional models for evaluating protein intake, solving the nutritional mysteries behind pellagra and rickets[318] as well as evaluating effects of diet on brain physiology.[319] Pet dogs are especially relevant for evaluating nutritional weight loss therapies because they experience naturally occurring weight gain in the same home and living environment as humans. Companion dogs have similarities to human disease when compared to colony dogs possibly due to the role of environmental factors, including variable diets.[320-322] Furthermore, recent reports identified biomarkers of canine obesity with similarities to human biomarkers such as blood lipid values that change with weight loss.[227; 323; 324]

[9] This chapter was originally published in *Journal of Obesity and Weight Loss Therapy* 2012:2(8):149. DOI: 10.4172/2165-7904.1000149. Authors are Genevieve M. Forster, Cadie A. Ollila, Jenna H. Burton, Dale Hill, John E. Bauer, Ann M. Hess, and Elizabeth P. Ryan.

While many nutritional intervention strategies exist for weight loss, epidemiological studies reveal that legume consumption promotes satiety and is more effective for weight loss than calorie restriction alone.[325] Beans have also been shown to improve nutrient intake levels and regulate body weights and waist circumferences in humans providing additional weight management benefits.[32] In addition to weight loss, beans have also shown to harbor chronic disease fighting properties for obesity related conditions such as heart disease[326; 327], diabetes[231], cancer[212; 328; 329], and dyslipidemia.[34; 39; 44] While studies investigating the role of beans for weight loss in dogs have not been previously performed, we have established the safety and digestibility of beans as a novel ingredient for inclusion in healthy adult dog diets.[330]

Changes in dietary patterns, such as a calorie restricted diet lead to weight reduction; however the role of diet composition during the weight loss process merits research attention.[325] Given that cooked beans are a novel food ingredient for dogs, this study was conducted to evaluate whether eating beans as a major staple diet ingredient will alter metabolic parameters compared to dogs not consuming beans while undergoing similar weight loss. We hypothesized that a 25 % weight/weight bean based diet improves the metabolic status of dogs compared to control diets during comparable weight loss.

MATERIALS AND METHODS

The Colorado State University Institutional Animal Care and Use Committee approved all clinical trial operations, animal care procedures, and collection of biological samples for analysis before beginning the study.

Study Design

Thirty overweight or obese, clinically healthy, client owned adult dogs of different breeds were individually randomized based on their baseline BCS into 1 of 3 diet groups: control (CON), black bean (BB), or navy bean (NB). Both the owner and clinician were blinded to which diet the dog was consuming. Dogs were transitioned onto their assigned study diet over a 4 day period; this was accomplished by mixing increasing proportions of the study diet with decreasing proportions for their normal diet over the 4 day period. Blood samples were collected at baseline and at 2 and 4 weeks post intervention. At the end of the 4 weeks dogs were transitioned over a 4 day period to their original diets.

A signed consent form and medical history was required before enrolling in the study. The owner was required to bring the dog to the CSU VTH for weekly body weight (BW) checks and biweekly for physical exams, BCS monitoring, and blood collection. Owners were required to feed the dog only the study diets in amounts that were calculated to achieve weight loss, and to record daily food intake and fecal scores. Exercise was recorded; however no changes in exercise activity were required for the study. A medical history form was completed by the owner at weeks 2 and 4 of the study to assess changes in the dogs' health and behavior, such as vomiting or diarrhea, flatulence, energy level, as well as to assess palatability of the study diet.

Inclusion/Exclusion criteria

Dogs between the ages of 2 to 7 years with a BW of at least 10 kg and a BCS of at least 6 on a 9 point scale [191] were eligible to participate in this clinical trial. Dogs were required to have normal biochemical and hematological values and normal thyroid function as determined by a screening total T4 test. If the total T4 was below the lower limit of normal, a full canine thyroid panel, including TSH, thyroid globulin autoantibodies, and free T4 by dialysis, was performed to

ensure adequate thyroid function. Dogs were excluded if they had hypothyroidism, dietary allergies, prior or current cancer history or other major medical illness, or had been administered antibiotics or analgesics within one month of starting the diet. Concomitant medications were not allowed while on study, with the exception of heartworm preventative.

Diet Compositions

All diets were formulated to meet the Association of American Feed Control Officials nutrient and energy requirements for adult dogs.[215] Table 24 shows the nutrient profiles of the control and 25 % weight/weight black and navy bean diets. The complete formulations are presented in Table 15. Staple ingredients such as wheat, corn, and pork and bone meal were adjusted to account for the inclusion of bean powders (Vegefull; ADM Edible Bean Specialties, Decatur, IL), and to match nutrient and energy density. The control diet was manufactured and processed in the same location and under the same conditions as the bean diets (ADM Alliance Nutrition Feed Research Pilot Plant, Quincy, IL; Applied Food Biotechnology Plant in St. Charles, MO). Navy and black beans were selected for investigation given their widespread consumption by humans and availability as cooked powders.

Table 24: Nutrient profiles of canine weight loss diets.

Nutrient	Control	Black Bean	Navy Bean
Moisture, %	4.98	4.41	5.04
Protein, %	27.99	28.14	27.70
Fat Acid Hydrolysis, %	8.84	8.47	8.42
Crude Fiber, %	4.10	4.50	3.90
Gross Energy, Kcal/g	4.74	4.57	4.61

Values are presented on a dry matter basis, with the exception of moisture, which is presented on an as-fed-basis.

Weight Loss Intervention

Total daily energy intake requirements for each dog were determined by the dog's BW and BCS at baseline. The BCS was determined by the clinician and study nurse using a 9-point Body Condition Scale.[191] Using this scale, a score of less than 4 is underweight, a score of either

4 or 5 is considered ideal BW, a score of 6 or 7 is overweight, and a score of 8 or 9 is considered obese. Ideal BW was determined using BCS. For a BCS of 6, a dog was considered 10 % over ideal BW, and for a BCS of 9, a dog is considered at least 40 % over ideal BW.[331] The total required daily caloric intake to maintain ideal body weight was calculated using the following formula: daily metabolizable energy (ME) requirement (kcal) = 110 x (ideal BW (kg) ^0.75). To achieve a weight loss rate of 2 % BW/week, dogs consumed 60 % of the energy calculated to maintain ideal BW. Dog owners were instructed to only feed the prescribed diet for the duration of the study according to the dog's normal feeding schedule. Daily food amounts were provided to the owners in pre-measured packets and determined by dividing the daily energy requirements of each dog for weight loss by the energy density of the diet. Water was provided *ad libitum*. Each dog owner maintained daily records of all food consumed, including any non-study consumed food. Any leftover or uneaten food was collected and weighed in the laboratory. The total amount consumed was calculated by subtracting the weight of the leftover food from the original weight of the food prescribed for that day. If dogs failed to achieve at least 0.5 % BW loss, caloric intake amounts were further decreased by 10 % at 2 weeks.

Dietary Intake Records and Fecal Scores

Owners recorded the total amount of food consumed each day and recorded a daily fecal score. A 5-point fecal scoring system was used: 1 = hard and dry, 2 = well formed, 3 = moist, 4 = no form, 5 = diarrhea. Space was also provided for any comments and owners were instructed to report any food intake outside of the prescribed diet. Study compliance was determined by total number of days that each dog consumed only the prescribed amount of food.

Blood Sample Collection and Analysis

Blood was collected after a 12 hour fast via jugular venipuncture at baseline, 2, and 4 weeks post intervention. Two mL of whole blood was collected into an evacuated red top tube without anticoagulant for biochemistry panel analysis and 4-6 mL was collected into a plasma separation tube with EDTA and used to determine lipid profiles.

The CSU Clinical Pathology Laboratory performed all blood analyses as previously described.[330] Briefly, the biochemistry panel was analyzed using a clinical chemistry analyzer (Hitachi 917; Roche Diagnostics, Indianapolis, IN), analytes evaluated include cholesterol, BUN, creatinine, total protein, albumin, globulin, ALP, ALT, AST, GGT, total bilirubin, glucose, calcium, chloride, magnesium, phosphorus, potassium, and sodium. Total T4 and endogenous TSH, and TgAA was analyzed using an immunology analyzer (Siemens Immulite 1000, Los Angeles, CA), TgAA was analyzed by ELISA (Oxford Laboratories, MI) and Free T4 was analyzed by equilibrium dialysis at the Endocrine Section, Animal Health Diagnostic Laboratory (Michigan State University) using previously reported methods.[332] The lipid analysis was performed by a Cobas c501 chemistry analyzer (Roche Healthcare Diagnostics, Indianapolis, IN) which measured total triglycerides (TG) and high-density lipoprotein (HDL). Low-density lipoprotein (LDL) levels were calculated to equal: total cholesterol – HDL – (TG / 5).[333] Cholesterol values were obtained from the biochemistry panel, performed at the time of collection.

Statistical Analysis

Statistical analysis was performed using SAS 9.2 (SAS Institute Inc. Cary, NC). A model was fit separately for each response variable (weight, lipids, etc) using proc mixed. The model included main effects for diet and week and a diet*week interaction term. Repeated measures on

dogs were captured using a random dog effect (where dog is nested within diet). Comparisons of interest were estimated and tested using contrasts of the model. TG, alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were log transformed to satisfy the assumption of normality. Data are reported as means ± SEM for all response variables. One dog in the black bean group had elevated levels of TG at 4 weeks, was deemed a statistical outlier and as the dog may have gotten into food before the blood draw this data point was removed from the 4 week TG analysis. Differences between groups in baseline, age, and weight were assessed using a one-way ANOVA (Graphpad V.5.2, La Jolla, CA). Differences between groups in sex and baseline BCS were analyzed using Fisher's exact test (R Project software, Vienna, Austria). Results were considered significant when $P \leq 0.05$.

RESULTS

Of the 40 dogs screened, 7 failed to meet inclusion criteria and three were dropped from the study between 1 and 2 weeks for food refusal, physical injury, and an owner's schedule not permitting follow up visits. Thirty dogs completed the study and no differences were observed in age, weight, sex, or BCS at baseline between 30 dogs in the control, black bean and navy bean diet groups (Table 25). The average age (years) in the control group was 5.8 ± 0.42, 4.3 ± 0.54 in the black bean group, and 4.6 ± 0.58 in the navy bean group ($P > 0.12$). In the control group, 7/10 dogs were female, 3/10 were male; In the black bean group 6/10 dogs were female and 4/10 were male; and in the navy bean group 4/10 dogs were female and 6/10 were male. All male dogs were castrated and all female dogs except one in the control group were spayed. Dogs with BCS of either 6 or 7 were considered overweight and dogs with BCS of either 8 or 9 were considered obese. In the control group 7/10 dogs were overweight while 3/10 were obese; in the

black bean group, 4/10 dogs were overweight and 6/10 were obese; and in the navy bean group 7/10 dogs were overweight and 3/10 were obese. No gastrointestinal discomfort or changes in flatulence were reported by owners. All owners reported adherence to the study diet, with a few exceptions (such as eating food scraps dropped by children, receiving cookies from the groomer, etc.) that were evenly distributed across groups. Some owners expressed uncertainty of the total amount of extra food their dog consumed. All owners were reasonably sure that their dog was fasted at the time of blood draw.

Table 25: Characteristics of thirty overweight dogs fed bean-based or control during weight loss.

	Control		**Black Bean**		**Navy Bean**		
Parameter	Mean	SEM	Mean	SEM	Mean	SEM	*P* Value
Age, years[1]	5.8	0.42	4.3	0.54	4.6	0.58	0.12
Weight, kg[1]	33.0	4.50	26.7	3.19	28.8	3.61	0.50
			Number of Dogs				
Sex[2]							0.53
Female	7		6		4		
Male	3		4		6		
BCS[3]							0.45
6-7	7		4		7		
8-9	3		6		3		

[1]Significant differences between groups were evaluated using a one-way ANOVA.
[2]All but one female participant in the control group was spayed and all males were castrated. Significance was evaluated using Fisher's exact test.
[3]Body Condition Score (BCS): Nine-point scale where BCS 4 and 5 are ideal, 6 and 7 are overweight, and 8 and 9 are obese (35). Significance was evaluated using Fisher's exact test and *P* value corresponds to the probability of a larger F statistic.

Effect of Caloric Restricted diets on Canine Weight Loss.

Daily caloric intake was calculated to achieve a loss of 0.5-2 % BW per week. Across all groups, 26/30 dogs achieved loss within this range. Figure 15 shows the average percent weight lost at 2 and 4 weeks post intervention. In all groups, weight loss was significant between baseline and 4 weeks. In the control group, average weight lost at 2 weeks was 3.2 % ± 0.59 and 4.2 % ± 0.88 at 4 weeks ($P < 0.001$). In the black bean group, average weight lost at 2 weeks was

3.3 % ± 0.75 and 5.2 % ± 0.92 at 4 weeks ($P < 0.001$). In the navy bean group, average weight lost at 2 weeks was 3.5 % ± 0.72 and 6.5 % ± 0.95 at 4 weeks ($P < 0.001$). No difference in percent weight loss was seen between groups ($P > 0.05$). The greatest percent lost was seen in the navy bean group > black bean group > control group.

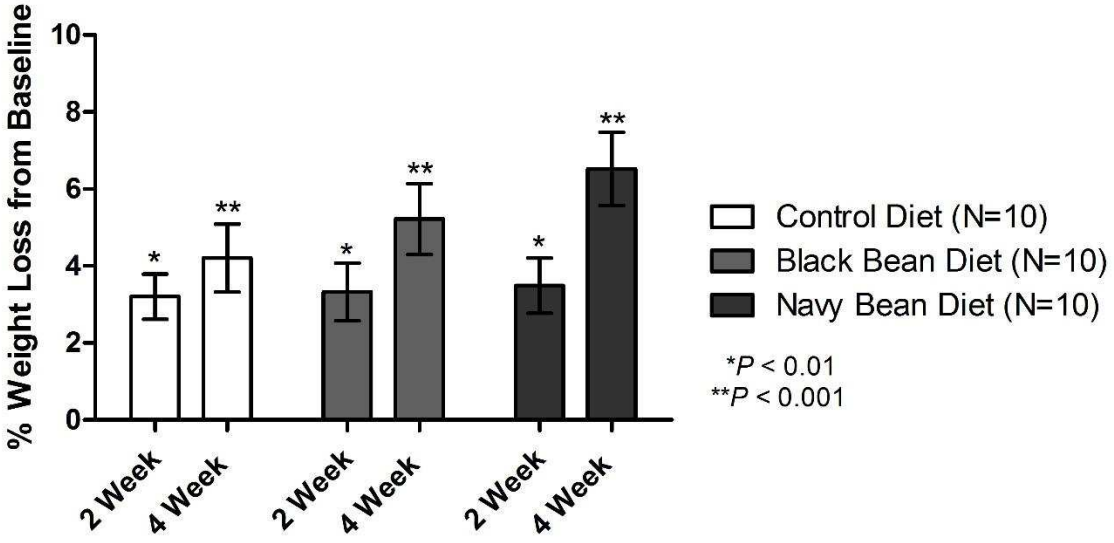

Figure 15: Percent weight loss of 30 adult dogs in a 4 week calorie restricted dietary intervention study. Dogs in all diet groups had significant weight reduction at 2 and 4 weeks, no significant differences were observed between groups, significant change is shown within each group compared to baseline values. Data are shown as mean and error bars represent SEM.

Navy and black beans differentially modulate blood lipids in dogs during weight loss.

Total serum cholesterol was significantly decreased in overweight dogs after 2 and 4 weeks of calorie restriction in the control and bean diet groups while TG, HDL, and LDL were also significantly reduced in at least one of the bean diet groups (Table 26). In the control group, total serum cholesterol was reduced by an average of 15 mg/dl after 2 weeks ($P < 0.05$), and showed a total average decrease of 17 mg/dl ($P < 0.02$) after 4 weeks. In the black bean group, total serum cholesterol was reduced by an average of 38 mg/dl after 2 weeks ($P < 0.001$), for a total average decrease of 40 mg/dl ($P < 0.001$) after 4 weeks. In the navy bean group, total serum

cholesterol was reduced by an average of 46 mg/dl after 2 weeks ($P < 0.001$), and 54 mg/dl after 4 weeks ($P < 0.001$). No significant differences in serum total cholesterol were observed between groups at 2 or 4 weeks. Total cholesterol was significantly higher in dogs consuming the black bean diet compared to control at baseline ($P = 0.05$).

Total serum TG were significantly decreased in all groups at 2 weeks, however only navy bean fed dogs continued to show a significant decrease at 4 weeks (Table 26). TG levels were not normally distributed and statistics were performed on log transformed variables and are reported as means ± (SEM). In the control group, total serum TG decreased from 123 mg/dl ± 21.16 at baseline to 89 mg/dl ± 28.62 at 2 weeks ($P < 0.03$) and 105 mg/dl ±24.65 at 4 weeks, for a total decrease of 18 mg/dl ($P = 0.09$). In the black bean group, total serum TG decreased from 127 mg/dl ± 21.84 at baseline to 94 mg/dl ± 12.24 at 2 weeks ($P = 0.05$) and 132 mg/dl ± 28.63 at 4 weeks for total increase of 5 mg/dl ($P = 0.22$). In the navy bean group, total serum TG decreased from 140 mg/dl ± 31.43 to 96 mg/dl ± 22.37 at 2 weeks ($P < 0.01$) and 77 mg/dl ± 11.04 at 4 weeks for a total decrease of 63 mg/dl ($P < 0.001$).

HDL and LDL were significantly reduced from baseline in both the black and navy bean diet at 2 and 4 weeks (Table 26). HDL of the dogs fed the control diet decreased by 5 mg/dl ($P = 0.45$) after 4 weeks. In black bean fed dogs HDL decreased an average of 23 mg/dl ($P < 0.001$), and in navy bean fed dogs HDL decreased an average of 27 mg/dl ($P < 0.001$) after 4 weeks. In control fed dogs LDL decreased an average of 9 mg/dl after 4 weeks ($P = 0.08$), in black bean fed dogs LDL decreased an average of 17 mg/dl after 4 weeks ($P < 0.001$), and in the navy bean fed dogs LDL decreased an average of 15 mg/dl after 4 weeks ($P < 0.01$).

Dietary Navy or Black Bean effects on Blood Chemistry and Electrolytes

Selected non-lipid serum analytes from the clinical biochemistry panel that have been associated with obesity and modulated by weight loss in both dogs and humans were assessed at baseline, 2, and 4 weeks post intervention in control, navy bean and black bean fed overweight and obese dogs (Table 27 and Table 28).

After 4 weeks, total protein was decreased an average of 0.21 g/dl ($P = 0.03$), AST was decreased an average of 9 IU/L ($P < 0.01$), and total bilirubin was decreased an average of 0.04 mg/dl ($P = 0.05$) in the dogs fed with the control diet. In the black bean fed dogs, after 4 weeks creatinine was increased by 0.06 mg/dl ($P = 0.05$) and ALP was decreased by 49 IU/L ($P = 0.01$), yet all values remained within clinically normal ranges. In the navy bean fed dogs, after 4 weeks blood urea nitrogen (BUN) was decreased by 5 mg/dl ($P <0.001$) and creatinine was increased 0.11 mg/dl ($P < 0.001$). At 4 weeks no changes were seen in albumin, ALT, gamma glutamyl transferase (GGT), globulin, or glucose within any group (Table 27).

Of the serum electrolytes measured, none were changed in the control group. In the black bean group, magnesium was increased 0.07 mg/dl ($P = 0.05$) and phosphorus was increased 0.39 mg/dl ($P = 0.04$). In the navy bean group, chloride was increased by 1.00 meq/L ($P =0.03$). No significant changes were observed in calcium, potassium, or sodium (Table 28)

Table 26: Blood lipid levels of thirty overweight dogs fed bean-based or control diets during weight loss.

Analyte[1] (Normal Range)[2]	Control					Black Bean					Navy Bean				
				Baseline vs 4 Week					Baseline vs 4 Week					Baseline vs 4 Week	
	Baseline	2 Week	4 Week	Change	*P*	Baseline	2 Week	4 Week	Change	*P*	Baseline	2 Week	4 Week	Change	*P*
Cholesterol (130-300 mg/dl)	208 (13.97)	193 (14.33)	191 (16.17)	-17	**0.02**	248 (15.87)	210 (12.62)	208 (14.28)	-40	**<0.001**	235 (11.14)	189 (12.77)	181 (11.03)	-54	**<0.001**
Triglycerides[3] (30-120 mg/dl)	123 (21.16)	89 (28.62)	105 (24.65)	-18	0.09	127 (21.84)	94 (12.24)	132 (28.63)	5	0.22	140 (31.43)	96 (22.37)	77 (11.04)	-63	**<0.001**
HDL[3] (112-131 mg/dl)	167 (6.93)	162 (8.10)	162 (11.24)	-5	0.45	200 (10.75)	179 (7.39)	177 (7.63)	-23	**<0.001**	182 (6.93)	158 (8.80)	155 (7.55)	-27	**<0.001**
LDL[3] (30-75 mg/dl)	17 (6.52)	14 (7.22)	8 (7.99)	-9	0.08	22 (8.77)	13 (5.24)	5 (7.27)	-17	**<0.001**	25 (3.56)	11 (3.24)	10 (2.53)	-15	**<0.01**

[1]Data are presented as mean (± SEM). [2]Normal ranges are from published reports.[3]Statistical analysis was performed on log transformed values to meet assumptions of normality, actual values are reported.

Table 27: Selected blood chemistry analytes of thirty overweight dogs fed bean-based or control diets during weight loss.

	Control					Black Bean					Navy Bean				
				Baseline vs 4 Week					Baseline vs 4 Week					Baseline vs 4 Week	
Analyte[1] (Normal Range)[2]	Baseline	2 Week	4 Week	Change	*P*	Baseline	2 Week	4 Week	Change	*P*	Baseline	2 Week	4 Week	Change	*P*
BUN (7-30 mg/dl)	17.4 (1.99)	15.2 (1.77)	16.5 (2.27)	-0.9	0.48	15.40 (1.26)	14.90 (1.27)	16.6 (1.92)	1.20	0.35	19.4 (2.54)	15.4 (1.18)	14.4 (1.47)	-5.00	**<0.001**
Creatinine (0.6-1.5 mg/dl)	1.10 (0.07)	1.12 (0.08)	1.12 (0.06)	0.02	0.5	0.90 (0.04)	0.96 (0.05)	0.96 (0.03)	0.06	**0.05**	1.00 (0.07)	1.10 (0.06)	1.11 (0.05)	0.11	**<0.001**
Total Protein (5.0-7.0 g/dl)	6.49 (0.09)	6.19 (0.14)	6.28 (0.08)	-0.21	**0.03**	6.18 (0.11)	6.18 (0.13)	6.09 (0.14)	-0.09	0.35	6.36 (0.15)	6.28 (0.18)	6.28 (0.1)	-0.08	0.41
Albumin (3.0-4.3 g/dl)	3.94 (0.10)	3.90 (0.06)	3.89 (0.07)	-0.05	0.36	3.97 (0.05)	3.94 (0.05)	3.88 (0.06)	-0.09	0.10	3.93 (0.11)	3.92 (0.12)	3.87 (0.11)	-0.06	0.27
Globulin (1.5-3.2 g/dl)	2.55 (0.07)	2.29 (0.09)	2.39 (0.06)	-0.16	0.10	2.21 (0.09)	2.24 (0.11)	2.21 (0.12)	0	1.00	2.43 (0.15)	2.36 (0.12)	2.41 (0.09)	-0.02	0.84
ALP[3] (15-140 IU/L)	143 (79.08)	111 (50.62)	84 (31.32)	-59	0.20	109 (42.99)	74 (22.43)	60 (12.05)	-49	**0.01**	44 (7.79)	37 (7.77)	35 (5.74)	-9.0	0.08
ALT[3] (10-90 IU/L)	49 (10.92)	42 (7.59)	42 (8.94)	-7.0	0.12	38 (4.52)	52 (16.06)	38 (5.59)	0	0.86	42 (4.93)	44 (6.53)	41 (5.17)	-1.0	0.61
AST[3] (16-50 IU/L)	34 (3.01)	23 (1.71)	25 (1.47)	-9.0	**<0.01**	25 (1.96)	23 (1.80)	23 (1.94)	-2.0	0.25	33 (6.93)	29 (3.47)	27 (3.59)	-6.0	0.14
GGT (0-9 IU/L)	0.60 (0.27)	1.20 (0.36)	0.40 (0.31)	-0.2	0.64	0.50 (0.27)	1.80 (0.51)	0.90 (0.413)	0.40	0.35	1.10 (0.31)	1.50 (0.34)	1.50 (0.34)	0.40	0.35
Total Bilirubin (0.0-0.3 mg/dl)	0.14 (0.02)	0.13 (0.02)	0.10 (0.00)	-0.04	**0.05**	0.12 (0.01)	0.14 (0.02)	0.11 (0.02)	-0.01	0.61	0.12 (0.02)	0.13 (0.02)	0.14 (0.02)	0.02	0.31
Glucose (70-115 mg/dl)	101 (5.59)	103 (3.79)	101 (4.21)	0	0.79	106 (3.33)	108 (2.40)	107 (2.03)	1.00	0.79	105 (3.09)	104 (2.76)	106 (2.72)	1.00	0.73

[1]Data are presented as mean (± SEM). [2]Normal ranges are in-house references.[3]Statistical analysis was performed on log transformed values to meet assumptions of normality, actual values are reported.

Table 28: Blood electrolytes for thirty overweight dogs fed bean-based or control diets during weight loss.

Analyte[1] (Normal Range)[2]	Control Diet					Black Bean					Navy Bean				
				Baseline vs 4 Week					Baseline vs 4 Week					Baseline vs 4 Week	
	Baseline	2 Week	4 Week	Change	*P* Value	Baseline	2 Week	4 Week	Change	*P* Value	Baseline	2 Week	4 Week	Change	*P* Value
Calcium (9.0-11.5 mg/dl)	10.75 (0.19)	10.50 (0.14)	10.64 (0.14)	-0.11	0.48	10.71 (0.10)	10.65 (0.14)	10.78 (0.11)	0.07	0.65	10.93 (0.13)	10.54 (0.25)	10.70 (0.14)	-0.23	0.15
Chloride (108-120 meq/l)	113 (0.53)	113 (0.54)	112 (0.55)	-1.00	0.74	112 (0.69)	112 (0.94)	112 (0.93)	0	0.50	111 (0.43)	113 (0.58)	112 (0.65)	1.00	**0.03**
Magnesium (1.9-2.7 mg/dl)	2.01 (0.05)	2.01 (0.04)	2.03 (0.05)	0.02	0.56	1.98 (0.03)	2.05 (0.05)	2.05 (0.03)	0.07	**0.05**	2.04 (0.04)	1.99 (0.02)	1.99 (0.03)	-0.05	0.15
Phosphorus (2.5-6.0 mg/dl)	3.58 (0.16)	3.36 (0.16)	3.63 (0.23)	0.05	0.79	3.30 (0.17)	3.27 (0.12)	3.69 (0.27)	0.39	**0.04**	3.47 (0.21)	3.42 (0.17)	3.50 (0.24)	0.03	0.87
Potassium (3.5-5.2 meq/l)	4.19 (0.10)	4.15 (0.10)	4.16 (0.11)	-0.03	0.76	4.28 (0.15)	4.24 (0.08)	4.36 (0.09)	0.08	0.41	4.11 (0.1)	4.13 (0.1)	4.24 (0.11)	0.13	0.18
Sodium (142-152 meq/l)	147 (0.49)	147 (0.52)	147 (0.50)	0	0.88	146 (0.21)	146 (0.65)	147 (0.52)	1.00	0.47	145 (1.01)	146 (0.65)	146 (0.42)	1.00s	0.47

[1]Data are presented as mean (± SEM). [2]Normal ranges are in-house references.

DISCUSSION

Laboratory, clinical, and epidemiological studies show that dry bean intake is associated with increased weight loss and lower body weights[32; 39; 334] Weight loss was intentionally achieved in the control diet fed dogs as well as for the experimental bean diets in this study due to caloric restriction. The navy and black bean diets showed a trend towards enhanced effects on weight loss after one month compared to the control. While this finding was not statistically significant, we postulate that a larger sample size and longer weight loss time may reveal more robust differences as previously reported with bean intake in humans.[39] Three of the four dogs (1/10 black bean, 1/10 navy bean, 1/10 control) who failed to achieve weight loss may have been due to dietary non-compliance as the owners expressed uncertainty of the dog's intake in spite of attempts to adhere to study diet. This highlights the challenges faced by owners and clinicians when undertaking a weight loss program for a companion animal and underscores the importance of developing weight management therapies that account for these challenges. The fourth dog (black bean diet) failed to lose weight in the 4 week period most likely due to resting energy requirements outside of the calculated required energy estimates as the owner was reasonably sure the dog was only consuming prescribed food. Other weight loss related measurements modulated by dry bean intake include decreased waist circumference and inflammatory biomarker expression.[32; 39; 326; 335] The macro and micronutrient composition of dry beans are thought to promote weight loss by providing low glycemic index sources of fiber, protein, minerals, and phytochemicals.[228; 334] Increased protein intake has been shown to improve weight loss [336] with plant proteins having higher satiety ratings than animal proteins[337]. Dry bean fiber may promote weight loss by improving satiety with altered transit time in the intestines, increased fermentation by gut microflora that results in higher short chain fatty acid production

that compete with protein and glucose for uptake and utilization[334], and enhanced release of cholecystokinin[40] which may have short term effects on energy intake and gastric emptying.[338] Bean phytochemicals such as phenolic compounds may interfere with glucose transport in the small intestine, phytic acid may delay glucose absorption leading to improved satiety and modulated energy uptake[334], and many phytosterols and saponins have been implicated in cholesterol reduction.[232; 339]

Lipid metabolism is an important component to weight loss as dyslipidemia underlies many of the comorbidities associated with obesity.[340] Weight loss has repeatedly been shown to reduce serum cholesterol levels in both humans and dogs [341; 342] and increased plant fiber intake has been shown to lower cholesterol levels after only 1 week with the same efficacy as first generation statins.[343] Our study shows serum cholesterol reduction occurred after 2 weeks of weight loss and was sustained throughout the study (Table 26). While not statistically significant, dogs consuming beans showed the largest change at 2 weeks whereas the decrease in the control group was similar between 2 and 4 weeks. This finding demonstrated that serum cholesterol reduction may be an early biomarker for a metabolic response to bean intake and weight loss wherein previous reports in dogs showed decreased serum cholesterol after 60 days[344], 90 days[341], and after the full amount of time required to reach ideal weight.[345; 346] Furthermore, TG, HDL and LDL were reduced in at least one of the bean diet groups, a finding consistent in human studies [44], with the exception of HDL which has been shown to increase in humans after bean consumption.[347] It should be noted, that LDL levels were determined indirectly using Friedewald's equation[333] and are therefore only indicative of trends between groups, and may not be reflective of actual values due to variance with HDL and LDL metabolism in dogs compared to humans.

Modulation of lipid metabolism by dry bean consumption has several proposed mechanisms which may work in conjunction with weight loss. Epidemiological studies have revealed inverse relationships between dietary protein sources and cholesterol levels.[348] Dietary fiber is thought to correct dyslipidemia by decreasing fat intake and increasing bile acid and cholesterol losses in the small intestines.[349] A meta-analysis of non-legume soluble fiber also revealed a slight improvement in lipid profiles.[350] In contrast, dry bean fiber has been shown to alter blood lipids by preventing micelle formation and preventing absorption of cholesterol and fatty acids making these compounds available for fermentation in the large intestine, and increasing the rate of removal of LDL.[351] *In vitro* studies have suggested that dry beans have a higher capacity for binding to bile acids than do soy proteins or wheat gluten[222; 352] and rat studies have shown higher bile acid synthesis and excretion with whole bean diets.[353; 354] Human studies have further demonstrated the interruption of the enterohepatic circulation of bile acids and increased excretion of acidic fecal sterols after prolonged bean consumption.[36] Characterization of bean fractions associated with lipid modulation in rats revealed that the starch and fiber component had the greatest lipid lowering effects [354], as well as upregulation of hepatic LDL receptor and cholesterol 7 alpha-hydroxylase expression.[355] These findings, taken together with the observed lipid metabolism changes in the bean groups suggest that there may be multiple compounds working in concert. Beans contain numerous bioactive components[356] and dietary intervention studies continue to show beneficial changes in blood lipids, regardless of underlying health conditions.[34; 325]

Non-lipid serum analytes previously reported to be associated with weight loss in both dogs and humans were also evaluated. BUN is decreased in overweight dogs compared to lean dogs [341], however changes in BUN after weight loss have been reported with inconsistent results,

namely no changes[344; 345], or increased levels of BUN.[341; 357] In this study, all dogs had BUN values within normal ranges, and only dogs on the navy bean diet showed an average decrease of 5 mg/dl ($P < 0.001$) during weight loss (Table 27). Creatinine levels have also been shown to be elevated in overweight dogs compared to lean dogs [341], however changes in serum creatinine levels have also been variably reported with increases and decreases after 60 and 90 days of weight loss.[341; 344; 345] Creatinine levels remained constant during the 4-week study in our control group and increased in both bean diet groups but remained within normal reference range. The precursor for creatinine, creatine, is generated from the urea cycle and has been touted as a supplement for stimulating muscle growth in athletes.[358] This candidate biomarker may have important implications in understanding the role of beans for maintaining muscle mass during weight loss. Total protein has been shown to be increased in overweight dogs compared to lean [341] and to decrease after 60 and 90 days of weight loss.[341; 344] In this study, total protein decreased in the control group ($P = 0.03$), however remained unchanged in the bean groups. Albumin has been demonstrated to be increased in overweight dogs [341] and reduced in dogs undergoing weight loss.[341; 344; 345] Serum albumin levels were not changed herein and may be reflective of the 4 week study time period of weight loss examined as previous studies have reported changes after 60-90 days. Enzymes ALP, ALT, AST, and GGT have all been positively associated with increased body fat in obese humans, with ALP dependent on gender.[359] ALP is elevated in overweight dogs and reduced in dogs undergoing weight loss.[341; 345] ALP was significantly reduced in dogs consuming black beans ($P = 0.01$) and had a decreasing trend in the navy bean group ($P = 0.08$). AST levels have not previously been shown to change with weight loss in dogs [346], however it was significantly decreased in the control group ($P < 0.01$). Bilirubin, while not elevated in overweight dogs [341], has been shown to be reduced during weight loss with specific

diets [344] as was observed in the control group. Glucose levels were not altered in any of the 3 diet groups and have not previously been shown to change with weight loss in dogs [341; 344; 346; 360], although increased glucose levels in overweight dogs have been reported.[341] No analyte was changed outside of normal ranges.

Beans are an effective staple food ingredient and a quality protein source during weight loss and these results suggest that consuming a diet with high dry bean intake improves lipid profiles along with other metabolic biomarkers when compared to a non-bean caloric restricted diet alone. Furthermore, dry bean consumption has been associated with increased longevity [234], reduced tumor growth in colon [361; 362], mammary tissue [41], upper digestive tract and stomach [329], and prostate [363], decreased risk for cardiovascular disease [347], and diabetes.[364; 365]

This study has demonstrated the ability of dietary bean intake to modulate blood lipids beyond what is expected from weight loss alone. Furthermore, the changes in blood biochemical analytes suggest a role for beans in liver and kidney function in dogs undergoing weight loss. This overweight dog trial was a practical and relevant approach to advance our understanding of the effects of dry bean consumption for lipid modulation during weight loss, because it accounts for variations in non-controlled living environments. Moreover, the companion animal dogs in this study represent a realistic reflection of serum lipid and biochemical profile variations and population based responses. Taken together, these results provide rationale for further explorations of the effects of dry bean consumption for chronic disease prevention, and regulation of lipid metabolism with weight loss.[10]

[10] We would like the Dry Bean Health Research Program, the Shipley Foundation, and the Animal Cancer Center for support of this study. We would also like to acknowledge Susan Lana and Kim Arnett in the ACC Clinical Trials Core for providing expertise and technical assistance.

CHAPTER 8: EFFECTS OF BEAN-BASED WEIGHT LOSS THERAPIES ON THE METABOLOME, INFLAMMATION, AND GUT HORMONES IN OVERWEIGHT AND OBESE DOGS

INTRODUCTION

Consumption of common beans (*Phaseolus vulgaris,* L.) has been correlated with increased longevity[31], reduced risk for cardiovascular disease[30], lower glycemic response to meals[366], and decreased risk for advanced adenoma recurrence.[367] Cooked common beans support weight loss in humans[368] and frequent consumption is associated with lower body weight.[32] Individuals consuming beans during weight loss have also experienced improvements in glucose homeostasis, decreased serum cholesterol, triglycerides, LDL, and increases in HDL that are not observed in caloric restricted groups without bean intake.[66; 369] Rebello, *et al.* recently reviewed the effects of bean consumption on human obesity and found that bean intake improves weight loss over caloric restriction alone, increases metabolic rates, improves satiety, reduces dyslipidemia, and improves metabolic syndrome parameters.[76] Cooked common beans comprise a unique combination of macro-, micro-, and phytonutrients that are associated with metabolic improvements in glucose, lipid, and carbohydrate metabolism as well as decreased inflammation, and increased satiety through the modulation of orexic gut hormones (reviewed in Chapter 1).

Obesity is the primary nutritional disorder in dogs in developed countries with over 40 % of dogs classified as overweight or obese in the U.S.[3], UK[4], Australia[5], and China.[6] Overweight dogs have reduced lifespans,[8] and are at higher risk for developing multiple chronic diseases and at significantly younger ages than normal weight or lean dogs.[8-10] Obesity is a complex

metabolic and endocrine disorder with white adipose tissue producing multiple inflammatory mediators, hormones, and growth factors.[370] Similar to humans, dogs with excess adipose tissue may have increased inflammation as indicated by higher levels of circulating CRP[175], TNF-alpha, and IL-6.[371] Overweight dogs also have alterations in gut hormone expression such that leptin is increased and adiponectin and ghrelin are decreased. We have demonstrated that overweight and obese dogs may have different serum biochemistry than normal weight dogs, and other canine studies have shown changes in serum lipid profiles, and liver enzymes (Chapter 3).[198] We have also demonstrated significant differences in the metabolome of normal weight versus overweight and obese dogs (**Figure 8**), and others have demonstrated that weight gain in dogs induces changes in carbohydrate, energy, and protein metabolome profiles.[372] The culmination of these aberrancies are detected as an increase in insulin resistance, hyperlipidemia, and endocrine dysfunction making overweight and obese dogs at higher risk for developing chronic diseases.[373]

Many of the metabolic aberrancies associated with canine obesity can be reversed with weight loss. Diet, lifestyle, and behavior based interventions to reduce adiposity have been successful in correcting dyslipidemia, reducing renal stress, normalizing endocrine function, and reducing inflammation.[344; 374-380] Therefore, inclusion of cooked common beans into nutritionally completed dog foods may potentiate the benefits of weight loss in dogs. Given that cooked common bean powder, when incorporated into an extruded, dry, kibble, is safe and digestible by dogs during weight loss[120] (Chapter 4), consumption of bean-based diets modulates serum lipids and analytes in overweight and obese dogs, independent of weight loss[95] (Chapter 7), and we found that in normal weight dogs consuming a bean-based diet for weight maintenance, carbohydrate, lipid, and protein metabolism were modulated[94] (Chapter 6), the effects of bean

intake on the metabolome, inflammation, and gut hormone expression during weight loss were evaluated.

The objectives of this research were 1) to evaluate the effects of weight loss on the metabolome, inflammasome, and gut hormones of clinically healthy, adult, companion dogs undergoing calorically restricted weight loss on nutrient matched BB and NB based and control (CON) diets and 2) determine the effects of cooked black (BB) and navy (NB) bean powders on the macro- and phyto-nutrient profiles of nutritionally complete, low fat dog foods. We hypothesize that dogs consuming the bean-based diets will have decreased inflammation, a shift in gut hormone expression to increases insulin sensitivity, as well experience changes in the metabolome consistent with improved carbohydrate and lipid metabolism. We also expect that the addition of bean powders to nutritionally complete, bean-based dog food formulations for weight loss, will increase the phytochemical diversity of the diets, and improve the macronutrient profiles for weight loss as was seen in diet formulations for weigh maintenance (Figure 11).

METHODS AND MATERIALS

Study Design/Dogs

Thirty clinically healthy, overweight or obese, adult, male and female, companion dogs were recruited to participate in a randomized, placebo controlled, double blinded, bean-based dietary weight loss study as previously reported (Chapter 7).[258] Dogs were calorically restricted to achieve a weight loss rate of 0.5 % to 2 % body weight per week and randomized based on body condition score (BCS) to one of two bean-based foods, or an iso-caloric, nutrient matched control dog food. There were no differences between groups in age, BCS, gender, or weight

(Table 14).[95] Clinical assessments and routine diagnostics were performed at baseline and throughout the study. Biological samples were collected at baseline, 2 weeks, and 4 weeks. Whole blood was collected via venipuncture after a 12 hour fast into glass tubes containing EDTA, urine was collected primarily by free catch, and owners were instructed to collect fecal samples within 5 hours of being voided. Samples were processed and stored at -80 °C as previously described.[94; 95; 120] There were no differences between the groups in percent body weight lost at 2 or 4 weeks.

The Colorado State University Institutional Animal Care and Use Committee approved all clinical trial operations, animal care procedures, and collection of biological samples for analysis before beginning the study (IACUC 13-4316A) and all owners provided informed written consent.

Diet formulations and analysis

A complete description of nutrient profiles, ingredients, and caloric content were previously reported (Table 15). The crude protein content, as-fed, was approximately 26 %; carbohydrate content (as calculated by nitrogen free extract) was about 48 %; crude fiber was about 4 %; and measured metabolizable energy was approximately 3,500 kcal/kg. All nutrients were equally digestible across diets with the exception of carbohydrates which were more digestible in both the NB and BB diets compared to CON, and protein and total dry matter which had higher digestibility in the BB diet compared to CON (Table 17).

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).

Fatty acids were extracted from 100 mg of ground diet with 3 ml of a 2:1 methanol-chloroform solution, and 25 µl of 10 µl/ml C17:0 (as an internal standard), vortexed and sonicated for 10 minutes. One ml of chloroform was added to each diet sample, vortexed and sonicated for an additional minute. Samples were then centrifuged for 7 min at 216 x g and the pellet re-extracted with 1 ml of chloroform. Supernatants from both extractions were pooled, vortexted and centrifuged for 5 minutes at 300 x g to separate the chloroform fraction. The chloroform fraction was filtered through sodium sulfate cartridge and rinsed with an additional 1 ml of chloroform. The filtered extract and rinsate were pooled and dried under nitrogen. Extracts were then derivatized and methylated in 1 ml hexane and 3 ml boron trifluoride in 14 % methanol at 100 °C for 30 minutes. Dervatized samples were cooled to room temperature and 1 ml of water was added. Samples were again vortexed and centrifuged to separate the hexane layer which was transferred to a gas chromatography (GC) sampler vial. 2 µl of sample was injected into a DB-Wax column (30 m, 0.25 i.d., 25 µm film thickness, Agilent Technologies: Santa Clara, CA). The initial starting temperature was 90 °C which was held for 2 min, ramped to 208 °C at 70 °C/min and then increased to 230 °C at 3 °C/min, then to 240 °C at 2 °C/min for a total run time of 16.5 minutes. Samples were injected into the injection port at 240 °C in splitless mode and scanned between 40-500 m/z.

Non-Targeted Metabolomic Profiles

Fecal, plasma, urine, and diet samples were prepared, extracted and derivatized as previously described.[94] Samples were analyzed by GC and liquid chromatography (LC) coupled to mass spectrometry (MS) under previously described conditions[281; 381]. Individual features, described by mass, charge and retention time were generated in XCMS. The average abundance of duplicate injections were normalized to total ion current and clustered together into individual

metabolites.[195] The relative abundance of each cluster was calculated based on the weighted sum of all features within the cluster and scaled to a median of one. Spectra were screened against in-house and external libraries including NIST v12 (www.nist.gov), Massbank, Metlin, and Golm (http://gmd.mpimp-golm.mpg.de) metabolite databases for annotation.

Gut Hormone Analysis

Canine gut hormones glucose-dependent insulinotropic peptide (GIP), peptide YY (PYY), pancreatic polypeptide (PP), leptin, insulin, and glucagon were detected using the Milliplex magnetic bead multiplex assay (Catalog #CGTMAG-98K: Billerica, MA) and analyzed on a Luminex 200 (Austin, TX) as described by the assay protocol. All samples were assayed in duplicate and quality controls run on every plate. Adiponectin was detected using an ELISA assay (Cat #EZCADP63K: Millipore, Billerica, MA) and performed as directed by the assay protocol.

Cytokine Detection

Canine plasma was analyzed for cytokines using a magnetic bead based multiplex assay (Cat#CCYTMG-90K, Millipore, Billerica, MA). Analytes examined were GM-CSF, IL-2, IL-6, IL-7, IL-8, IL-15, IL-18, IP-10, KC-like, and MCP-1. CRP was analyzed using a well validated, single ELISA (PHASE canine CRP Assay:TP-803, Tridelta, Kildare, Ireland). All assays were performed according to specific protocol instructions. Levels of IL-6, MCP-1, TNF-α, IFN-γ, and GM-CSF are being analyzed and verified using canine specific single ELISAs (Cat#s: DY1609, DY1774, DY1507, DY781B, DY1546, respectively. R&D Systems: Minneapolis, MN).

Statistical Analysis

To determine the magnitude of differences in compounds across the CON, NB, and BB diets, the absolute difference of the relative abundance of a compound between a specific bean diet and CON was normalized to the relative abundance of CON with the following formula: (|BB or NB – CON|/CON) x 100 = % difference from CON. To determine differences between NB and BB, BB was arbitrarily selected as the reference value. Compounds that were more than 10 % different were reported. A principal components analysis (PCA) was used to determine the overall variance between diets and the clusters (metabolites) contributing to that variance. A one way ANOVA was used to determine clusters that varied in relative abundance between diets, however because this variance is only based on technical replicates, only clusters with an ANOVA p-value < 0.05 and at least 10 % difference in abundance, or were significant contributors to the variance in a PC, are reported individually.

All metabolomic profiles were evaluated with a PCA 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, plasma 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 [284] in R.[196] 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.[197] An adjusted p-value < 0.1 was accepted as significant. Metabolites that were significant by both time and diet are reported and post-hoc ANOVAs were performed to determine which diet group a significant change over time occurred (GraphPad Prism version 5.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

RESULTS

Fasted Canine Plasma Gut Hormones and Inflammatory Biomarkers after Four Weeks of Weight Loss on Bean-Based or Control Diets

Six canine gut hormones were evaluated in the plasma of all dogs at baseline and after 2 and 4 weeks of weight loss and dietary intervention with a multiplex assay. Hormones assayed included GIP, glucagon, insulin, leptin, PP, and PYY (Figure 16). All hormone levels were similar across groups at baseline with the exception of GIP which was elevated in the NB group. At baseline, the median fasted plasma level of GIP was 7.0 pg/ml, 5.4 pg/ml, and 32.0 pg/ml for dogs in the CON, BB, and NB diet groups, respectively. The median values did not change over time in any group except for NB, in which the median level decreased by 32.0 pg/ml to 4.7 pg/ml and at 4 weeks was not different from CON or BB. Median glucagon plasma levels at baseline were 24.2 pg/ml, 26.8 pg/ml, and 34.2 pg/ml; median insulin plasma levels were 360.2 pg/ml, 594.2 pg/ml, and 394.8 pg/ml; median leptin plasma levels were 4,955.4 pg/ml, 5,445.3 pg/ml, and 5,258.3 pg/ml; median PP plasma levels were 36.7 pg/ml, 35.5 pg/ml, and 63.0 pg/ml; median adiponectin plasma levels were 2.4 ng/ml, 3.2 ng/ml, and 2.5 ng/ml for CON, BB, and NB, respectively and did not change over time in any group. Median PYY serum levels were 117.5 pg/ml, 101.9 pg/ml, and 122.2 pg/ml at baseline for CON, BB, and NB, respectively, and

in the CON group, decreased to 93.9 pg/ml at 2 weeks, and then increased back to 108.3 pg/ml at 4 weeks and was not different from baseline. PYY did not change in either the BB or NB group.

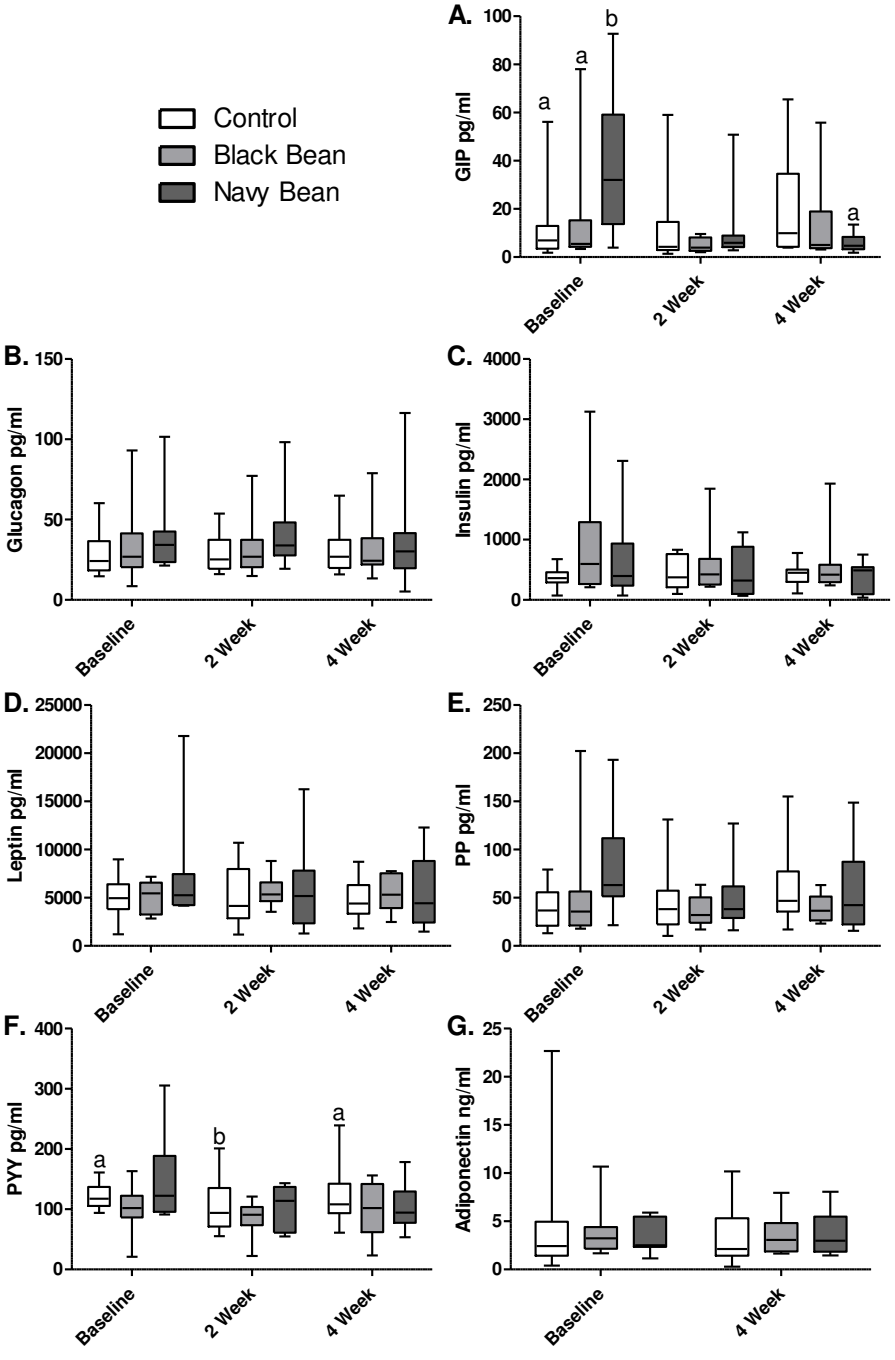

Figure 16: Fasted canine gut hormone levels in plasma at baseline and after 2 and 4 week of weight loss while consuming a control diet (clear bars), black bean diet (gray bars) or navy bean diet (dark gray bars). GIP (A), glucagon (B), insulin (C), leptin (D), PP (E), and PYY (F) were all evaluated together using a multiplex assay. Adiponectin (G) was evaluated alone using a single ELISA.

A panel of 11 inflammatory mediators was used to evaluate systemic inflammation in all 30 dogs at baseline and 4 weeks and 5 dogs from each group were also evaluated at 2 weeks. Inflammatory biomarkers included GM-CSF, IL-2, IL-6, IL-7, IL-8, IL-15, IL-18, IP-10, KC-like protein, and MCP-1. The number of dogs assayed at each timepoint and within diet group, and the number of samples with detectable amounts of each cytokine are shown in Table 29.

Table 29: Canine plasma samples with inflammatory biomarker levels above the limits of quantitation (detected/total assayed).

Diet	Control			Black Bean			Navy Bean		
Cytokine	Baseline	2wk	4wk	Baseline	2wk	4wk	Baseline	2wk	4wk
CRP	7/7	N/A	7/7	7/7		7/7	8/8		8/8
GM-CSF	4/10	3/5	4/10	5/10	3/5	6/10	4/10	3/6	5/10
IL-2	3/10	3/10	3/10	5/10	3/5	6/10	4/10	2/6	2/10
Il-6	3/10	2/5	3/10	4/10	3/5	5/10	4/10	2/6	2/10
Il-7	5/10	3/5	3/10	6/10	2/5	6/10	7/10	1/6	3/10
Il-8	10/10	5/5	10/10	9/10	4/5	10/10	10/10	6/6	9/10
Il-15	5/10	4/5	6/10	8/10	3/5	7/10	6/10	4/6	6/6
IL-18	8/10	4/5	8/10	10/10	5/5	8/10	9/10	5/6	9/10
IP-10	2/10	2/5	2/10	0/10	0/5	0/10	2/10	2/6	3/10
KC-Like	9/10	5/5	8/10	9/10	3/5	9/10	9/10	4/5	9/10
MCP-1	9/10	5/5	8/10	8/10	3/5	8/10	8/10	5/6	7/10

Multiple analytes were below limits of detection in over 50 % of the dogs. Of the detected analytes, the predominant inflammatory cytokine detected was IL-8 (detected in 96 % of the dogs), followed by IL-18 (87 %), and KC-like protein (86 %). No differences over time or between diet groups were observed. C reactive protein (CRP) has been evaluated in the majority of the 30 overweight dogs who completed the 4 week study, before and after weight loss. Within this subset, no significant differences in plasma levels of CRP were observed, before or after weight loss (Table 30). A non-parametric, one-way ANOVA was used to determine if there were significant differences between diet groups at each timepoint and Dunn's Multiple Comparison test was used to determine specific differences between groups. At 4 weeks, GM-CSF was

elevated in dogs in the BB diet group compared to dogs in the CON and NB diet groups, although there were no significant differences between baseline and 4 weeks in any of the dogs. IP-10 was the only other analyte significantly different between groups and at baseline was significantly higher in the CON group compared to the navy bean group (not detected in the black bean group) and at 2 weeks was higher in the navy bean group compared to the CON group; levels were not significantly different at 4 weeks between any diet group.

Detection of TNF-α and IFN-γ and verification of IL-6, MCP-1, and GM-CSF are currently underway with single ELISAs.

Table 30: Detectable levels of plasma inflammatory biomarkers in 30 overweight or obese dogs at baseline and after 2 and 4 weeks of calorically restricted weight loss.

	Control				Black Bean				Navy Bean			
Cytokine	0wk	2wk	4wk	p-value[a]	0wk	2wk	4wk	p-value[a]	0wk	2wk	4wk	p-value[a]
CRP	5.819 ± 0.76	n/a	5.68 ± 0.76	0.81	7.492 ± 2.42	n/a	7.61 ± 2.67	0.58	8.406 ± 6.73	n/a	6.11 ± 1.04	0.84
GM-CSF	49.96 ± 19.80	52.22 ± 21.76	36.69 ± 13.30	0.45	1223 ± 2540	94.22 ± 9.13	1106 ± 2482	0.99	56.56 ± 29.95	42.08 ± 33.58	41.53 ± 6.398	0.49
IL-2	68.94 ± 34.32	68.33 ± 47.43	45.76 ± 20.42	0.59	705.5 ± 1456	129.7 ± 98.87	707.8 ± 1544	0.76	61.77 ± 28.35	20.72 ± 15.87	69.86 ±5.96	0.25
Il-6	91.84 ± 115.7	147.3 ± 3.274	55.92 ± 59.09	0.36	432.4 ± 543.8	176.4 ± 124.9	415.3 ± 524.6	0.88	77.89 ± 27.75	42.88± 20.17	57.27 ± 25.26	0.09
Il-7	61.43 ± 33.01	72.0 ± 19.97	78.97 ± 11.46	0.87	302.1 ± 514.9	141.1 ± 45.35	357.2 ± 569.5	0.81	73.64 ± 39.14	51.91 ± n/a	109.9 ± 60.61	0.34
Il-8	10,010± 17,440	9,500 ± 13,852	8,476 ± 14,189	0.75	4,053 ± 5,487	2,992 ± 2,547	3,408 ± 3,150	0.75	3,345 ± 2,598	3,478 ± 4,683	3,309 ± 3,354	0.8
Il-15	253.1 ± 90.67	323.0 ± 127.6	155 ± 81.68	0.05	2,157 ± 5,213	326.5 ± 99.62	2,610 ± 6,014	0.99	419.30± 421.60	349.3 ± 468.3	401.3 ± 332.5	0.55
IL-18	79.42 ± 74.10	147.0 ± 86.66	55.47 ± 43.01	0.06	1,394 ± 4,128	105.2 ± 110.2	1,779 ± 4,622	0.51	91.07 ± 100.2	65.47 ± 61.00	57.62 ± 32.26	0.86
IP-10	55.73 ± 18.58	54.83 ± 15.66	56.28 ± 16.74	0.87	ND	ND	ND		47.61 ± 15.59	107.7 ± 58.29	74.73 ± 61.18	0.3
KC-Like	330.4 ± 585.5	181.5 ± 103.9	119.3 ± 89.09	0.64	97.56 ± 91.66	48.02 ± 25.54	90.27 ± 85.20	0.5	103.0 ± 67.52	94.16 ± 60.68	55.27 ± 36.61	0.09
MCP-1	189.5 ± 111.2	144.5 ± 64.22	180.5 ± 167.9	0.78	291.3 ± 317.5	241.2 ± 24.62	329.3 ± 350.3	0.88	155.9 ± 64.92	130.5 ± 42.72	130.6 ± 59.61	0.76

Data are presented as mean ± SD. The number of samples assayed and detected are given in **Table 29**.

[a]A one-way ANOVA with no repeated measures was used to analyze these data. Because all dogs were not detected at all timepoints a repeated measures test is not possible, each measurement is therefore treated like an independent variable. GM-CSF, IL-2, IL-6, IL-7, IL-8, IL-15, IL-18, IL-15, IL-18, IP-10, KC-Like, and MCP-1 were evaluated together in a multiplex assay. CRP was evaluated alone is single ELISA. ND = not detected; n/a = timepoint not assayed.

Non-targeted Metabolomic Analysis of the Metabolome of Overweight and Obese Dogs after Four Weeks of Calorically Restricted Weight Loss

Fecal, plasma, and urine samples were analyzed by GC- and LC-MS for changes in the metabolome in response to 4 weeks of weight loss (Figure 17). To define changes in the metabolome associated with time during weight loss, PCA components from each matrix significant by the time term were analyzed to determent the compounds or clusters that contributed to the weight loss/time effect. For fecal LC-MS data, PC1 (10.7 % variance), PC3 (8.0 % variance), PC6 (5.1 % variance), and PC7 (4.3 % variance) were all significant ($p < 0.05$). Figure 17A shows the total variance in LC detected fecal between baseline, 2 weeks, and 4 weeks between PC1 and PC3. Within PC1, 14 metabolites significantly explained the total variance associated with time, and 16 explained the variance in PC3, 7 of which were similar with PC1. In total, 37 clusters explained the variance associated with time. For fecal GC-MS data, PC7 (4.1 % variance) and PC12 (1.6 %) were significant with respect to time ($p < 0.05$), but only described a very small amount of the total variance (Figure 17B). PC7 contained 14 compounds, PC12 contained 27, 22 of which were unique to PC12.

Plasma LC components that were significant with respect to time were PC2 (12.2 % variance), PC6 (2.7 % variance), PC8 (2.0 % variance), PC10 (1.7 %) and PC17 (0.7 % variance). PC2 and PC6 are plotted in Figure 17C. PC2 contained 7 significant compounds, 4 of which were unique; PC6 contained 3 significant compounds, 1 of which was unique; PC 8 contained 3 significant compounds, 2 of which were unique; PC17 contained 7 unique compounds, 5 of which were unique. Plasma GC components that were significant with respect to time were PC6 (3.9 % variance), PC9 (2.9 % variance), and PC15 (0.09 % variance). PC6 and PC9 are plotted in Figure 17D. PC6 contained 13 significant compounds, 6 of which were unique to PC6; 14 compounds contributed to the variance in PC9, 9 of which were unique to PC9, and in

PC15, 10 compounds contributed to the variance, 5 of which were unique. In total, 29 compounds significantly presented to the variation over time.

In urine samples analyzed by LC variance over time with weight loss was explained in PC4 (5.9 % variance), PC7 (4.0 % variance), and PC15 (1.4 % variance). Figure 17E shows the variance over time in PC4 versus PC7. PC4 contained 8 compounds that contributed significantly to the variation, 1 of which was unique; PC7 contained 13 significant compounds, 4 of which were unique; PC15 contained 24 unique compounds, 17 of which were unique. In urine compounds detected by GC, components PC7 (2.4 % variance) and PC20 (0.6 %) were significant by time and are plotted in Figure 17F.

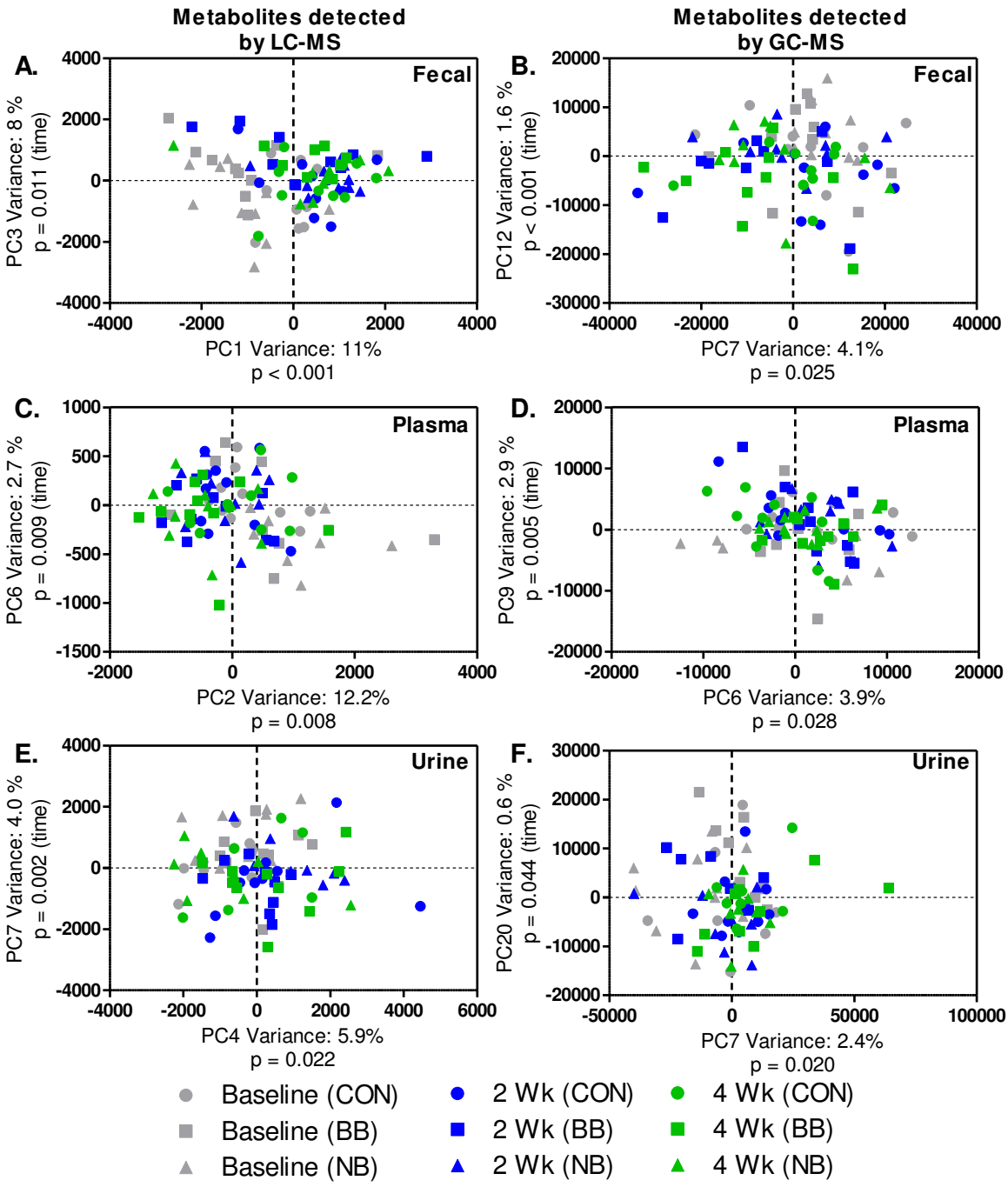

Figure 17: Orthogonal variation in the metabolome of 30 adult dogs undergoing calorically restricted weight loss for 4 weeks. Variation in the fecal metabolome with weight loss by LC-MS (A) and GC-MS (B). Variation in the canine plasma metabolome by LC-MS (C) and GC-MS (D). Variation in the canine urine metabolome with weight loss by LC-MS (E) and GC-MS (F). Different time points are indicated by color (baseline = gray, 2 weeks = blue and 4 weeks = green) and diet groups are indicated by shape (circle = control, square = black bean, and triangle = navy bean).

The number of compounds or clusters detected by LC- or GC-MS in canine feces, plasma, and urine that significantly contributed to variation by diet (CON, BB, or NB) and weight loss (time) are summarized in Figure 18. Also included are data from Chapter 3, which describe the variation in the canine metabolome by weight class (normal weight, overweight or obese). There were 152 metabolites that varied by weight loss, 139 that varied by diet, and 78 that varied only by weight class. Ten metabolites varied by diet, weight loss, and weight class; 43 metabolites describe variation by both weight loss and diet; and 20 by adiposity and weight loss.

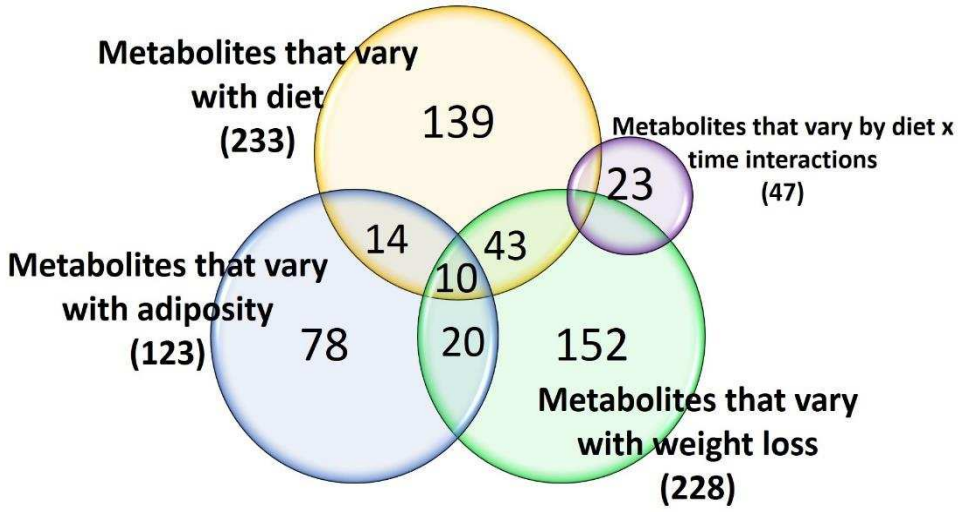

Figure 18: Number of compounds that contribute to the orthogonal variation in the canine fecal, plasma, and urine metabolome by diet, weight classification, and time with weight loss. Numbers in parenthesis indicate the total number of compounds that contribute to PC significantly associated with weight class (normal weight, overweight, or obese); time with weight loss (0, 2, or 4 week time points); and diet, CON, BB, or NB. The small circle indicates a special statistical case where the diet and time with weight loss interactions were significant: 23 of the 47 metabolites were only different by the interaction term.

Effects of Bean-Consumption on the Canine Metabolome during Calorically Restricted Weight Loss

To determine which metabolites were differentially altered in the bean consuming dogs compared to dogs consuming the CON diet, the relative abundance of each individual metabolite was analyzed for variation using mixed model regression. The total number of detected and significant clusters are presented in Table 31**.** GC-MS platforms detected the highest number of compounds across all three matrices, however LC-MS compounds had the highest proportion of significantly different compounds; 35 clusters were associated with time, 411 clusters were associated with time and 48 clusters were significant by diet x time interactions.

Table 31: Total number and number of significant metabolite clusters detected by GC- and LC-MS in canine fecal, plasma, and urine.

		Number of Clusters by Matrix		Number by Factor and Interaction		
Matrix		*GC*	*LC*	*Diet*	*Time*	*Diet x Time*
Fecal		**1,112**	**795**			
	Diet	0	21	**21**		
	Time	2	173		**175**	
	Diet x Time	0	25			**25**
Plasma		**554**	**348**			
	Diet	0	2	**2**		
	Time	17	39		**56**	
	Diet x Time	3	11			**14**
Urine		**1,139**	**1,318**			
	Diet	0	12	**12**		
	Time	1	179		**180**	
	Diet x Time	0	9			**9**
Total		**23**	**471**	**35**	**411**	**48**

Total number of detected and significant clusters are indicated by bolding. Significance was determined with mixed model linear regression and FDR corrected p values < 0.10 were accepted as significant.

To define the metabolites that were differentially modulated in the bean diet groups, the 48 compounds or clusters were further evaluated to determine the time and group the differences occurred in. The plasma compounds were primarily different between the NB and CON groups at baseline (data not shown). Compounds that were similar between all three groups at baseline and only changed in bean-consuming dogs are summarized in Figure 19. Three compounds

increased in both the BB and NB fecal samples (Figure 19A), 3 increased in urine samples (Figure 19C), while 4 compounds decreased in fecal samples (Figure 19B). Identification of these compounds is underway, but one fecal compound (C58, Figure 19A, middle panel) has been putatively identified as uvaol, a triterpenic daialcohol.

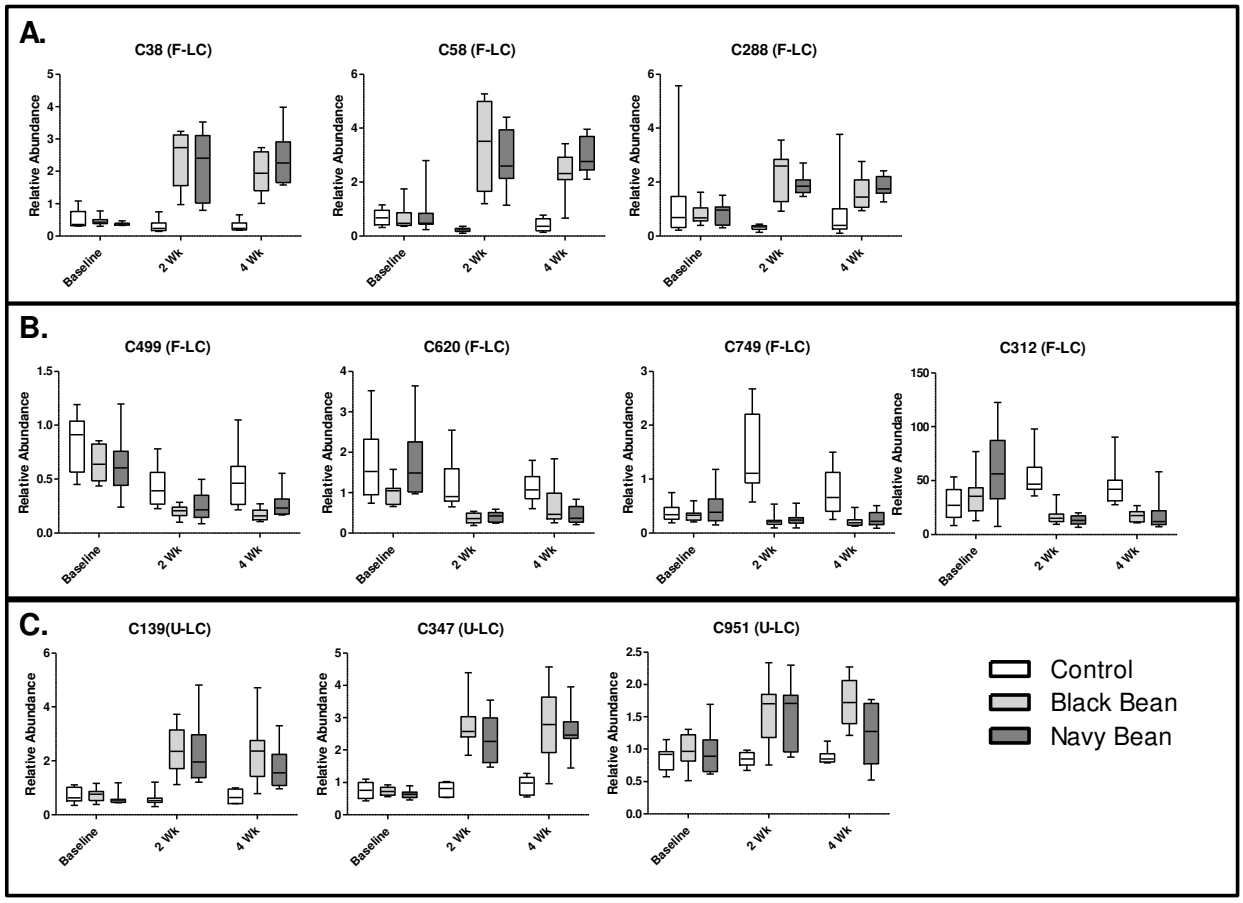

Figure 19: Differentially modulated metabolites by time in the overweight canine metabolome during weight loss. A) Fecal (F) metabolites detected by UPLC-MS that increased in both bean BB and NB diet groups and were significantly different from CON ($p < 0.05$); B) fecal metabolites that decreased in both the BB and NB group compared to CON; C) Urine metabolites that increased in the BB and NB groups compared to CON. The CON group is indicated by clear boxes, the BB group by light gray boxes, and the NB group by dark gray boxes. Samples were collected at baseline, and 2 and 4 weeks after beginning a 4 week, calorically restricted weight loss intervention. Each compound is labeled with its cluster number (C), matrix (F=fecal, U=urine), and detection platform (LC or GC).

Targeted Macronutrient and Non-Targeted Metabolomic Analysis of Diets Reveals Diversity in Amino Acids, Lipids, and Small Molecule Profiles of Bean-Based Dog Foods

We previously reported that the CON, NB, and BB diets were nutrient matched and isocaloric (Table 15) and that the bean-based diets were digestible compared to CON (Table 17). The total crude protein content in all three diets was approximately 26 % as-fed. Total amino acid content as a percent of total diet is shown in Figure 20 in order of descending abundance relative to CON. Glutamic acid was the most abundant amino acid (3.72 %) followed by proline (2.13 %), glycine (1.89 %), leucine (1.71 %), arginine (1.55 %), aspartic acid (1.54 %), alanine (1.50 %), valine (1.48 %), lysine (1.3 %), isoleucine (0.97 %), threonine (0.96 %), tyrosine (0.96 %), phenylalanine (0.85 %), serine (0.82 %), histidine (0.69 %), methionine (0.49 %), cysteine (0.36 %), tryptophan (0.32 %, and other amino acids such as taurine (2.96 %). The BB diet had 6 amino acids that were at least 10 % different from and higher than CON, in order of difference: serine (52 %), tryptophan (31 %), aspartic acid (27 %), lysine (18 %), threonine (14 %), and valine (14 %); unidentified amino acids were 37 % lower than CON. The NB had 10 amino acids that were at least 10 % different from CON. In order of difference, amino acids that were higher in NB were cysteine (50 %), tryptophan (38 %), lysine (22 %), phenylalanine (15 %), tyrosine (15 %), valine (14 %), and leucine (11 %). Amino acids that were lower in the NB compared to CON were proline (25 %), glycine (-16 %), histidine (15 %), and other amino acids (25 %). Nine amino acids were at least 10 % different between BB and NB: the BB diet had higher levels of serine (33 %), proline (23 %), glycine (17 %), histidine (16 %), and aspartic acid (13 %). Amino acids that were higher in NB compared to BB were cysteine (59 %), phenylalanine (21 %), leucine (17 %), isoleucine (13 %), and other amino acids (20 %). The summed magnitude of difference in amino acids different by greater than 10 % between CON and BB was 193 %, 245 % between CON and NB, and 232 % between BB and NB.

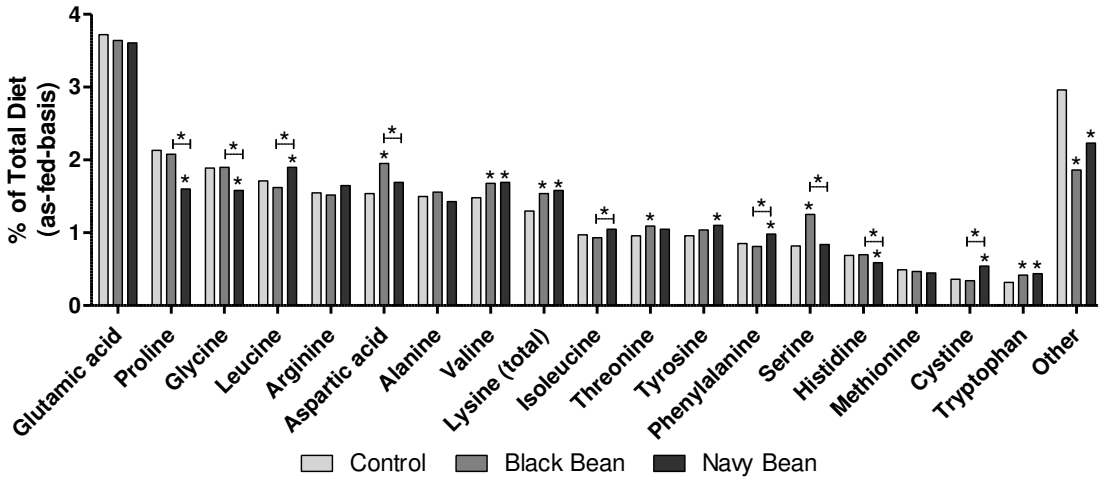

Figure 20: Total amino acid profiles of low-fat CON (gray bars), BB (dark gray bars) and NB (black bars) dog foods as a percent of total diet. "Other" amino acids include non-protein amino acids, such as s-methyl cysteine, conditionally essential amino acids such as taurine. Amino acids that were at least 10 % different from CON are indicated by asterisks (*) and amino acids that have at least a 10 % difference between BB and NB are indicated with bars and asterisks (|-*-|).

Total crude fat content of the diets was between 8 % - 8.4 % (Table 15). Fatty acid content is presented as the relative abundance of total fat (Figure 21): the most abundant unsaturated fatty acids in CON were oleic acid (29.42 %), linoleic acid (26.02 %), α-linolenic acid (3.28 %), arachidonic acid (0.76 %), gondic acid (075 %), eicosapentaenoic acid (0.26 %), and eicosadienoic acid (0.09 %). In order of abundance, saturated fatty acids were palmitic acid (26.03 %), stearic acid (7.13 %), palmitoleic acid (4.73 %), myristic acid (0.85 %), margaric acid (0.21 %), arachidic acid (0.16 %), myristoleic acid (0.14 %), and pentadecylic acid (0.16 %). In the BB and NB diets, α-linoenic acid was increased 66 % and 100 %; eicosapentaenoic acid was increased by 38 % and 31 %; arachidic acid was increased by 31 % and 38 %; palmitoleic acid was decreased by 28 % and 14 %; myristic acid was decreased by 27 % and 28 %; and gondic acid was decreased by 31 % and 16 % difference, respectively. Compared to CON, the BB diet was higher in linoleic acid (23 % difference); myristoleic acid, arachidonic acid, and eicosadienoic acid were not detected. Compared to CON, the NB bean diet was higher in

arachidonic acid (26 % difference), and lower in stearic acid (12 %), pentadecylic acid (14 %), and myristoleic acid (25 %). The NB diet also contained the unique fatty acids behenic acid (0.19 % relative abundance) and eicosatrienoic acid (0.20 %). Between the BB and NB diets, the BB diet contained higher amounts of pentadecylic acid (14 % increase), margaric acid (13 % increase), and linoleic acid (12 % increase); compared to BB, the NB diet contained higher amounts of gondic acid (21 % increase), α-linolenic acid (20 % increase), and palmitoleic acid (19 % increase).

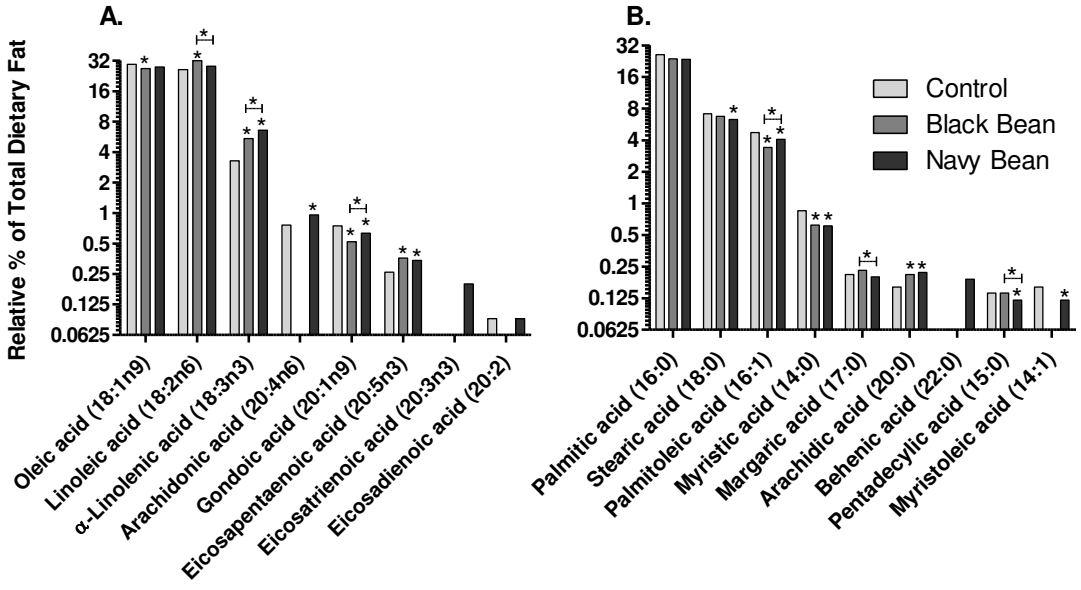

Figure 21: Fatty acid profiles of low-fat CON (gray bars), BB (dark gray bars) and NB (black bars) dog foods as a relative percent of total dietary fat. Fatty acids that were at least 10 % different from CON are indicated by asterisks (*) and amino acids that have at least a 10 % difference between BB and NB are indicated with bars and asterisks (|-*-|).

Non-targeted metabolomics was used to detect small molecules and phytochemicals in the CON, BB, and NB dog foods. One thousand, two hundred two clusters were detected by LC-MS and a PCA reveals a significant variation of 54 % ($p < 0.001$) between the CON and bean-based diets (PC1), and a variation 16 % ($p < 0.001$) between BB and NB diets (Figure 22A). Twenty-seven LC clusters significantly ($p < 0.05$) describe the variation in PC1 between bean

CON foods and 15 LC clusters significantly describe the variation in PC2 between the two different bean diets. One thousand, eight hundred twenty-one clusters were detected by GC-MS and a PCA reveals a significant variation of 75 % ($p < 0.001$) between the CON and bean-based diets (PC1), and a non-significant variation of 13.7 % ($p = 0.052$) between BB and NB diets (PC2, Figure 22B). Thirty GC clusters significantly ($p < 0.05$) describe the variation in PC1 between the CON and bean-based diets. One of the primary metabolites currently identified in PC1 was 2-piperidinecarboxylic acid. This compound contributed to the variation between the CON and bean diets, but was not a contributor in PC2 (differences between the bean diets).

Metabolites that were at least 10 % higher in both bean groups, compared to CON are expected to included carbohydrates, amino acids, peptides, lipids, and phytochemicals such as trigonelline and s-methylcysteine.

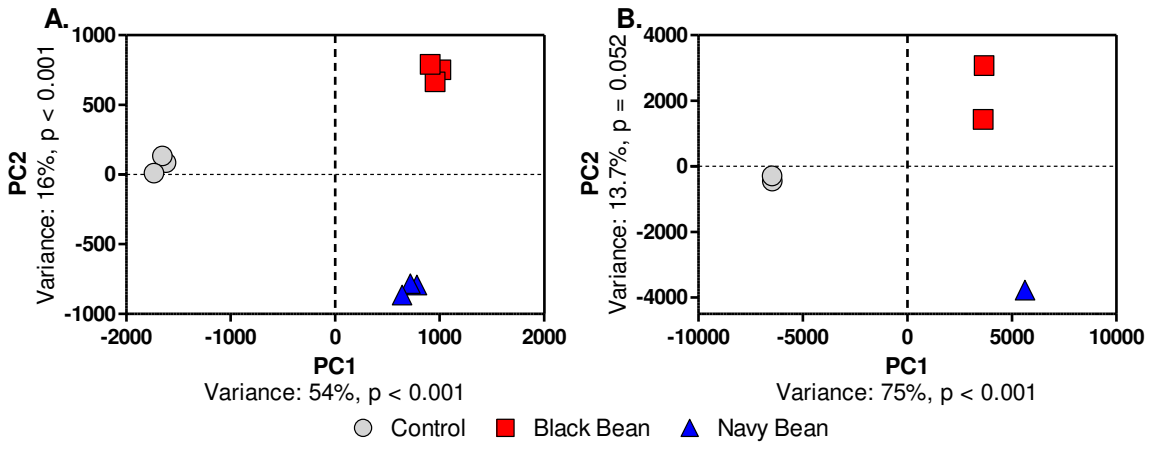

Figure 22: Principal components analysis (PCA) of dog food metabolites detected by non-targeted LC-MS (A) and GC-MS (B). Control diet replicates are indicated by gray circles, black bean diets are indicated by red squares, and navy bean diets are indicated by blue triangles. P values indicate the level of significance each component contributes to the variation between samples.

DISCUSSION

Bean Intake Was Not Strongly Associated with Changes in Canine Gut Hormone Expression and Inflammatory Biomarkers Were Below Limits of Detection

A panel of gut hormones and inflammatory cytokines was assayed in the plasma of overweight and obese dogs undergoing calorically restricted weight loss. Gut hormones were detected in all samples, but did not appear to be modulated overtime, regardless of diet. The exception to this was GIP, which was higher in the NB group at baseline compared to both CON and BB groups, but was significantly reduced by 4 weeks so that there were no differences between groups. GIP, previously gastric inhibitory peptide, has been renamed as glucose-dependent insulinotropic peptide to reflect its role in increasing insulin secretion. GIP expression has been shown to be important in coupling excess nutrient intake to obesity[382] and decreasing GIP expression in mice has been shown to prevent obesity in the context of a high fat diet.[383] The elevation GIP in the NB group may indicate a concentration of dogs with higher risk for metabolic syndrome than in the CON and BB group. This hypothesis is somewhat supported by the observation that the majority of plasma metabolites that were significant by time and diet interactions, were different between the NB and CON groups at baseline. The observation that modulation of GIP only occurred in dogs with elevated levels is consistent with multiple other studies showing that bean-derived phytonutrients have discriminatory activity, such that physiological effects are only observed when abnormal perturbations are already present, such as in the case of cancer.[384-391] Because this effect was only seen in the NB group where GIP levels were different at baseline, it is difficult to determine whether this effect occurred as an effect of weight loss, bean intake, or a combination of both, but does provide additional rationale for the continued investigation of the role of bean intake in modulating metabolic aberrancies associated

with canine obesity. PYY was slightly increased in the CON group after 2 weeks of weight loss and then went back to baseline levels at 4 weeks. PYY is an anorexic hormone, and increases postprandially, in humans, for up to 6 hours.[392] Given the state of caloric deficit of these dogs, anorexic hormones would be expected to decreases, this finding may reflect a true physiological effect; however it seems more likely some of the CON dogs may have inadvertently had shorter fasting times before the blood draw at the two week time point. The rationale for measuring serum levels of these gut hormones was to determine if a bean-based diet was able to increase satiety and insulin sensitivity in dogs undergoing weight loss. These data support that in dogs with abnormal PYY expression, a bean diet may play a role in regulation. Further studies are warranted to determine the effects of bean intake on canine gut hormone expression postprandially. While gut hormone levels are a relatively simple way to approximate satiety, the majority of endocrine molecules have very short half-lives and are most dynamic immediately after food consumption, such as GIP, which has a half-life of minutes. Other molecules may be enzymatically degraded, as is the case with amylin, ghrelin, and GLP-1. To appropriately detect these analytes in plasma, enzyme inhibitors must be present in the blood collection tube at the time of collection. As enzyme inhibitors were not used at the time these samples were collected, we were unable to accurately detect other, important gut hormones. In addition to measuring changes in gut hormones postprandially, future studies should also employ the use of direct measurable outcomes of satiety, such as amount of food consumed as a second meal, as satiety can be influenced by multiple factors outside of hormone expression. Insulin sensitivity could also be assessed directly via euglycemic clamp or glucose tolerance methods after a period of dietary bean intake. In order to control for fasting compliance, future study designs should also

utilize a testing structure that allows companion dogs to be fed and sampled in a controlled environment.

For the majority of the samples, inflammatory cytokine concentrations were below the limits of quantification for the multiplex assay. While this platform has been well validated for other species, only performance parameters with recombinant protein in an artificial plasma matrix have been validated for canine specific analytes. One of the advantages to using the multiplex assay is that a minimum amount of sample is needed to detect multiple analytes, however detection can be affected by the plasma matrix making it difficult to determine if circulating cytokines were truly low, or if the plasma matrix interfered with detection. Given the small number of samples in which inflammatory cytokines were detected, a valid statistical measurement of changes over time was not possible. CRP was analyzed in a subset of the dogs and was detected in all samples; however levels did not change after 4 weeks.

Previous studies have shown significant reductions in circulating canine cytokines after 3 and 6 months of weight loss using this same panel[374] and other studies have reported decreases in CRP and MCP-1 after completion of a weight loss program.[393] Interestingly, in intact female research dogs, inflammation indices did not change with weight loss.[394] While multiple examples of discrepancies are present in published research regarding the relationship of canine obesity and gut hormones, adipocytes, and inflammatory biomarkers, and the ability of weight loss to modify these parameters, current published studies typically have small sample sizes and utilize diverse populations.[395] Changes in gut hormones and inflammatory biomarkers have not been reported with only 4 weeks of weight loss in dogs. Aside from the low detection frequency in inflammatory biomarkers, the lack of change in this study could be a function of the short amount of time assayed and the relatively low level of body weight lost, sample size or the

sampling time in relation to the last meal. In human studies, a single bean-based meal has been shown to alter glucose metabolism, reduce inflammatory biomarkers, and modulate gut hormones[70] and in this cohort of dogs, lipids were modulated after only 2 weeks of bean consumption with weight loss[396], indicating metabolic pathways were altered. These observations support that changes in adipokine expression may be occurring in these dogs and provides the rationale for the further investigating inflammatory biomarker concentrations with assays less sensitive to the plasma matrix and sampling collections after meal consumption for gut hormone analysis.

Non-targeted Metabolomics Reveals Changes in the Metabolome of Overweight and Obese Dogs after Four Weeks of Calorically Restricted Weight Loss

Changes in the canine metabolome over time revealed a large number of compounds that changed over the 4 week period regardless of diet. Compounds detected in fecal, plasma, and urine samples contributed to the variation between baseline, 2 and 4 week samples (Figure 19) and 411 compounds were significant by time in at least one of the diet groups (Table 31).While these compounds have yet to be individually identified, metabolomic studies in humans undergoing weight loss have revealed changes in serum lipids, such that saturated and monounsaturated fatty acids are decreased.[397] Other studies have found that individuals with a greater weight loss response can be differentiated by decreased levels of citric acid, succinic acid, dodecanol, threonic acid, and coumaric acid and increased levels of arachidonic acid.[398] It is therefore reasonable to expect the metabolite changes in this cohort of dogs will be associated with serum lipids and energy metabolism. These data indicate that the canine metabolome is highly responsive to weight loss and that changes can be detectable after 2 and 4 weeks.

Additional analyses and future studies in dogs may be able to define metabolites associated with greater weight loss to determine the efficacy of weight loss interventions for individual dogs. [286]

Consumption of Bean-based Diets during Weight Loss Modulates Individual Metabolites within the Canine Metabolome

Ten metabolites changed in both bean diets over time and were different from CON. Three compounds increased in fecal samples, one of which has been identified as uvaol, a compound usually described in olive oil, ubiquitous in plants[399], but not yet described in common beans. Uvaol may have cardiovascular protective, chemopreventive, antioxidative, and anti-inflammatory properties[400] and may represent a novel mechanism by which beans improve health outcomes. Further studies are needed to confirm the contribution of uvaol by the bean powders; however, as the only ingredient difference between the CON and bean-based dog foods were the cooked bean powders, it is reasonable to attribute the presence of uvaol in the feces of dogs consuming the bean-based diets to the bean powders themselves. As the metabolite profiles of the diets are now available, they can be interrogated for the presence of uvaol, however metabolomic analysis of the navy bean powder used in the NB diet did not reveal the presence of this compound (data not shown).

Four fecal compounds decreased in the bean groups over time and compared to CON. Previous metabolites that decreased in bean fed dogs were vitamin E isoforms and bile acids, both of which were detected by GC-MS.

Three compounds increased in the urine of dogs consuming a bean-based diet. Based on results from previous studies in dogs consuming a navy bean diet for weight maintenance, we hypothesize that these compounds will represent bean phytochemicals that are biomarkers of bean intake such as trigonelline.[94]

In addition to these compounds that changed over time in both bean groups and compared to CON, additional compounds may have changed in only one bean group. Additional interrogation of this data set could reveal changes that are specific to one bean diet group. One factor complicating the interpretation of these data is that for the fecal, plasma, and urine metabolome analysis, baseline data from an additional 15 overweight and obese dogs are currently included. Because the individual ID was accounted for in the time course analysis, this should have minimal effects, but could dilute or exaggerate any true baseline variation between the diet groups. Eliminating these dogs from future analysis could reveal more metabolites that vary by the time x diet interaction.

Cooked Bean Powder Dog Foods Have Amino Acid Profiles Associated with Increased Satiety and Weight Reduction

Total protein content is associated with increased satiety, however amino acid profiles have been shown to play an important role as well.[59; 401] Overall, compared to CON, bean-based diets were higher in tryptophan, lysine, and valine; BB diets were lower in unidentified amino acids, but higher in serine, aspartic acid, and threonine; NB diets were higher in cysteine, phenylalanine, tyrosine, and leucine, but lower in histidine, glycine, proline, and other amino acids (Figure 20). Furthermore, the two bean-based diets were different from each other with respect to serine, proline, glycine, histidine, aspartic acid, cysteine, phenylalanine, leucine, isoleucine, and other, unidentified amino acids.

Tryptophan had a relative increase of 31 % and 38 % in the BB and NB diets, respectively. Tryptophan supplementation has been associated with increased satiety[402] as it can be metabolized into serotonin.[403] However, in obese humans undergoing weight loss, circulating levels of tryptophan do not correlate with satiety[401] possibly because in obese adults, the

kynurenine pathway is over expressed[404] and tryptophan is metabolized into kynurenine, kynurenic acid, xanturenic acid, and quinolinic acid.[403] Tryptophan metabolism has been shown to change in dogs undergoing rapid weight gain[372] and serotonin levels are lower in obese dogs than normal weight dogs.[405] While multiple factors determine metabolism, higher content of dietary tryptophan could lead to higher levels of serotonin synthesis.

Common beans are rich in branched chain amino acids, and leucine and valine were higher in the NB, and NB and CON diets, respectively. When administered centrally, leucine has been shown to induce anorexia, enhance expression of leptin, and reduce body weight.[406] While studies with oral supplementation of leucine have shown mixed anorexic effects, convincing evidence supports that increased dietary leucine may decrease adiposity by increasing metabolic rates and improve glycemic control.[406] Overall, higher levels of dietary branched chain amino acid intake is associated with reduced rates of obesity[407] and may represent a beneficial bean-related amino acid profile for regulating a healthy body weight.

Interestingly, total arginine and glutamate (as glutamic acid) were not different between any of the diets (average relative abundance 1.57 %, and 3.67 %, respectively) although beans have been shown to be relatively high in these amino acids. Glutamine is the primary amino acid across multiple dietary proteins and is highly associated with satiety[408] thus, the relative potential contribution of glutamine to satiety was equivalent between diets. While beans are limited in sulfur containing amino acids, methionine only had minimal differences between the CON and bean-based diets, but lysine was 18 % and 22 % higher in the BB and NB diets, respectively. While restricting dietary methionine has been shown to exert beneficial metabolic effects in obese adults[409], these data indicate that replacement of 25 % weight/weight of the diet ingredients with cooked bean powder does not result in limiting essential amino acids, and

results in an amino acid profile that, independent of other nutrients, provide additional benefits to weight management.

Lipid Profiles of Bean-Based Diets Support Reduced Inflammation, Dyslipidemia and Insulin Resistance

While the total fat profiles of the CON, BB, and NB diets were similar, individual fatty acids varied both with the inclusion of bean powders and by the type of bean powder (Figure 21). Bean-based diets were higher in the omega-3 fatty acids α-linolenic acid and eicosapentaenoic acid while only the NB diet contained eicosatrienoic acid. Omega-3 fatty acids are strongly associated with decreasing body weight, modulating gut hormones, and reducing inflammation and insulin resistance.[410; 411] In humans and lab animals, α-linoleic acid is inversely associated with adiposity[412], eicosapentoaenoic acid is associated with improving insulin sensitivity and reducing inflammation.[413] While excessive supplementation of omega-3 fatty acids can have undesired consequences in dogs[414], dietary levels are associated with improvements in osteoarthritis[415] and inflammation[416]. While common beans do contain relatively high levels of the omega-3 fatty acid α-linoleic acid, because the overall fat content is so low, they are generally regarded as having a minor role in overall dietary contribution.[417] The primary source of the remaining fatty acids was most likely the fish meal, which was composed 1 % weight/weight of each of the dog food formulations (Table 15). In general, the bean based diets were lower in saturated fatty acids than CON (with the exception of arachidic acid and margaric acid): both bean diets were lower in palmitoleic acid, myristic acid, and myristoleic acid (which not detected in the BB diet; the NB diet was lower in stearic acid, and pentadecylic acid, but contained behenic acid, which was not detected in either the CON or BB diet. Lower levels of dietary saturated fatty acids are associated with improvements in circulating lipid levels and may

improve cardiovascular health[418], and while of lower concern in dogs, obese dogs are at higher risk for cardio-respiratory disease than normal weight dogs.[419] Overall, the lipid profiles of the bean based diets are consistent with profiles associated with reducing inflammation, improving serum lipid profiles, and beneficially modulating glucose homeostasis.

CONCLUSIONS

This study evaluated the effects of including a cooked bean powder at 25 % weight/weight on the nutrient profiles of macro and micronutrient matched CON, BB, and NB extruded dog foods and the metabolic effects of these diets on dogs undergoing weight loss. Targeted analysis of the amino acid and lipid contents of the diets demonstrated profiles consistent with beneficial effects on metabolism and inflammation. The non-targeted metabolomic analysis of the diets revealed a subset of compounds that together described up to 75 % variance between the CON and BB and NB diets, while variance between the BB and NB was also significant with 16 % of the variance explained. 2-piperdinecarboxylic acid was one of the identified diet metabolites that has previously been described as biomarker of bean intake in humans and mice. The metabolomes of dogs changed significantly with weight loss, regardless of diet and compounds were identified that differentiated dogs consuming a bean-based diet from dogs consuming a CON diet. While weight loss and bean consumption were not associated with changes in detectable inflammatory biomarkers or gut hormones in this study, the metabolome of these dogs offers the opportunity to discover metabolites associated with improvements in lipid and carbohydrate metabolism and other potential, small molecule mediators of inflammation. Further studies are needed to determine the ideal sample collection period to detect changes in canine gut hormones and to optimize detection of low level circulating inflammatory biomarkers.

This study has demonstrated the beneficial effects of including cooked bean powders in dog foods formulated for weight loss and the magnitude of canine metabolites that are responsive to weight loss.

CHAPTER 9: CONCLUSIONS AND FUTURE DIRECTIONS

Summary of Major Findings

- Companion dogs are exposed to potential endocrine and metabolic disruptors through environmental pollutants and obesity.
- Bean-based diets are safe, digestible, and well tolerated in companion dogs.
- Metabolic aberrancies in overweight dogs can be modulated with weight loss.
- Modulation of lipid and carbohydrate metabolism is enhanced in companion dogs consuming beans.

Our hypotheses that companion dogs are exposed to low dose mixtures of pesticides, and that overweight and obese dogs have altered clinical serum biochemical analytes and hemograms, altered gut microflora, and metabolic aberrancies compared to normal weight dogs were partially supported:

- Low levels of atrazine were detected in the urine of all 21, normal weight dogs.
- Dogs were exposed to a novel mixture of 15 parent pesticide compounds from the environment with the potential to disrupt metabolic and endocrine function.
- Diet was not a driving factor for pesticide exposure in this cohort of dogs.
- The proportions of overweight and obese dogs with hemolysis, CK, and AST levels outside reference ranges were higher than normal weight dogs.
- The fecal microbiomes of all dogs were highly diverse and not different between normal weight, overweight, or obese dogs.
- The metabolomes of overweight and obese dogs were significantly different from normal dogs. The greatest variation was in plasma metabolites detected by the LC platform.

Our hypotheses that nutritionally complete bean-based dog foods are digestible, safe, and nutritionally adequate for adult dog weight maintenance and support calorically restricted weight loss were supported:

- For weight maintenance, the navy bean-based dog food was equally as digestible as the matched control diet.

- For weight loss, low fat, black and navy bean-based dog foods were as digestible as the nutrient matched control diet.

- Protein digestibility was higher in the black bean dog food and carbohydrate digestibility was higher in both the black and navy bean-based dog foods, compared to control.

- Total percentage of body weight loss was equivalent between all calorically restricted weight loss groups.

- All dogs maintained indices of nutritional adequacy as defined by AAFCO.

- None of the owners reported noticeable changes in their dog's flatulence, regardless of diet.

Our hypotheses that dogs consuming a bean based diet will have alterations in their lipid and carbohydrate metabolism and that inclusion of 25 % weight/weight cooked bean powder nutritionally complete dog foods increases phytonutrient diversity were supported:

- After consuming a navy bean based dog food for 4 weeks, normal weight dogs had lower cholesterol than dogs consuming the nutrient matched control diet.

- Only dogs consuming a navy or black bean based diets had reductions in triglycerides, HDL and LDL levels at 4 weeks.

- Fecal glucose and steroid-like excretion increased in bean consuming normal weight dogs.

- Urine metabolites included validated biomarkers of bean intake.

- Fasted gut hormone levels and detected inflammatory biomarkers did not change with weight loss or bean intake.

- The urine and fecal metabolome of overweight and obese dogs shifted significantly in response to weight loss, independent of diet. However, 10 fecal and urine metabolites differentiated metabolic changes in both bean groups over time compared to the control group.

- Bean-based diets had distinct lipid and amino acids profiles compared to control diets and bean type. The navy bean diet comprised a distinct carbohydrate and phytonutrient profile that included known lipid-lowering molecules.

- Small molecule variation between bean based diets was significant by both LC- and GC-MS, but only LC metabolites described significant variation between the black and navy bean diets.

Limitations

- One of the limitations of these studies was the significant baseline variation between dogs. The diversity of the canine participants was intentional to represent the physiological variations in companion dogs; however, it limited our ability to detect subtle changes. For example, serum cholesterol was different in the short-term weigh loss study in the black bean diet group compared to both navy bean and control, but dogs were randomized based on BCS, not cholesterol. This may have masked subtle differences in cholesterol metabolism differences between bean and control groups. Future studies may need to increase the randomization criteria and sample size or use cross-over study designs and run-in periods to minimize variation and increase power for detecting subtle effects on metabolic parameters.

- A second limitation to these studies was owner compliance and is inherent to studies in companion animals. Several owners reported that their dogs may not have been appropriately fasted, and when metabolic analyses supported this (such elevated triglyceride levels or metabolome outliers) data would require exclusion. In other cases, owners were unable to complete fecal and urine sample collections at home or mixed samples. To control for food intake prior to sample collection, future studies could implement study designs that required overnight housing to insure compliance and increase the integrity of sample collection.

- Especially in the long-term weight loss study, several owners requested to move into a weight maintenance phase before ideal weight was reached and one participant was withdrawn from the study because the owner was unwilling to stop feeding treats. The primary source of follow up for the weight loss studies were weight checks every 2 weeks. Given the known challenges associated with successful weight loss in companion dogs, compliance to weight loss protocols could be potentially enhanced by more frequent interactions with owners to track compliance.

- This study was not able to accurately evaluate ghrelin, amylin, or glucagon-like peptide-1 as they have short half-lives, are enzymatically degraded, and can only be reliably detected when enzymatic inhibitors are added to plasma at the time of the draw.

- In this cohort of 66 dogs, overweight dogs were more likely to have hemolytic blood draws which could further decrease the sensitivity of clinical blood analysis.

Conclusions

- Clinical biochemical and hemogram analysis may lack sensitivity in detecting metabolic aberrancies in dogs that may increase disease risk and support expanded use of metabolomic platforms to assess metabolic dysfunction in dogs.

- Overweight dogs have altered metabolomes compared to normal weight dogs and future interrogation of this data may reveal similarities between cancer and obese metabolic profiles.

- Serum chloride levels were different between normal weight, overweight, and obese dogs but normalized in the navy bean group with 4 weeks of weight loss. These data indicate that more pronounced effects of bean intake may be observed in the presence of dysregulated metabolism.

- The bean-based diet associated modifications of the canine metabolome are most likely the result of small molecule or phytochemical content of beans as the macro and micronutrients were controlled.

- Dog foods containing 25 % w/w cooked bean powder contain adequate amounts of phytochemicals to detect bean-associated biomarkers of intake in dogs.

- The plasma metabolomes described the greatest variation between normal weight and overweight dogs while the fecal and urine metabolomes contained the greatest number of metabolites that were responsive to diet and weight loss interventions. These data may indicate that excreted, versus circulating molecules are most useful for detecting responses to interventions while circulating molecules may be more useful for detecting established metabolic dysfunction.

- Our hypothesis that bean intake improves gut hormone profiles was not supported by the results from this study. While it is possible that the amount of weight lost after 4 weeks was not sufficient to induce detectable changes, it is also possible that hormone expression changed postprandially and was therefore not detected in fasted samples. In addition to postprandial effects of bean intake on satiety and insulin sensitivity related hormones, future studies should also directly assess the effects of bean intake on glucose homeostasis and insulin responsiveness.

FUTURE DIRECTIONS

Further Interrogation of Metabolomic Data

The next step for evaluating the obesity-associated differences, and diet and weight-loss modifiable compounds in the canine metabolome, is to annotate, or identify the metabolites that

were different between normal weight, overweight and obese dogs and determine metabolic pathways that are disturbed in overweight dogs and modulated with weight loss. To begin, specific metabolite biomarkers associated with obesity will be assessed. For example, in obese humans, higher levels of circulating amino acids have been detected and may be linked to increasing insulin resistance by mTOR activation[420] and associated with increases in obesity associated gut-permeability.[421] We will be able to determine if overweight/obese dogs have higher levels of circulating amino acids and detect previously described biomarkers of mTOR activation such as phosphatidylcholine and lysophosphatidic acids metabolites.[422] Elevated levels of mannitol or xylitol in plasma samples would provide evidence for increased gut permeability[423], but, in this particular cohort of dogs, could be confounded by dietary intake.

Human metabolomic studies have also reported a strong relationship between obesity and circulating levels of energy and glycolysis related compounds, such that glucose, lactate, pyruvate, and glycerol are elevated along with small molecule indices of inflammation, such as glycoprotein acetyles.[420] To globally determine specific pathways with potential disruption in overweight and obese dogs, annotated compounds will be uploaded to Metaboanalyst[424] (http://www.metaboanalyst.ca/), a metabolomic pathway analysis tool that links individual metabolites with their respective metabolic pathways. This will allow us to determine the full range of metabolic pathways differentially affected in overweight and obese dogs and to cross reference them with pathway disruptions associated with cancer. In addition to providing a greater understanding of the metabolic perturbances associated with canine obesity, these data will also provide novel insight into the mechanisms underlying obesity associated co-morbidities and has the potential to show a metabolic link between obesity and cancer in dogs.

The amino acid and lipid profiles of the bean-based diets have been associated with decreased inflammation and increased insulin sensitivity and may have affected metabolic pathways upstream from gut hormone expression, such as mTOR activation. We will annotate the small molecules in the bean diets and determine their potential bioactivity. Future studies are needed to quantify the presence of bioactive molecules in bean-based diets and to correlate intake with modulation of metabolic parameters. Once annotations have been completed in the canine metabolome, we will also determine metabolic pathways altered during weight loss, with and without bean intake. Of particular interest will be modification of aberrant pathways shared between obesity and cancer. We expect to see increases in fecal lipid excretion, decreases in circulating amino acids and lipids, and increase expression of bean-associated intake biomarkers in urine indicating pathway level changes in glucose homeostasis, inflammation, and lipid metabolism.

Future Studies

Study 1: This dissertation has provided evidence that companion dogs are exposed to environmental pollutants, however the extent and route of this exposure remains unknown. To further investigate the extent of environmental exposures in dogs, future studies need to address the following questions:

1. What are the relevant chemicals and their metabolites to study to in dogs?
2. What environmental chemicals are dogs exposed to?
3. What are the routes of exposure in companion dogs to environmental pollutants?

The first step will be to develop a panel of environmentally and biologically relevant pesticides to detect. This dissertation has provided information on 15 parent pesticides that can be detected in dogs and can be used to determine biologically relevant metabolites of these

residues to look for. To expand this list, compounds currently monitored in human populations should also be evaluated in dogs, and targeted methods to both detect and quantify exposures will be useful in determining if environmental exposures have a causative role in canine obesity and disease. Once this detection panel has been created and validated in canine samples, a cross sectional study across companion dogs of different breeds, ages, gender, and living environments (rural vs. suburban, etc.) can be performed. In addition to detecting excreted chemicals, the study design should also incorporate diet and water sample analysis, as well as full pesticide use data, and detailed medical records with detailed anthelmintic usage information. These studies could be designed to determine baseline exposures in healthy dogs, normal weight verses overweight dogs, or could sample across different diseases, e.g. cancer type to determine relationships between exposures and disease outcomes.

Study 2: Beans have been shown to have potent anti-tumorigenic activities in humans and rodents, but not yet in dogs. This dissertation has demonstrated the potential of bean-based diets to modulate aberrant canine metabolism and the ability to confirm bean intake with bean-associated biomarker detection. Studies to determine if a bean-based diet can modulate cancer associated metabolic disruptions in dogs are warranted. To more directly assess the potential of bean-based diets to reduce cancer risk in dogs, both mechanistic *in vitro* and longitudinal studies are needed to answer the following questions:

1. Do bean-derived phytonutrients have direct tumoricidal activity against canine cancer cells *in vitro*?
2. Is the *ex vivo* tumoricidal activity of canine white blood cells and plasma improved after bean consumption?
3. Is cancer risk reduced in dogs consuming a bean-based diet?

Study 3: Conflicting evidence exists in the literature regarding the causative role of obesity in cancer in dogs. This dissertation has demonstrated the utility of metabolomic platforms to detect changes in metabolomic profiles. Literature and data-based comparisons can be used to determine similarities in metabolic disturbances associated with cancer and obesity, however direct comparisons are needed to determine a physiological relationship between canine obesity and cancer and answer the following questions:

1. What metabolic aberrancies are shared between obese dogs and dogs with cancer that are not shared with normal weight dogs?
2. Are obese dogs with identified metabolic pathway dysfunctions associated with obesity and cancer at higher risk for developing cancer than obese that do not have the same metabolic aberrancies?
3. If yes, does modulation of the dysfunction result in reduced risk of developing cancer?

Study 4: One finding of potential clinical importance in this dissertation is that the proportion of overweight dogs with hemolytic values was higher than normal weight dogs. While previous studies have reported serum analytes in lean and obese dogs, none of these studies reported either the degree of hemolysis in samples or the proportion of hemolytic samples from lean and obese dogs. Excess free hemoglobin has the potential to alter accurate detection of multiple analytes and further investigation of the relationship between hemolytic venipuncture and obesity in dogs, even retrospectively, is warranted.

REFERENCES

(*1*). Ogden, C. L., M. D. Carroll, B. K. Kit, and K. M. Flegal. **2014**. PRevalence of childhood and adult obesity in the united states, 2011-2012. *Jama* 311: 806-814.

(*2*). Klausner, J., and E. Lund. **2012**. State of Pet Health. Accessed June 6th, 2012 2012.

(*3*). German, A. J. **2006**. The Growing Problem of Obesity in Dogs and Cats. *The Journal of Nutrition* 136: 1940S-1946S.

(*4*). Courcier, E., R. Thomson, D. Mellor, and P. Yam. **2010**. An epidemiological study of environmental factors associated with canine obesity. *Journal of small animal practice* 51: 362-367.

(*5*). McGreevy, P., P. Thomson, C. Pride, A. Fawcett, T. Grassi, and B. Jones. **2005**. Prevalence of obesity in dogs examined by Australian veterinary practices and the risk factors involved. *The Veterinary Record*: 695-702.

(*6*). Mao, J. F., Z. F. Xia, J. N. Chen, and J. H. Yu. **2013**. Prevalence and risk factors for canine obesity surveyed in veterinary practices in Beijing, China. *Prev Vet Med* 112: 438-442.

(*7*). Finkelstein, E. A., J. G. Trogdon, J. W. Cohen, and W. Dietz. **2009**. Annual Medical Spending Attributable To Obesity: Payer-And Service-Specific Estimates. *Health Affairs* 28: w822-w831.

(*8*). Kealy, R. D., D. F. Lawler, J. M. Ballam, S. L. Mantz, D. N. Biery, E. H. Greeley, G. Lust, M. Segre, G. K. Smith, and H. D. Stowe. **2002**. Effects of diet restriction on life span and age-related changes in dogs. *J Am Vet Med Assoc* 220: 1315-1320.

(*9*). German, A. J., V. H. Ryan, A. C. German, I. S. Wood, and P. Trayhurn. **2010**. Obesity, its associated disorders and the role of inflammatory adipokines in companion animals. *Veterinary Journal* 185: 4-9.

(*10*). Linder, D. E. **2014**. Top 5 Clincial Consequences of Obesity. *Clinician's Brief*: 19-21.

(*11*). Despres, J.-P., and I. Lemieux. **2006**. Abdominal obesity and metabolic syndrome. *Nature* 444: 881-887.

(*12*). Tvarijonaviciute, A., J. J. Ceron, S. L. Holden, D. J. Cuthbertson, V. Biourge, P. J. Morris, and A. J. German. **2012**. Obesity-related metabolic dysfunction in dogs: a comparison with human metabolic syndrome. *Bmc Veterinary Research* 8.

(*13*). Verkest, K. R. **2014**. Is the metabolic syndrome a useful clinical concept in dogs? A review of the evidence. *Veterinary Journal* 199: 24-30.

(*14*). Sandoe, P., C. Palmer, S. Corr, A. Astrup, and C. R. Bjornvad. **2014**. Canine and feline obesity: a One Health perspective. *Veterinary Record* 175: 610-616.

(*15*). Switonski, M., and M. Mankowska. **2013**. Dog obesity - The need for identifying predisposing genetic markers. *Research in veterinary science* 95: 831-836.

(*16*). Lasram, M. M., I. B. Dhouib, A. Annabi, S. El Fazaa, and N. Gharbi. **2014**. A review on the molecular mechanisms involved in insulin resistance induced by organophosphorus pesticides. *Toxicology* 322: 1-13.

(*17*). Langer, P., J. Ukropec, A. Kocan, B. Drobna, Z. Radikova, M. Huckova, R. Imrich, D. Gasperikova, I. Klimes, and T. Trnovec. **2014**. Obesogenic and diabetogenic impact of high organochlorine levels (HCB, p,p'-DDE, PCBs) on inhabitants in the highly polluted Eastern Slovakia. *Endocrine regulations* 48: 17-24.

(*18*). Peris-Sampedro, F., M. Cabre, P. Basaure, I. Reverte, J. L. Domingo, and M. Teresa Colomina. **2015**. Adulthood dietary exposure to a common pesticide leads to an obese-like phenotype and a diabetic profile in apoE3 mice. *Environ Res* 142: 169-176.

(*19*). Mailloux, R. J., M. Florian, Q. Chen, J. Yan, I. Petrov, M. C. Coughlan, M. Laziyan, D. Caldwell, M. Lalande, D. Patry, C. Gagnon, K. Sarafin, J. Truong, H. M. Chan, N. Ratnayake, N. Li, W. G. Willmore, and X. Jin. **2014**. Exposure to a northern contaminant mixture (NCM) alters hepatic energy and lipid metabolism exacerbating hepatic steatosis in obese JCR rats. *PloS one* 9: e106832.

(*20*). Arrebola, J. P., M. F. Fernandez, P. Martin-Olmedo, J. P. Bonde, J. L. Martin-Rodriguez, J. Exposito, A. Rubio-Dominguez, and N. Olea. **2015**. Historical exposure to persistent organic pollutants and risk of incident hypertension. *Environ Res* 138: 217-223.

(*21*). Under, D., and M. Mueller. **2014**. Pet Obesity Management Beyond Nutrition. *Vet Clin N Am-Small* 44: 789-+.

(*22*). Brooks, D., J. Churchill, K. Fein, D. Linder, K. E. Michel, K. Tudor, E. Ward, and A. Witzel. **2014**. 2014 AAHA Weight Management Guidelines for Dogs and Cats*†. *J Am Anim Hosp Assoc* 50: 1-11.

(*23*). Linder, D. E., L. M. Freeman, S. L. Holden, V. Biourge, and A. J. German. **2013**. Status of selected nutrients in obese dogs undergoing caloric restriction. *BMC veterinary research* 9: 219.

(*24*). Linder, D. E., L. M. Freeman, P. Morris, A. J. German, V. Biourge, C. Heinze, and L. Alexander. **2012**. Theoretical evaluation of risk for nutritional deficiency with caloric restriction in dogs. *Vet Quart* 32: 123-129.

(*25*). Broughton, W. J., G. Hernandez, M. Blair, S. Beebe, P. Gepts, and J. Vanderleyden. **2003**. Beans (Phaseolus spp.)–model food legumes. *Plant and soil* 252: 55-128.

(*26*). Swanson, K. S., C. M. Grieshop, G. M. Clapperr, R. G. Shields, T. Belay, N. R. Merchen, and G. C. Fahey. **2001**. Fruit and vegetable fiber fermentation by gut microflora from canines. *J Anim Sci* 79: 919-926.

(*27*). **2011**. The Dry Bean Book. In: U. S. D. o. Agriculture (ed.). U.S. Dry Edible Beans, Washington, DC.

(*28*). Wu, G., J. Fanzo, D. D. Miller, P. Pingali, M. Post, J. L. Steiner, and A. E. Thalacker-Mercer. **2014**. Production and supply of high-quality food protein for human consumption: sustainability, challenges, and innovations. *Annals of the New York Academy of Sciences* 1321: 1-19.

(*29*). Swanson, K. S., R. A. Carter, T. P. Yount, J. Aretz, and P. R. Buff. **2013**. Nutritional Sustainability of Pet Foods. *Advances in Nutrition* 4: 141-150.

(*30*). Chang, W. C., M. L. Wahlqvist, H. Y. Chang, C. C. Hsu, M. S. Lee, W. S. Wang, and C. A. Hsiung. **2012**. A bean-free diet increases the risk of all-cause mortality among Taiwanese women: the role of the metabolic syndrome. *Public Health Nutrition* 15: 663-672.

(*31*). Darmadi-Blackberry, I., M. L. Wahlqvist, A. Kouris-Blazos, B. Steen, W. Lukito, Y. Horie, and K. Horie. **2004**. Legumes: the most important dietary predictor of survival in older people of different ethnicities. *Asia Pacific journal of clinical nutrition* 13: 217-220.

(*32*). Papanikolaou, Y., and V. L. Fulgoni. **2008**. Bean Consumption Is Associated with Greater Nutrient Intake, Reduced Systolic Blood Pressure, Lower Body Weight, and a Smaller Waist Circumference in Adults: Results from the National Health and Nutrition Examination Survey 1999-2002. *J Am Coll Nutr* 27: 569-576.

(*33*). Broughton, W. J., G. Hernandez, M. Blair, S. Beebe, P. Gepts, and J. Vanderleyden. **2003**. Beans (Phaseolus spp.) - model food legumes. *Plant and Soil* 252: 55-128.

(*34*). Bazzano, L. A., A. M. Thompson, M. T. Tees, C. H. Nguyen, and D. M. Winham. **2011**. Non-soy legume consumption lowers cholesterol levels: A meta-analysis of randomized controlled trials. *Nutrition Metabolism and Cardiovascular Diseases* 21: 94-103.

(*35*). Mensack, M. M., J. N. McGinley, and H. J. Thompson. **2012**. Metabolomic analysis of the effects of edible dry beans (Phaseolus vulgaris L.) on tissue lipid metabolism and carcinogenesis in rats. *British Journal of Nutrition* 108: S155-S165.

(*36*). Duane, W. C. **1997**. Effects of legume consumption on serum cholesterol, biliary lipids, and sterol metabolism in humans. *J Lipid Res* 38: 1120-1128.

(*37*). Oseguera-Toledo, M. E., E. G. de Mejia, V. P. Dia, and S. L. Amaya-Llano. **2011**. Common bean (Phaseolus vulgaris L.) hydrolysates inhibit inflammation in LPS-induced macrophages through suppression of NF-kappa B pathways. *Food Chemistry* 127: 1175-1185.

(*38*). Esmaillzadeh, A., and L. Azadbakht. **2012**. Legume Consumption Is Inversely Associated with Serum Concentrations of Adhesion Molecules and Inflammatory Biomarkers among Iranian Women. *Journal of Nutrition* 142: 334-339.

(*39*). Hermsdorff, H. H. M., M. A. Zulet, I. Abete, and J. A. Martinez. **2011**. A legume-based hypocaloric diet reduces proinflammatory status and improves metabolic features in overweight/obese subjects. *Eur J Nutr* 50: 61-69.

(*40*). Bourdon, I., B. Olson, R. Backus, B. D. Richter, P. A. Davis, and B. O. Schneeman. **2001**. Beans, as a source of dietary fiber, increase cholecystokinin and apolipoprotein B48 response to test meals in men. *Journal of Nutrition* 131: 1485-1490.

(*41*). Thompson, M. D., H. J. Thompson, M. A. Brick, J. N. McGinley, W. Q. Jiang, Z. J. Zhu, and P. Wolfe. **2008**. Mechanisms Associated with Dose-Dependent Inhibition of Rat Mammary Carcinogenesis by Dry Bean (Phaseolus vulgaris, L.). *Journal of Nutrition* 138: 2091-2097.

(*42*). Mollard, R. C., B. L. Luhovyy, S. Panahi, M. Nunez, A. Hanley, and G. H. Anderson. **2012**. Regular consumption of pulses for 8 weeks reduces metabolic syndrome risk factors in overweight and obese adults. *British Journal of Nutrition* 108: S111-S122.

(*43*). Thompson, M. D., M. M. Mensack, W. Q. Jiang, Z. J. Zhu, M. R. Lewis, J. N. McGinley, M. A. Brick, and H. J. Thompson. **2012**. Cell signaling pathways associated with a reduction in mammary cancer burden by dietary common bean (Phaseolus vulgaris L.). *Carcinogenesis* 33: 226-232.

(*44*). Finley, J. W., J. B. Burrell, and P. G. Reeves. **2007**. Pinto bean consumption changes SCFA profiles in fecal fermentations, bacterial populations of the lower bowel, and lipid profiles in blood of humans. *Journal of Nutrition* 137: 2391-2398.

(*45*). McCrory, M. A., B. R. Hamaker, J. C. Lovejoy, and P. E. Eichelsdoerfer. **2010**. Pulse Consumption, Satiety, and Weight Management. *Advances in Nutrition* 1: 17-30.

(*46*). Sathe, S. K. **2002**. Dry bean protein functionality. *Critical reviews in biotechnology* 22: 175.

(*47*). Kleintop, A. E., D. Echeverria, L. A. Brick, H. J. Thompson, and M. A. Brick. **2013**. Adaptation of the AOAC 2011.25 integrated total dietary fiber assay to determine the dietary fiber and oligosaccharide content of dry edible beans. *Journal of agricultural and food chemistry* 61: 9719-9726.

(*48*). **2015**. USDA National Nutrient Database for Standard Reference, Release 27. US Department of Agriculture, Agricultural Research Service, Nutrient Data Laboratory.

(*49*). Rebello, C. J., F. L. Greenway, and J. W. Finley. **2014**. Whole Grains and Pulses: A Comparison of the Nutritional and Health Benefits. *Journal of Agricultural and Food Chemistry* 62: 7029-7049.

(*50*). Campos-Vega, R., B. D. Oomah, G. Loarca-Piña, and H. A. Vergara-Castañeda. **2013**. Common Beans and Their Non-Digestible Fraction: Cancer Inhibitory Activity—An Overview. *Foods* 2: 374-392.

(*51*). Fialho, L. D., V. M. Guimaraes, E. G. De Barros, M. A. Moreira, L. A. D. Dias, M. G. D. Oliveira, I. C. Jose, and S. T. De Rezende. **2006**. Biochemical composition and indigestible oligosaccharides in Phaseolus vulgaris L. seeds. *Plant Foods for Human Nutrition* 61: 87-89.

(*52*). Vargas-Torres, A., P. Osorio-Díaz, J. Tovar, O. Paredes-López, J. Ruales, and L. A. Bello-Pérez. **2004**. Chemical Composition, Starch Bioavailability and Indigestible fraction of Common Beans (Phaseolus Vulgaris L.). *Starch - Stärke* 56: 74-78.

(*53*). Araya, H., N. Pak, G. Vera, and M. Alvina. **2003**. Digestion rate of legume carbohydrates and glycemic index of legume-based meals. *International journal of food sciences and nutrition* 54: 119-126.

(*54*). Holm, J., and I. Björck. **1992**. Bioavailability of starch in various wheat-based bread products: evaluation of metabolic responses in healthy subjects and rate and extent of in vitro starch digestion. *The American journal of clinical nutrition* 55: 420-429.

(*55*). Hutchins, A. M., D. M. Winham, and S. V. Thompson. **2012**. Phaseolus beans: impact on glycaemic response and chronic disease risk in human subjects. *The British journal of nutrition* 108 Suppl 1: S52-65.

(*56*). Slavin, J. **2003**. Impact of the proposed definition of dietary fiber on nutrient databases. *Journal of Food Composition and Analysis* 16: 287-291.

(*57*). Slavin, J. L. **2005**. Dietary fiber and body weight. *Nutrition* 21: 411-418.

(*58*). Bosch, G., A. Verbrugghe, M. Hesta, J. J. Holst, A. F. B. van der Poel, G. P. J. Janssens, and W. H. Hendriks. **2009**. The effects of dietary fibre type on satiety-related hormones and voluntary food intake in dogs. *British Journal of Nutrition* 102: 318-325.

(*59*). Weber, M., T. Bissot, E. Servet, R. Sergheraert, V. Biourge, and A. J. German. **2007**. A high-protein, high-fiber diet designed for weight loss improves satiety in dogs. *J Vet Intern Med* 21: 1203-1208.

(*60*). Jewell, D. E., P. W. Toll, M. J. Azain, R. D. Lewis, and G. L. Edwards. **2006**. Fiber but not conjugated linoleic: Acid influences adiposity in dogs. *Vet Ther* 7: 78-85.

(*61*). Mitsuhashi, Y., K. Bigley, and J. E. Bauer. 2008. Post-feeding satiety and weight loss effect of a vegetable-based fiber supplement in Beagles. In: 26th Annual ACVIM Forum, San Antonio, TX. p 743 (abstract #142).

(*62*). German, A. J., S. L. Holden, T. Bissot, P. J. Morris, and V. Biourge. **2010**. A high protein high fibre diet improves weight loss in obese dogs. *Vet J* 183: 294-297.

(*63*). Ramírez-Jiménez, A. K., R. Reynoso-Camacho, M. E. Tejero, F. León-Galván, and G. Loarca-Piña. **2015**. Potential role of bioactive compounds of Phaseolus vulgaris L. on lipid-lowering mechanisms. *Food Research International* http://dx.doi.org/10.1016/j.foodres.2015.01.002.

(*64*). Feregrino-Perez, A. A., C. Pinol-Felis, X. Gomez-Arbones, R. G. Guevara-Gonzalez, R. Campos-Vega, J. Acosta-Gallegos, and G. Loarca-Pina. **2014**. A Non-digestible Fraction of the Common Bean (Phaseolus vulgaris L.) Induces Cell Cycle Arrest and Apoptosis During Early Carcinogenesis. *Plant Foods for Human Nutrition* 69: 248-254.

(*65*). Cruz-Bravo, R. K., R. G. Guevara-Gonzalez, M. Ramos-Gomez, B. D. Oomah, P. Wiersma, R. Campos-Vega, and G. Loarca-Pina. **2014**. The fermented non-digestible fraction of common bean (Phaseolus vulgaris L.) triggers cell cycle arrest and apoptosis in human colon adenocarcinoma cells. *Genes Nutr* 9.

(*66*). da Costa, T. H. M., C. E. G. Reis, F. V. P. da Silva, and L. A. Casulari. **2014**. Improvement in metabolic parameters in obese subjects after 16 weeks on a Brazilian-staple calorie-restricted diet. *Nutrition Research and Practice* 8: 410-416.

(*67*). Mitchell, D. C., F. R. Lawrence, T. J. Hartman, and J. M. Curran. **2009**. Consumption of Dry Beans, Peas, and Lentils Could Improve Diet Quality in the US Population. *J. Am. Diet. Assoc.* 109: 909-913.

(*68*). da, S. Q.-M. K., G. E. Costa, N. da Silva, S. M. Reis, and A. C. de Oliveira. **2005**. Bifidogenic effect of dietary fiber and resistant starch from leguminous on the intestinal microbiota of rats. *Nutrition* 21: 602-608.

(*69*). Kerr, K. R., G. Forster, S. E. Dowd, E. P. Ryan, and K. S. Swanson. **2013**. Effects of dietary cooked navy bean on the fecal microbiome of healthy companion dogs. *Plos One* 8: e74998.

(*70*). Nilsson, A., E. Johansson, L. Ekstrom, and I. Bjorck. **2013**. Effects of a Brown Beans Evening Meal on Metabolic Risk Markers and Appetite Regulating Hormones at a Subsequent Standardized Breakfast: A Randomized Cross-Over Study. *PloS one* 8.

(*71*). Marinangeli, C. P. F., and P. J. H. Jones. **2012**. Pulse grain consumption and obesity: effects on energy expenditure, substrate oxidation, body composition, fat deposition and satiety. *British Journal of Nutrition* 108: S46-S51.

(*72*). Kumar, V., A. K. Sinha, H. P. Makkar, G. de Boeck, and K. Becker. **2012**. Dietary roles of non-starch polysaccharides in human nutrition: a review. *Crit Rev Food Sci Nutr* 52: 899-935.

(*73*). Arora, T., R. Sharma, and G. Frost. **2011**. Propionate. Anti-obesity and satiety enhancing factor? *Appetite* 56: 511-515.

(*74*). Boye, J. I., and A. Maltais. **2011**. Pulses A novel protein source. *Agro Food Industry Hi-Tech* 22: 24-26.

(*75*). Lajolo, F. M., and M. I. Genovese. **2002**. Nutritional significance of lectins and enzyme inhibitors from legumes. *Journal of Agricultural and Food Chemistry* 50: 6592-6598.

(*76*). Rebello, C., F. Greenway, and J. Finley. **2014**. A review of the nutritional value of legumes and their effects on obesity and its related co-morbidities. *Obes Rev* 15: 392-407.

(*77*). Barrett, M. L., and J. K. Udani. **2011**. A proprietary alpha-amylase inhibitor from white bean (Phaseolus vulgaris): a review of clinical studies on weight loss and glycemic control. *Nutr J* 10: 24.

(*78*). Petry, N., I. Egli, B. Campion, E. Nielsen, and R. Hurrell. **2013**. Genetic reduction of phytate in common bean (Phaseolus vulgaris L.) seeds increases iron absorption in young women. *The Journal of nutrition* 143: 1219-1224.

(*79*). Helbig, E., A. C. de Oliveira, S. Queiroz Kda, and S. M. Reis. **2003**. Effect of soaking prior to cooking on the levels of phytate and tannin of the common bean (Phaseolus vulgaris, L.) and the protein value. *Journal of nutritional science and vitaminology* 49: 81-86.

(*80*). Jariwalla, R. J. **1999**. Inositol hexaphosphate (IP6) as an anti-neoplastic and lipid-lowering agent. *Anticancer research* 19: 3699-3702.

(*81*). Kalogeropoulos, N., A. Chiou, M. Ioannou, V. T. Karathanos, M. Hassapidou, and N. K. Andrikopoulos. **2010**. Nutritional evaluation and bioactive microconstituents (phytosterols, tocopherols, polyphenols, triterpenic acids) in cooked dry legumes usually consumed in the Mediterranean countries. *Food Chemistry* 121: 682-690.

(*82*). Zhang, C., J. M. Monk, J. T. Lu, L. Zarepoor, W. Wu, R. Liu, K. P. Pauls, G. A. Wood, L. Robinson, R. Tsao, and K. A. Power. **2014**. Cooked navy and black bean diets improve biomarkers of colon health and reduce inflammation during colitis. *The British journal of nutrition* 111: 1549-1563.

(*83*). Khan, N., O. Khymenets, M. Urpi-Sarda, S. Tulipani, M. Garcia-Aloy, M. Monagas, X. Mora-Cubillos, R. Llorach, and C. Andres-Lacueva. **2014**. Cocoa polyphenols and inflammatory markers of cardiovascular disease. *Nutrients* 6: 844-880.

(*84*). Terao, J. **2009**. Dietary flavonoids as antioxidants. *Forum of nutrition* 61: 87-94.

(*85*). Lila, M. A. **2007**. From Beans to Berries and Beyond. *Annals of the New York Academy of Sciences* 1114: 372-380.

(*86*). Chao, J., T. I. Huo, H. Y. Cheng, J. C. Tsai, J. W. Liao, M. S. Lee, X. M. Qin, M. T. Hsieh, L. H. Pao, and W. H. Peng. **2014**. Gallic Acid Ameliorated Impaired Glucose and Lipid Homeostasis in High Fat Diet-Induced NAFLD Mice. *PloS one* 9.

(*87*). Ros, E. **2010**. Health benefits of nut consumption. *Nutrients* 2: 652-682.

(*88*). Campos-Vega, R., G. Loarca-Piña, and B. D. Oomah. **2010**. Minor components of pulses and their potential impact on human health. *Food Research International* 43: 461-482.

(*89*). Wang, S., N. Moustaid-Moussa, L. Chen, H. Mo, A. Shastri, R. Su, P. Bapat, I. Kwun, and C. L. Shen. **2014**. Novel insights of dietary polyphenols and obesity. *J Nutr Biochem* 25: 1-18.

(*90*). Kitano-Okada, T., A. Ito, A. Koide, Y. Nakamura, K.-H. Han, K. Shimada, K. Sasaki, K. Ohba, S. Sibayama, and M. Fukushima. **2012**. Anti-obesity role of adzuki bean extract containing polyphenols: in vivo and in vitro effects. *Journal of the Science of Food and Agriculture* 92: 2644-2651.

(*91*). Gylling, H., J. Plat, S. Turley, H. N. Ginsberg, L. Ellegard, W. Jessup, P. J. Jones, D. Lutjohann, W. Maerz, L. Masana, G. Silbernagel, B. Staels, J. Boren, A. L. Catapano, G. De Backer, J. Deanfield, O. S. Descamps, P. T. Kovanen, G. Riccardi, L. Tokgozoglu, M. J. Chapman, and P. European Atherosclerosis Society Consensus Panel on. **2014**. Plant sterols and plant stanols in the management of dyslipidaemia and prevention of cardiovascular disease. *Atherosclerosis* 232: 346-360.

(*92*). Forster, G. M., D. G. Brown, G. P. Dooley, R. L. Page, and E. P. Ryan. **2014**. Multiresidue analysis of pesticides in urine of healthy adult companion dogs. *Environmental science & technology* 48: 14677-14685.

(*93*). Forster, G. M., D. Hill, G. Gregory, K. M. Weishaar, S. Lana, J. E. Bauer, and E. P. Ryan. **2012**. Effects of cooked navy bean powder on apparent total tract nutrient digestibility and safety in healthy adult dogs1. *J. Anim. Sci.* 90: 2631-2638.

(*94*). Forster, G. M., A. L. Heuberger, C. D. Broeckling, J. E. Bauer, and E. P. ryan. **2015**. Consumption of Cooked Navy Bean Powders Modulate the Canine Fecal and Urine Metabolome. *Current Metabolomics* 3: 90-101.

(*95*). Forster, G. M., C. A. Ollila, J. H. Burton, D. Hill, J. E. Bauer, A. M. Hess, and E. P. Ryan. **2012**. Nutritional Weight Loss Therapy with Cooked Bean Powders Regulates Serum Lipids and Biochemical Analytes in Overweight and Obese Dogs *Obesity and Weight Loss Therapy* 2.

(*96*). Adams, V. J., K. M. Evans, J. Sampson, and J. L. Wood. **2010**. Methods and mortality results of a health survey of purebred dogs in the UK. *The Journal of small animal practice* 51: 512-524.

(*97*). **2011**. Global status report on noncommunicable diseases 2010. 9789241564229, World Health Organization, Geneva, Switzerland.

(*98*). Mostafalou, S., and M. Abdollahi. **2013**. Pesticides and human chronic diseases: evidences, mechanisms, and perspectives. *Toxicology and applied pharmacology* 268: 157-177.

(*99*). Nawaz, A., A. Razpotnik, P. Rouimi, G. de Sousa, J. P. Cravedi, and R. Rahmani. **2014**. Cellular impact of combinations of endosulfan, atrazine, and chlorpyrifos on human primary hepatocytes and HepaRG cells after short and chronic exposures. *Cell Biol Toxicol* 30: 17-29.

(*100*). Zhu, W., D. R. Schmehl, C. A. Mullin, and J. L. Frazier. **2014**. Four Common Pesticides, Their Mixtures and a Formulation Solvent in the Hive Environment Have High Oral Toxicity to Honey Bee Larvae. *PloS one* 9.

(*101*). Coleman, M. D., J. D. O'Neil, E. K. Woehrling, O. B. A. Ndunge, E. J. Hill, A. Menache, and C. J. Reiss. **2012**. A Preliminary Investigation into the Impact of a Pesticide Combination on Human Neuronal and Glial Cell Lines In Vitro. *PloS one* 7.

(*102*). Rouimi, P., N. Zucchini-Pascal, G. Dupont, A. Razpotnik, E. Fouche, G. De Sousa, and R. Rahmani. **2012**. Impacts of low doses of pesticide mixtures on liver cell defense systems. *Toxicology in vitro : an international journal published in association with BIBRA* 26: 718-726.

(*103*). Darbre, P. D., and P. W. Harvey. **2014**. Parabens can enable hallmarks and characteristics of cancer in human breast epithelial cells: a review of the literature with reference to new exposure data and regulatory status. *Journal of applied toxicology : JAT* 34: 925-938.

(*104*). Rider, C. V., D. J. Carlin, M. J. DeVito, C. L. Thompson, and N. J. Walker. **2013**. Mixtures research at NIEHS: An evolving program. *Toxicology* 313: 94-102.

(*105*). Khanna, C., K. Lindblad-Toh, D. Vail, C. London, P. Bergman, L. Barber, M. Breen, B. Kitchell, E. McNeil, J. F. Modiano, S. Niemi, K. E. Comstock, E. Ostrander, S. Westmoreland, and S. Withrow. **2006**. The dog as a cancer model. *Nature biotechnology* 24: 1065-1066.

(*106*). Ranieri, G., C. D. Gadaleta, R. Patruno, N. Zizzo, M. G. Daidone, M. G. Hansson, A. Paradiso, and D. Ribatti. **2013**. A model of study for human cancer: Spontaneous occurring tumors in dogs. Biological features and translation for new anticancer therapies. *Critical reviews in oncology/hematology* 88: 187-197.

(*107*). Schmidt, P. L. **2009**. Companion animals as sentinels for public health. *The Veterinary clinics of North America. Small animal practice* 39: 241-250.

(*108*). Glickman, L. T., L. M. Domanski, T. G. Maguire, R. R. Dubielzig, and A. Churg. **1983**. Mesothelioma in Pet Dogs Associated with Exposure of Their Owners to Asbestos. *Environmenal Research* 32: 305-313.

(*109*). Venier, M., and R. A. Hites. **2011**. Flame retardants in the serum of pet dogs and in their food. *Environmental science & technology* 45: 4602-4608.

(*110*). Reynolds, P. M., J. S. Reif, H. S. Ramsdell, and J. D. Tessari. **1994**. Canine exposure to herbicide-treated lawns and urinary excretion of 2,4-dichlorophenoxyacetic acid. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 3: 233-237.

(*111*). Knapp, D. W., W. A. Peer, A. Conteh, A. R. Diggs, B. R. Cooper, N. W. Glickman, P. L. Bonney, J. C. Stewart, L. T. Glickman, and A. S. Murphy. **2013**. Detection of herbicides

in the urine of pet dogs following home lawn chemical application. *The Science of the total environment* 456-457: 34-41.

(*112*). Ali, N., R. N. Malik, T. Mehdi, S. A. Eqani, A. Javeed, H. Neels, and A. Covaci. **2013**. Organohalogenated contaminants (OHCs) in the serum and hair of pet cats and dogs: biosentinels of indoor pollution. *The Science of the total environment* 449: 29-36.

(*113*). Glickman, L. T., M. Raghavan, D. W. Knapp, P. L. Bonney, and M. H. Dawson. **2004**. Herbicide exposure and the risk of transitional cell carcinoma of the urinary bladder in Scottish Terriers. *Journal of the American Veterinary Medical Association* 224: 1290-1297.

(*114*). Takashima-Uebelhoer, B. B., L. G. Barber, S. E. Zagarins, E. Procter-Gray, A. L. Gollenberg, A. S. Moore, and E. R. Bertone-Johnson. **2012**. Household chemical exposures and the risk of canine malignant lymphoma, a model for human non-Hodgkin's lymphoma. *Environ Res* 112: 171-176.

(*115*). Glickman, L. T., F. S. Schofer, L. J. McKee, J. S. Reif, and M. H. Goldschmidt. **1989**. Epidemiologic study of insecticide exposures, obesity, and risk of bladder cancer in household dogs. *Journal of toxicology and environmental health* 28: 407-414.

(*116*). Andrade, F. H., F. C. Figueiroa, P. R. Bersano, D. Z. Bissacot, and N. S. Rocha. **2010**. Malignant mammary tumor in female dogs: environmental contaminants. *Diagnostic pathology* 5: 45.

(*117*). Arrebola, J. P., E. Mutch, M. Rivero, A. Choque, S. Silvestre, N. Olea, R. Ocana-Riola, and L. A. Mercado. **2012**. Contribution of sociodemographic characteristics, occupation, diet and lifestyle to DDT and DDE concentrations in serum and adipose tissue from a Bolivian cohort. *Environment international* 38: 54-61.

(*118*). Hernandez-Valero, M. A., A. P. Herrera, S. H. Zahm, and L. A. Jones. **2007**. Community-Based Participatory Research and Gene-Environment Interaction Methodologies Addressing Environmental Justice among Migrant and Seasonal Farmworker Women and Children in Texas: "From Mother to Child Project". *Californian journal of health promotion* 5: 114-127.

(*119*). Jamin, E. L., N. Bonvallot, M. Tremblay-Franco, J.-P. Cravedi, C. Chevrier, S. Cordier, and L. Debrauwer. **2014**. Untargeted profiling of pesticide metabolites by LC-HRMS: an exposomics tool for human exposure evaluation. *Anal Bioanal Chem* 406: 1149-1161.

(*120*). Forster, G. M., D. Hill, G. Gregory, K. M. Weishaar, S. Lana, J. E. Bauer, and E. P. Ryan. **2012**. Effects of cooked navy bean powder on apparent total tract nutrient digestibility and safety in healthy adult dogs. *Journal of animal science* 90: 2631-2638.

(*121*). Ad Hoc Committee on Dog and Cat Nutrition, C. o. A. N., Board on Agriculture and Natural Resources, Division on Earth and Life Studies, National Research Council.

2006 Nutrient Requirements of Dogs and Cats, National Research Council, Washington, D.C.

(*122*). AAFCO. **2010**. 2010 Official Publication. Association of American Feed Control Officials Inc, West Lafayette, IN.

(*123*). **2003**. Interim Reregistration Eligibility Decision for Atrazine. In: P. a. T. S. Office of Prevention (ed.)Case No. 0062.

(*124*). **1996**. Registrations Eligibility Decision: Bromacil. In: P. a. T. S. Office of Prevention (ed.).

(*125*). EPA. **2005**. Notice of Pesticide Registration: Clopyralid MEA AG. In: O. o. P. P. R. Division (ed.)Registration #: 42750-89. U.S. Environmental Protection Agency.

(*126*). **2004**. Registrations Eligibility Decision: Ethoxyquin. In: P. a. T. S. Office of Prevention (ed.).

(*127*). EPA. **2008**. Imidacloprid Summary Document
Registration Review: Initial Docket. In: O. o. P. P. R. Division (ed.)EPA-HQ-OPP-2008-0844. U.S. Environmental Protection Agency.

(*128*). **2008**. Registrations Eligibility Decision: Nicotine. In: P. a. T. S. Office of Prevention (ed.).

(*129*). **1995**. Registrations Eligibility Decision: Propamocarb Hydrochloride. In: P. a. T. S. Office of Prevention (ed.).

(*130*). **1997**. Registrations Eligibility Decision: Propoxur. In: P. a. T. S. Office of Prevention (ed.).

(*131*). **2000**. Fact Sheet: Pymetrozine. In: P. a. T. S. Office of Prevention (ed.).

(*132*). EPA. **2011**. Spinosad and Spinetoram Summary Document
Registration Review: Initial Docket. In: O. o. P. P. R. Division (ed.). U.S. Environmental Protection Agency.

(*133*). **2002**. Registrations Eligibility Decision: Thiabendazole. In: P. a. T. S. Office of Prevention (ed.)738-R-02. Office of Prevention, Pesticides and Toxic Substances.

(*134*). EPA. **2012**. Triasulfuron Summary Document
Registration Review: Initial Docket. In: O. o. P. P. R. Division (ed.)EPA-HQ-OPP-2010-0115. U.S. Environmental Protection Agency.

(*135*). Catenacci, G., F. Barbieri, M. Bersani, A. Fereoli, D. Cottica, and M. Maroni. **1993**. Biological monitoring of human exposure to atrazine. *Toxicol Lett* 69: 217-222.

(*136*). Hutson, D. H., P. W. Lee, P. H. Nichols, and J. R. Plimmer. **1998**. 1,3,5-Triazines. In: T. R. Roberts, D. H. Hutson, P. W. Lee, P. H. Nichols and J. R. Plimmer (eds.) Metabolic Pathways of Agrochemicals. p 623-656. The Royal Society of Chemistry, Cambridge.

(*137*). **1998**. Metabolic pathways of agrochemicals. Royal Society of Chemistry, Cambridge.

(*138*). Talcott, P. A. **2013**. Miscellaneous Herbicides, Fungicides, and Nematocides. In: M. E. Peterson, Talcott, Patricia A. (ed.) Small Animal Toxicology10.1016/B978-1-4557-0717-1.02001-9. p 401–408. Elsevier, St. Louis.

(*139*). Hukkanen, J., P. Jacob, and N. L. Benowitz. **2005**. Metabolism and disposition kinetics of nicotine. *Pharmacol Rev* 57: 79-115.

(*140*). Kaufman, D. D. **1975**. Herbicides: chemistry, degradation, and mode of action. 2d ed., rev. and expanded ed. M. Dekker, New York.

(*141*). Hutson, D. H., P. W. Lee, P. H. Nichols, and J. R. Plimmer. **1998**. Sulfonylureas. In: T. R. Roberts, D. H. Hutson, P. W. Lee, P. H. Nichols and J. R. Plimmer (eds.) Metabolic Pathways of Agrochemicals. p 451-482. The Royal Society of Chemistry, Cambridge.

(*142*). Boler, B. M. V., T. A. Faber, L. L. Bauer, K. S. Swanson, S. Smiley, P. J. Bechtel, and G. C. Fahey. **2012**. Acute satiety response of mammalian, avian and fish proteins in dogs. *British Journal of Nutrition* 107: 146-154.

(*143*). Beloshapka, A. N., A. K. Wolff, and K. S. Swanson. **2012**. Effects of feeding polydextrose on faecal characteristics, microbiota and fermentative end products in healthy adult dogs. *The British journal of nutrition* 108: 638-644.

(*144*). Liu, M., B. Bienfait, O. Sacher, J. Gasteiger, R. J. Siezen, A. Nauta, and J. M. Geurts. **2014**. Combining chemoinformatics with bioinformatics: in silico prediction of bacterial flavor-forming pathways by a chemical systems biology approach "reverse pathway engineering". *PloS one* 9: e84769.

(*145*). Bonanno, A., G. Tornambe, A. Di Grigoli, V. Genna, V. Bellina, G. Di Miceli, and D. Giambalvo. **2012**. Effect of legume grains as a source of dietary protein on the quality of organic lamb meat. *Journal of the science of food and agriculture* 92: 2870-2875.

(*146*). Rothwell, J. A., Y. Fillatre, J. F. Martin, B. Lyan, E. Pujos-Guillot, L. Fezeu, S. Hercberg, B. Comte, P. Galan, M. Touvier, and C. Manach. **2014**. New biomarkers of coffee consumption identified by the non-targeted metabolomic profiling of cohort study subjects. *PloS one* 9: e93474.

(*147*). Brambillasca, S., A. Britos, C. Deluca, M. Fraga, and C. Cajarville. **2013**. Addition of citrus pulp and apple pomace in diets for dogs: influence on fermentation kinetics, digestion, faecal characteristics and bacterial populations. *Archives of animal nutrition* 67: 492-502.

(*148*). Beloshapka, A. N., S. E. Dowd, J. S. Suchodolski, J. M. Steiner, L. Duclos, and K. S. Swanson. **2013**. Fecal microbial communities of healthy adult dogs fed raw meat-based diets with or without inulin or yeast cell wall extracts as assessed by 454 pyrosequencing. *Fems Microbiol Ecol* 84: 532-541.

(*149*). Balazadeh, S., J. Schildhauer, W. L. Araujo, S. Munne-Bosch, A. R. Fernie, S. Proost, K. Humbeck, and B. Mueller-Roeber. **2014**. Reversal of senescence by N resupply to N-starved Arabidopsis thaliana: transcriptomic and metabolomic consequences. *Journal of experimental botany* 65: 3975-3992.

(*150*). Avondo, M., R. I. Pagano, A. De Angelis, and P. Pennisi. **2013**. Diet choice by goats as effect of milk production level during late lactation. *Animal : an international journal of animal bioscience* 7: 1113-1118.

(*151*). Abeysekara, S., P. D. Chilibeck, H. Vatanparast, and G. A. Zello. **2012**. A pulse-based diet is effective for reducing total and LDL-cholesterol in older adults. *British Journal of Nutrition* 108: S103-S110.

(*152*). Zhang, P. C. **2012.** The Effects of Cooked Common Beans on DSS-induced Colitis in Mice.

(*153*). Suchodolski, J. S., M. E. Markel, J. F. Garcia-Mazcorro, S. Unterer, R. M. Heilmann, S. E. Dowd, P. Kachroo, I. Ivanov, Y. Minamoto, E. M. Dillman, J. M. Steiner, A. K. Cook, and L. Toresson. **2012**. The fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. *PloS one* 7: e51907.

(*154*). Campbell, C., D. Grapov, O. Fiehn, C. J. Chandler, D. J. Burnett, E. C. Souza, G. A. Casazza, M. B. Gustafson, N. L. Keim, J. W. Newman, G. R. Hunter, J. R. Fernandez, W. T. Garvey, M. E. Harper, C. L. Hoppel, J. K. Meissen, K. Take, and S. H. Adams. **2014**. Improved metabolic health alters host metabolism in parallel with changes in systemic xeno-metabolites of gut origin. *PloS one* 9: e84260.

(*155*). Campos-Vega, R., T. Garcia-Gasca, R. Guevara-Gonzalez, M. Ramos-Gomez, B. D. Oomah, and G. Loarca-Pina. **2012**. Human Gut Flora-Fermented Nondigestible Fraction from Cooked Bean (Phaseolus vulgaris L.) Modifies Protein Expression Associated with Apoptosis, Cell Cycle Arrest, and Proliferation in Human Adenocarcinoma Colon Cancer Cells. *Journal of Agricultural and Food Chemistry* 60: 12443-12450.

(*156*). Garcia-Galvan, A., R. Belmar-Casso, L. Sarmiento-Franco, and C. A. Sandoval-Castro. **2012**. Evaluation of the metabolizable energy value for growing lambs of the Mucuna pruriens seed and the whole pod. *Tropical animal health and production* 44: 843-847.

(*157*). Collino, S., F. P. Martin, L. G. Karagounis, M. N. Horcajada, S. Moco, C. Franceschi, M. Kussmann, and E. Offord. **2014**. Reprint of: Musculoskeletal system in the old age and the demand for healthy ageing biomarkers. *Mechanisms of ageing and development* 136-137: 94-100.

(*158*). Singh, J., and P. S. Basu. **2012**. Non-nutritive bioactive compounds in pulses and their impact on human health: an overview.

(*159*). Mendoza-Diaz, S., M. D. Ortiz-Valerio, E. Castano-Tostado, J. D. Figueroa-Cardenas, R. Reynoso-Camacho, M. Ramos-Gomez, R. Campos-Vega, and G. Loarca-Pina. **2012**. Antioxidant Capacity and Antimutagenic Activity of Anthocyanin and Carotenoid Extracts from Nixtamalized Pigmented Creole Maize Races (Zea mays L.). *Plant Foods for Human Nutrition* 67: 442-449.

(*160*). Martins Rodrigues, A. G., R. P. da Costa Proenca, M. C. Marino Calvo, and G. M. Rataichesck Fiates. **2012**. Overweight/obesity is associated with food choices related to rice and beans, colors of salads, and portion size among consumers at a restaurant serving buffet-by-weight in Brazil. *Appetite* 59: 305-311.

(*161*). Liu, Y. **2012.** Acute Effects of Navy Bean Powder, Lentil Powder and Chickpea Powder on Postprandial Glycaemic Response and Subjective Appetite in Healthy Young Men.

(*162*). Hooda, S., Y. Minamoto, J. S. Suchodolski, and K. S. Swanson. **2012**. Current state of knowledge: the canine gastrointestinal microbiome. *Animal health research reviews / Conference of Research Workers in Animal Diseases* 13: 78-88.

(*163*). Heilmann, R. M., A. E. Jergens, M. R. Ackermann, J. W. Barr, J. S. Suchodolski, and J. M. Steiner. **2012**. Serum calprotectin concentrations in dogs with idiopathic inflammatory bowel disease. *American Journal of Veterinary Research* 73: 1900-1907.

(*164*). de Sales, P. M., P. M. de Souza, L. A. Simeoni, P. d. O. Magalhaes, and D. Silveira. **2012**. alpha-Amylase Inhibitors: A Review of Raw Material and Isolated Compounds from Plant Source. *Journal of Pharmacy and Pharmaceutical Sciences* 15: 141-183.

(*165*). Hang, I., T. Rinttila, J. Zentek, A. Kettunen, S. Alaja, J. Apajalahti, J. Harmoinen, W. M. de Vos, and T. Spillmann. **2012**. Effect of high contents of dietary animal-derived protein or carbohydrates on canine faecal microbiota. *BMC veterinary research* 8: 90.

(*166*). Gupta, L., R. A. Kumar, T. Ghanshyam, D. Rajesh, and R. Garg. **2012**. Effect of feeding different proportions of groundnut haulms (Arachis hypogaea) and cluster bean straw (Cyamopsis tetragonoloba) on nutrient utilisation and serum biochemical parameters in dromedary camels. *Tropical animal health and production* 44: 1689-1695.

(*167*). Gnanandarajah, J. S., T. J. Johnson, H. B. Kim, J. E. Abrahante, J. P. Lulich, and M. P. Murtaugh. **2012**. Comparative faecal microbiota of dogs with and without calcium oxalate stones. *Journal of applied microbiology* 113: 745-756.

(*168*). Garcia-Mazcorro, J. F., J. S. Suchodolski, K. R. Jones, S. C. Clark-Price, S. E. Dowd, Y. Minamoto, M. Markel, J. M. Steiner, and O. Dossin. **2012**. Effect of the proton pump inhibitor omeprazole on the gastrointestinal bacterial microbiota of healthy dogs. *FEMS microbiology ecology* 80: 624-636.

(*169*). Garcia-Mazcorro, J. F., S. E. Dowd, J. Poulsen, J. M. Steiner, and J. S. Suchodolski. **2012**. Abundance and short-term temporal variability of fecal microbiota in healthy dogs. *MicrobiologyOpen* 1: 340-347.

(*170*). Rouimi, P., N. Zucchini-Pascal, G. Dupont, A. Razpotnik, E. Fouche, G. De Sousa, and R. Rahmani. **2012**. Impacts of low doses of pesticide mixtures on liver cell defence systems. *Toxicology in Vitro* 26: 718-726.

(*171*). Sturla, S. J., A. R. Boobis, R. E. FitzGerald, J. Hoeng, R. J. Kavlock, K. Schirmer, M. Whelan, M. F. Wilks, and M. C. Peitsch. **2014**. Systems Toxicology: From Basic Research to Risk Assessment. *Chem Res Toxicol* 27: 314-329.

(*172*). Kealy, R. D., D. F. Lawler, J. M. Ballam, S. L. Mantz, D. N. Biery, E. H. Greeley, G. Lust, M. Segre, G. K. Smith, and H. D. Stowe. **2002**. Effects of diet restriction on life span and age-related changes in dogs. *Journal of the American Veterinary Medical Association* 220: 1315-1320.

(*173*). Verkest, K. R., J. S. Rand, L. M. Fleeman, and J. M. Morton. **2012**. Spontaneously obese dogs exhibit greater postprandial glucose, triglyceride, and insulin concentrations than lean dogs. *Domestic Animal Endocrinology* 42: 103-112.

(*174*). Handl, S., A. J. German, S. L. Holden, S. E. Dowd, J. M. Steiner, R. M. Heilmann, R. W. Grant, K. S. Swanson, and J. S. Suchodolski. **2013**. Faecal microbiota in lean and obese dogs. *Fems Microbiol Ecol* 84: 332-343.

(*175*). Eirmann, L. A., L. M. Freeman, D. P. Laflamme, K. E. Michel, and E. Satyaraj. **2009**. Comparison of Adipokine Concentrations and Markers of Inflammation in Obese Versus Lean Dogs. *Int J Appl Res Vet M* 7: 196-205.

(*176*). Park, H. J., S. E. Lee, J. H. Oh, K. W. Seo, and K. H. Song. **2014**. Leptin, adiponectin and serotonin levels in lean and obese dogs. *Bmc Veterinary Research* 10.

(*177*). Zhang, A., H. Sun, and X. Wang. **2013**. Power of metabolomics in biomarker discovery and mining mechanisms of obesity. *Obes Rev* 14: 344-349.

(*178*). Gogna, N., M. Krishna, A. M. Oommen, and K. Dorai. **2015**. Investigating correlations in the altered metabolic profiles of obese and diabetic subjects in a South Indian Asian population using an NMR-based metabolomic approach. *Mol Biosyst* 11: 595-606.

(*179*). Ding, W., and R. H. Mak. **2015**. Early markers of obesity-related renal injury in childhood. *Pediatr Nephrol* 30: 1-4.

(*180*). Yu, Z. R., Y. Ning, H. Yu, and N. J. Tang. **2014**. A HPLC-Q-TOF-MS-based urinary metabolomic approach to identification of potential biomarkers of metabolic syndrome. *J Huazhong U Sci-Med* 34: 276-283.

(*181*). Wiklund, P. K., S. Pekkala, R. Autio, E. Munukka, L. T. Xu, J. Saltevo, S. M. Cheng, U. M. Kujala, M. Alen, and S. L. Cheng. **2014**. Serum metabolic profiles in overweight and obese women with and without metabolic syndrome. *Diabetol Metab Syndr* 6.

(*182*). Rodriguez-Gallego, E., S. Fernandez-Arroyo, M. Riera-Borrull, A. Hernandez-Aguilera, M. Guirro, R. Marine-Casado, F. Luciano-Mateo, A. Garcia-Heredia, A. A. Marti-Lindez, R. Beltran-Debon, and J. Joven. **2014**. Metabolomics Reveals That Alterations in Circulating a-Ketoglutarate May Be Relevant in the Assessment of Morbid Obesity-Associated Nonalcoholic Fatty Liver Disease. *Atherosclerosis* 235: E65-E65.

(*183*). Rauschert, S., O. Uhl, B. Koletzko, and C. Hellmuth. **2014**. Metabolomic Biomarkers for Obesity in Humans: A Short Review. *Ann Nutr Metab* 64: 314-324.

(*184*). Perng, W., M. W. Gillman, A. F. Fleisch, R. D. Michalek, S. M. Watkins, E. Isganaitis, M. E. Patti, and E. Oken. **2014**. Metabolomic Profiles and Childhood Obesity. *Obesity* 22: 2570-2578.

(*185*). Floegel, A., A. Wientzek, U. Bachlechner, S. Jacobs, D. Drogan, C. Prehn, J. Adamski, J. Krumsiek, M. B. Schulze, T. Pischon, and H. Boeing. **2014**. Linking diet, physical activity, cardiorespiratory fitness and obesity to serum metabolite networks: findings from a population-based study. *International Journal of Obesity* 38: 1388-1396.

(*186*). Calvani, R., E. Brasili, G. Pratico, F. Sciubba, M. Roselli, A. Finamore, F. Marini, E. Marzetti, and A. Miccheli. **2014**. Application of NMR-based Metabolomics to the Study of Gut Microbiota in Obesity. *J Clin Gastroenterol* 48: S5-S7.

(*187*). Kumar, J., R. Karlsson, C. D Broeckling, M.-G. Hong, J. A Prince, J. E Prenni, E. Ingelsson, and F. Wiklund. **2014**. Associations of Body Mass Index and Obesity-Related Genetic Variants with Serum Metabolites. *Current Metabolomics* 2: 27-36.

(*188*). Zhou, Y., L. Qiu, Q. Xiao, Y. Wang, X. Y. Meng, R. Xu, S. Y. Wang, and R. Na. **2013**. Obesity and diabetes related plasma amino acid alterations. *Clin Biochem* 46: 1447-1452.

(*189*). Munoz, J. M. O. **2013**. Predictors of obesity: the "power" of the omics. *Nutr Hosp* 28: 63-72.

(*190*). Kim, O. Y., J. H. Lee, and G. Sweeney. **2013**. Metabolomic profiling as a useful tool for diagnosis and treatment of chronic disease: focus on obesity, diabetes and cardiovascular diseases. *Expert review of cardiovascular therapy* 11: 61-68.

(*191*). Laflamme, D. **1997**. Development and validation of a body condition score system for dogs. *Canine Practice*: 10-15.

(*192*). German, A. J., S. L. Holden, T. Bissot, P. J. Morris, and V. Biourge. **2009**. Use of starting condition score to estimate changes in body weight and composition during weight loss in obese dogs. *Research in veterinary science* 87: 249-254.

(*193*). Forster, G. M., D. Hill, G. Gregory, K. M. Weishaar, S. Lana, J. E. Bauer, and E. P. Ryan. **2012**. Effects of cooked navy bean powder on apparent total tract nutrient digestibility and safety in healthy adult dogs. *J Anim Sci* 90: 2631-2638

(*194*). Heuberger, A. L., C. D. Broeckling, K. R. Kirkpatrick, and J. E. Prenni. **2014**. Application of nontargeted metabolite profiling to discover novel markers of quality traits in an advanced population of malting barley. *Plant Biotechnology Journal* 12: 147-160.

(*195*). Broeckling, C. D., F. A. Afsar, S. Neumann, A. Ben-Hur, and J. E. Prenni. **2014**. RAMClust: A Novel Feature Clustering Method Enables Spectral-Matching-Based Annotation for Metabolomics Data. *Analytical Chemistry* 86: 6812-6817.

(*196*). R-Core-Team. **2014**. R: A language and envrionment for statistical computing. http://www.R-project.org/ Accessed 3/2/2015.

(*197*). Benjamini, Y., and Y. Hochberg. **1995**. Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. *J Roy Stat Soc B Met* 57: 289-300.

(*198*). Pena, C., L. Suarez, I. Bautista, J. A. Montoya, and M. C. Juste. **2008**. Relationship between analytic values and canine obesity. *Journal of Animal Physiology and Animal Nutrition* 92: 324-325.

(*199*). Usui, S., H. Yasuda, and Y. Koketsu. **2015**. Lipoprotein cholesterol and triglyceride concentrations associated with dog body condition score; effect of recommended fasting duration on sample concentrations in Japanese private clinics. *The Journal of veterinary medical science / the Japanese Society of Veterinary Science* 10.1292/jvms.15-0032.

(*200*). Park, H. J., S. E. Lee, H. B. Kim, R. E. Isaacson, K. W. Seo, and K. H. Song. **2015**. Association of obesity with serum leptin, adiponectin, and serotonin and gut microflora in beagle dogs. *Journal of veterinary internal medicine / American College of Veterinary Internal Medicine* 29: 43-50.

(*201*). Ricci, R., F. Gottardo, J. C. Ferlito, A. Stefani, L. Ravarotto, and I. Andrighetto. **2007**. Body condition score (BCS) and metabolic status of shelter dogs. *Italian Journal of Animal Science* 6: 3.

(*202*). Usui, S., Y. Mizoguchi, H. Yasuda, N. Arai, and Y. Koketsu. **2014**. Dog Age and Breeds Associated with High Plasma Cholesterol and Triglyceride Concentrations. *Journal of Veterinary Medical Science* 76: 269-U123.

(*203*). Paulson, J. N., O. C. Stine, H. C. Bravo, and M. Pop. **2013**. Robust methods for differential abundance analysis in marker gene surveys. *Nature methods* 10: 1200-1202.

(*204*). United States. Public Health Service. Office of the Surgeon General. **1988**. The Surgeon General's report on nutrition and health. U.S. Dept. of Health and Human Services For sale by the Supt. of Docs., U.S. G.P.O., Washington, DC.

(*205*). Hand, M. S. **2000**. Small animal clinical nutrition. 4th ed. Mark Morris Institute, Topeka, Kan.

(*206*). Fortes, C. M. L. S., A. C. Carciofi, N. K. Sakomura, I. M. Kawauchi, and R. S. Vasconcellos. **2010**. Digestibility and metabolizable energy of some carbohydrate sources for dogs. *Anim. Feed. Sci. Tech.* 156: 121-125.

(*207*). Bednar, G. E., A. R. Patil, S. M. Murray, C. M. Grieshop, N. R. Merchen, and G. C. Fahey, Jr. **2001**. Starch and fiber fractions in selected food and feed ingredients affect their small intestinal digestibility and fermentability and their large bowel fermentability in vitro in a canine model. *J. Nutr.* 131: 276-286.

(*208*). Dust, J. M., C. M. Grieshop, C. M. Parsons, L. K. Karr-Lilienthal, C. S. Schasteen, J. D. Quigley, N. R. Merchen, and G. C. Fahey. **2005**. Chemical composition, protein quality, palatability, and digestibility of alternative protein sources for dogs. *J. Anim. Sci.* 83: 2414-2422.

(*209*). Zentek, J., and R. Mischke. **1997**. Soya and casein as dietary proteins for dogs. *Journal of Animal Physiology and Animal Nutrition-Zeitschrift Fur Tierphysiologie Tierernahrung Und Futtermittelkunde* 77: 139-148.

(*210*). Geil, P. B., and J. W. Anderson. **1994**. Nutrition and Health Implications of Dry Beans - a Review. *J. Am. Coll. Nutr.* 13: 549-558.

(*211*). Mentor-Marcel, R. A., G. Bobe, K. G. Barrett, M. R. Young, P. S. Albert, M. R. Bennink, E. Lanza, and N. H. Colburn. **2009**. Inflammation-Associated Serum and Colon Markers as Indicators of Dietary Attenuation of Colon Carcinogenesis in ob/ob Mice. *Cancer Prev. Res. (Phila.)* 2: 60-69.

(*212*). Thompson, M. D., M. A. Brick, J. N. McGinley, and H. J. Thompson. **2009**. Chemical Composition and Mammary Cancer Inhibitory Activity of Dry Bean. *Crop Sci* 49: 179-186.

(*213*). Yamka, R. M., D. L. Harmon, W. D. Schoenherr, C. Khoo, K. L. Gross, S. J. Davidson, and D. K. Joshi. **2006**. In vivo measurement of flatulence and nutrient digestibility in dogs fed poultry by-product meal, conventional soybean meal, and low-oligosaccharide low-phytate soybean meal. *Am. J. Vet. Res.* 67: 88-94.

(*214*). Laflamme, D. **1997**. Development and validation of a body condition score system for dogs. *Canine Practice* 22: 10-15.

(*215*). AAFCO. **2010**. Official Publication. Association of American Feed Control Officials Inc, Oxford, IN.

(*216*). National Research Council (U.S.). Ad Hoc Committee on Dog and Cat Nutrition. **2006**. Nutrient requirements of dogs and cats. National Academies Press, Washington, D.C.

(*217*). Osborne, C. A. **1981**. Handbook of Canine and Feline Urinalysis. Ralston Purina Company, Saint Louis, Missouri.

(*218*). AOAC. **2000**. Official Methods of Analysis AOAC International, Gaithersburg, MD.

(*219*). Dunbar, B. L., K. E. Bigley, and J. E. Bauer. **2010**. Early and Sustained Enrichment of Serum n-3 Long Chain Polyunsaturated Fatty Acids in Dogs Fed a Flaxseed Supplemented Diet. *Lipids* 45: 1-10.

(*220*). Brown, R. G. **1997**. A comparison of certified and noncertified pet foods. *Can. Vet. J.* 38: 707-712.

(*221*). Krogdahl, A., O. Ahlstrom, and A. Skrede. **2004**. Nutrient digestibility of commercial dog foods using mink as a model. *J. Nutr.* 134: 2141-2144.

(*222*). Kahlon, T. S., and C. L. Woodruff. **2002**. In vitro binding of bile acids by soy protein, pinto beans, black beans and wheat gluten. *Food Chemistry* 79: 425-429.

(*223*). Messina, M. J. **1999**. Legumes and soybeans: overview of their nutritional profiles and health effects. *Am. J. Clin. Nutr.* 70: 439-450.

(*224*). Mallillin, A. C., T. P. Trinidad, R. Raterta, K. Dagbay, and A. S. Loyola. **2008**. Dietary fibre and fermentability characteristics of root crops and legumes. *Br. J. Nutr.* 100: 485-488.

(*225*). Fahey, G. C., E. A. Flickinger, C. M. Grieshop, and K. S. Swanson. **2004**. The role of dietary fibre in companion animal nutrition. *Dietary Fibre*: 295-328.

(*226*). Middelbos, I. S., B. M. V. Boler, A. Qu, B. A. White, K. S. Swanson, and G. C. Fahey. **2010**. Phylogenetic Characterization of Fecal Microbial Communities of Dogs Fed Diets with or without Supplemental Dietary Fiber Using 454 Pyrosequencing. *PloS one* 5.

(*227*). German, A. J. **2006**. The growing problem of obesity in dogs and cats. *Journal of Nutrition* 136: 1940s-1946s.

(*228*). Mitsuhashi, Y., D. Nagaoka, K. Ishioka, K. E. Bigley, M. Okawa, K. Otsuji, and J. E. Bauer. **2010**. Postprandial lipid-related metabolites are altered in dogs fed dietary diacylglycerol and low glycemic index starch during weight loss. *J Nutr* 140: 1815-1823.

(*229*). Gupta, S. C., J. H. Kim, S. Prasad, and B. B. Aggarwal. **2010**. Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals. *Cancer Metastasis Rev.* 29: 405-434.

(*230*). Venn, B. J., and J. I. Mann. **2004**. Cereal grains, legumes and diabetes. *Eur. J. Clin. Nutr.* 58: 1443-1461.

(*231*). Villegas, R., Y. T. Gao, G. Yang, H. L. Li, T. A. Elasy, W. Zheng, and X. O. Shu. **2008**. Legume and soy food intake and the incidence of type 2 diabetes in the Shanghai Women's Health Study. *Am. J. Clin. Nutr*. 87: 162-167.

(*232*). Shi, J., K. Arunasalam, D. Yeung, Y. Kakuda, G. Mittal, and Y. M. Jiang. **2004**. Saponins from edible legumes: Chemistry, processing, and health benefits. *J. Med. Food* 7: 67-78.

(*233*). Zhou, Z. K., D. L. Topping, M. K. Morell, and A. R. Bird. **2010**. Changes in starch physical characteristics following digestion of foods in the human small intestine. *Br. J. Nutr*. 104: 573-581.

(*234*). Darmadi-Blackberry, I., M. L. Wahlqvist, A. Kouris-Blazos, B. Steen, W. Lukito, Y. Horie, and K. Horie. **2004**. Legumes: the most important dietary predictor of survival in older people of different ethnicities. *Asia Pac. J. Clin. Nutr*. 13: 217-220.

(*235*). Mensack, M. M., V. K. Fitzgerald, M. R. Lewis, and H. J. Thompson. **2010**. Characterization of Low Molecular Weight Chemical Fractions of Dry Bean (Phaseolus vulgaris) for Bioactivity Using Caenorhabditis elegans Longevity and Metabolite Fingerprinting. *J. Agric. Food Chem.* 58: 6697-6705.

(*236*). Park, Y., A. F. Subar, A. Hollenbeck, and A. Schatzkin. **2011**. Dietary Fiber Intake and Mortality in the NIH-AARP Diet and Health Study. *Arch. Intern. Med.* 171: 1061-1068.

(*237*). Jamroz, D., and J. Kubizna. **2008**. Harmful substances in legume seeds - their negative and beneficial properties. *Pol. J. Vet. Sci.* 11: 389-404.

(*238*). Martin-Cabrejas, M. A., Y. Aguilera, M. M. Pedrosa, C. Cuadrado, T. Hernandez, S. Diaz, and R. M. Esteban. **2009**. The impact of dehydration process on antinutrients and protein digestibility of some legume flours. *Food Chem.* 114: 1063-1068.

(*239*). Rehman, Z. U., and W. H. Shah. **2005**. Thermal heat processing effects on antinutrients, protein and starch digestibility of food legumes. *Food Chem.* 91: 327-331.

(*240*). German, A. **2010**. Obesity in companion animals. *In Practice* 32: 42-50.

(*241*). Linder, D. E., L. M. Freeman, S. L. Holden, V. Biourge, and A. J. German. **2013**. Status of selected nutrients in obese dogs undergoing caloric restriction. *BMC Veterinary Research* 9: 219.

(*242*). Linder, D. E. 2014. Top 5 Clincial Consequences of Obesity. Clinician's Brief http://www.cliniciansbrief.com/article/top-5-clinical-consequences-obesity Accessed 7/7/2015.

(*243*). NRC. **2006**. Nutrient Requirements of Dogs and Cats. The National Academies Press, Washington, DC.

(*244*). Butterwick, R., and P. Markwell. **1997**. Effect of amount and type of dietary fiber on food intake in energy-restricted dogs. *American journal of veterinary research* 58: 272-276.

(*245*). Weber, M., T. Bissot, E. Servet, R. Sergheraert, V. Biourge, and A. J. German. **2007**. A High-Protein, High-Fiber Diet Designed for Weight Loss Improves Satiety in Dogs. *J Vet Intern Med* 21: 1203-1208.

(*246*). Shai, I., D. Schwarzfuchs, Y. Henkin, D. R. Shahar, S. Witkow, I. Greenberg, R. Golan, D. Fraser, A. Bolotin, H. Vardi, O. Tangi-Rozental, R. Zuk-Ramot, B. Sarusi, D. Brickner, Z. Schwartz, E. Sheiner, R. Marko, E. Katorza, J. Thiery, G. M. Fiedler, M. Blüher, M. Stumvoll, and M. J. Stampfer. **2008**. Weight Loss with a Low-Carbohydrate, Mediterranean, or Low-Fat Diet. *New England Journal of Medicine* 359: 229-241.

(*247*). Rayalam, S., M. A. Della-Fera, and C. A. Baile. **2008**. Phytochemicals and regulation of the adipocyte life cycle. *The Journal of Nutritional Biochemistry* 19: 717-726.

(*248*). Deibert, P., D. Konig, A. Schmidt-Trucksaess, K. S. Zaenker, I. Frey, U. Landmann, and A. Berg. **2004**. Weight loss without losing muscle mass in pre-obese and obese subjects induced by a high-soy-protein diet. *International Journal of Obesity* 28: 1349-1352.

(*249*). Mollard, R., B. Luhovyy, S. Panahi, M. Nunez, A. Hanley, and G. Anderson. **2012**. Regular consumption of pulses for 8 weeks reduces metabolic syndrome risk factors in overweight and obese adults. *British Journal of Nutrition* 108: S111-S122.

(*250*). Borresen, E. C., K. A. Gundlach, M. Wdowik, S. Rao, R. J. Brown, and E. P. Ryan. **2014**. Feasibility of Increased Navy Bean Powder Consumption for Primary and Secondary Colorectal Cancer Prevention. *Current Nutrition and Food Science* 10: 112-119.

(*251*). McCrory, M. A., B. R. Hamaker, J. C. Lovejoy, and P. E. Eichelsdoerfer. **2010**. Pulse consumption, satiety, and weight management. *Advances in Nutrition: An International Review Journal* 1: 17-30.

(*252*). Mudryj, A. N., N. Yu, and H. M. Aukema. **2014**. Nutritional and health benefits of pulses. *Applied Physiology, Nutrition, and Metabolism* 39: 1197-1204.

(*253*). Hayat, I., A. Ahmad, T. Masud, A. Ahmed, and S. Bashir. **2014**. Nutritional and health perspectives of beans (Phaseolus vulgaris L.): an overview. *Critical reviews in food science and nutrition* 54: 580-592.

(*254*). Barrett, M. L., and J. K. Udani. **2011**. A proprietary alpha-amylase inhibitor from white bean (Phaseolus vulgaris): A review of clinical studies on weight loss and glycemic control. *Nutrition Journal* 10.

(*255*). Chávez-Santoscoy, R. A., A. R. Tovar, S. O. Serna-Saldivar, N. Torres, and J. A. Gutiérrez-Uribe. **2014**. Conjugated and free sterols from black bean (Phaseolus vulgaris L.) seed coats as cholesterol micelle disruptors and their effect on lipid metabolism and cholesterol transport in rat primary hepatocytes. *Genes & Nutrition* 9: 367.

(*256*). Ramírez-Jiménez, A. K., R. Reynoso-Camacho, M. E. Tejero, F. León-Galván, and G. Loarca-Piña. **2015**. Potential role of bioactive compounds of Phaseolus vulgaris L. on lipids-lowering mechanisms. *Food Research International* ahead-of-print.

(*257*). Forster, G. M., D. Hill, G. Gregory, K. M. Weishaar, S. Lana, J. E. Bauer, and E. P. Ryan. **2012**. Effects of cooked navy bean powder on apparent total tract nutrient digestibility and safety in healthy adult dogs. *Journal of Animal Science* 90: 2631-2638.

(*258*). Forster, G. M., C. A. Ollila, J. H. Burton, D. Hill, J. E. Bauer, A. M. Hess, and E. P. Ryan. **2012**. Nutritional weight loss therapy with cooked bean powders regulates serum lipids and biochemical analytes in overweight and obese dogs. *Journal of Obesity & Weight Loss Therapy* 2: 149.

(*259*). Prosky, L., N. G. Asp, T. F. Schweizer, J. W. Devries, and I. Furda. **1992**. Determination of Insoluble and Soluble Dietary Fiber in Foods and Food-Products - Collaborative Study. *J Aoac Int* 75: 360-367.

(*260*). Laflamme, D. P. **2012**. Obesity in dogs and cats: What is wrong with being fat? *Journal of Animal Science* 90: 1653-1662.

(*261*). Yamka, R. M., K. G. Friesen, and N. Z. Frantz. **2006**. Identification of Canine Markers Related to Obesity and the Effects of Weight Loss on the Markers of Interest. *International Journal of Applied Research in Veterinary Medicine* 4: 10.

(*262*). Hayat, I., A. Ahmad, T. Masud, A. Ahmed, and S. Bashir. **2014**. Nutritional and health perspectives of beans (*Phaseolus vulgaris* L.): an overview. *Crit Rev Food Sci Nutr* 54: 580-592.

(*263*). Geil, P. B., and J. W. Anderson. **1994**. Nutrition and health implications of dry beans: a review. *Journal of the American College of Nutrition* 13: 549-558.

(*264*). Chavez-Santoscoy, R. A., A. R. Tovar, S. O. Serna-Saldivar, N. Torres, and J. A. Gutierrez-Uribe. **2014**. Conjugated and free sterols from black bean (Phaseolus vulgaris L.) seed coats as cholesterol micelle disruptors and their effect on lipid metabolism and cholesterol transport in rat primary hepatocytes. *Genes Nutr* 9: 367.

(*265*). Bouchenak, M., and M. Lamri-Senhadji. **2013**. Nutritional Quality of Legumes, and Their Role in Cardiometabolic Risk Prevention: A Review. *J Med Food* 16: 185-198.

(*266*). Aparicio-Fernandez, X., G. G. Yousef, G. Loarca-Pina, E. de Mejia, and M. A. Lila. **2005**. Characterization of polyphenolics in the seed coat of Black Jamapa bean (Phaseolus vulgaris L.). *Journal of agricultural and food chemistry* 53: 4615-4622.

(*267*). Anderson, J. W., B. M. Smith, and C. S. Washnock. **1999**. Cardiovascular and renal benefits of dry bean and soybean intake. *The American journal of clinical nutrition* 70: 464S-474S.

(*268*). Mensack, M. M., V. K. Fitzgerald, M. R. Lewis, and H. J. Thompson. **2010**. Characterization of low molecular weight chemical fractions of dry bean (Phaseolus vulgaris) for bioactivity using Caenorhabditis elegans longevity and metabolite fingerprinting. *Journal of agricultural and food chemistry* 58: 6697-6705.

(*269*). Daniell, E. L., E. P. Ryan, M. A. Brick, and H. J. Thompson. **2012**. Dietary dry bean effects on hepatic expression of stress and toxicity-related genes in rats. *The British journal of nutrition* 108 Suppl 1: S37-45.

(*270*). Zhu, Z., W. Jiang, and H. J. Thompson. **2012**. Edible dry bean consumption (Phaseolus vulgaris L.) modulates cardiovascular risk factors and diet-induced obesity in rats and mice. *The British journal of nutrition* 108 Suppl 1: S66-73.

(*271*). Winham, D. M., and A. M. Hutchins. **2007**. Baked bean consumption reduces serum cholesterol in hypercholesterolemic adults. *Nutrition Research* 27: 380-386.

(*272*). Birketvedt, G. S., A. Travis, B. Langbakk, and J. R. Florholmen. **2002**. Dietary supplementation with bean extract improves lipid profile in overweight and obese subjects. *Nutrition* 18: 729-733.

(*273*). Kirwan, J. **2013**. Metabolomics for the practising vet. *In Practice* 35.

(*274*). Jones, O. A., and V. L. Cheung. **2007**. An introduction to metabolomics and its potential application in veterinary science. *Comparative medicine* 57: 436-442.

(*275*). Whitfield, P. D., A. J. German, and P. J. Noble. **2004**. Metabolomics: an emerging post-genomic tool for nutrition. *The British journal of nutrition* 92: 549-555.

(*276*). O'Gorman, A., and L. Brennan. **2015**. Metabolomic applications in nutritional research: a perspective. *Journal of the Science of Food and Agriculture* 10.1002/jsfa.7070: n/a-n/a.

(*277*). Ryan, E. P., A. L. Heuberger, C. D. Broeckling, E. C. Borresen, C. Tillotson, and J. E. Prenni. **2013**. Advances in Nutritional Metabolomics. *Curr Metabolomics* 1: 109-120.

(*278*). **2006** Nutrient Requirements of Dogs and Cats, National Research Council, Washington, D.C.

(*279*). Heuberger, A. L., M. R. Lewis, M. H. Chen, M. A. Brick, J. E. Leach, and E. P. Ryan. **2010**. Metabolomic and functional genomic analyses reveal varietal differences in bioactive compounds of cooked rice. *PloS one* 5: e12915.

(*280*). Weir, T. L., D. K. Manter, A. M. Sheflin, B. A. Barnett, A. L. Heuberger, and E. P. Ryan. **2013**. Stool microbiome and metabolome differences between colorectal cancer patients and healthy adults. *PloS one* 8: e70803.

(*281*). Heuberger, A. L., C. D. Broeckling, M. R. Lewis, L. Salazar, P. Bouckaert, and J. E. Prenni. **2012**. Metabolomic profiling of beer reveals effect of temperature on non-volatile small molecules during short-term storage. *Food Chem* 135: 1284-1289.

(*282*). Smith, C., E. Want, O. M. G, R. Abagyan, and G. Siuzdak. **2006**. Processing Mass Spectrometry Data for Metabolite Profiling Using Nonlinear Peak Alignment, Matching, and Identification. *Analytical Chemistry* 78: 779-787.

(*283*). Sumner, L. W., A. Amberg, D. Barrett, M. H. Beale, R. Beger, C. A. Daykin, T. W. Fan, O. Fiehn, R. Goodacre, J. L. Griffin, T. Hankemeier, N. Hardy, J. Harnly, R. Higashi, J. Kopka, A. N. Lane, J. C. Lindon, P. Marriott, A. W. Nicholls, M. D. Reily, J. J. Thaden, and M. R. Viant. **2007**. Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics* 3: 211-221.

(*284*). Bates, D., M. Mächler, B. Bolker, and S. Walker. **2014**. Fitting linear mixed-effects models using lme4. arXiv.

(*285*). Li, C.-J., D. M. Brownson, T. J. Mabry, C. Perera, and E. Arthur Bell. **1996**. Nonprotein amino acids from seeds of Cycas circinalis and Phaseolus vulgaris. *Phytochemistry* 42: 443-445.

(*286*). Perera, T., M. R. Young, Z. Zhang, G. Murphy, N. H. Colburn, E. Lanza, T. J. Hartman, A. J. Cross, and G. Bobe. **2015**. Identification and monitoring of metabolite markers of dry bean consumption in parallel human and mouse studies. *Mol Nutr Food Res* 10.1002/mnfr.201400847.

(*287*). Guertin, K. A., S. C. Moore, J. N. Sampson, W. Y. Huang, Q. Xiao, R. Z. Stolzenberg-Solomon, R. Sinha, and A. J. Cross. **2014**. Metabolomics in nutritional epidemiology: identifying metabolites associated with diet and quantifying their potential to uncover diet-disease relations in populations. *The American journal of clinical nutrition* 100: 208-217.

(*288*). Fernández-Marín, B., R. Milla, N. Martín-Robles, E. Arc, I. Kranner, J. M. Becerril, and J. I. García-Plazaola. **2014**. Side-effects of domestication: cultivated legume seeds contain similar tocopherols and fatty acids but less carotenoids than their wild counterparts. *BMC Plant Biology* 14: 1599.

(*289*). Ryan, E., K. Galvin, T. P. O'Connor, A. R. Maguire, and N. M. O'Brien. **2007**. Phytosterol, squalene, tocopherol content and fatty acid profile of selected seeds, grains, and legumes. *Plant Foods for Human Nutrition* 62: 85-91.

(*290*). Biesiekierski, J. R., O. Rosella, R. Rose, K. Liels, J. S. Barrett, S. J. Shepherd, P. R. Gibson, and J. G. Muir. **2011**. Quantification of fructans, galacto-oligosacharides and other short-chain carbohydrates in processed grains and cereals. *Journal of Human Nutrition and Dietetics* 24: 154-176.

(*291*). Guillon, F., and M.-J. Champ. **2002**. Carbohydrate fractions of legumes: uses in human nutrition and potential for health. *British Journal of Nutrition* 88: 293-306.

(*292*). Kelkar, S., M. Siddiq, J. B. Harte, K. D. Dolan, G. Nyombaire, and H. Suniaga. **2012**. Use of low-temperature extrusion for reducing phytohemagglutinin activity (PHA) and oligosaccharides in beans (Phaseolus vulgaris L.) cv. Navy and Pinto. *Food Chemistry* 133: 1636-1639.

(*293*). Forster, G. M., C. A. Ollila, J. H. Burton, D. Hill, J. E. Bauer, A. M. Hess, and E. P. Ryan. **2012**. Nutritional Weight Loss Therapy with Cooked Bean Powders Regulates Serum Lipids and Biochemical Analytes in Overweight and Obese Dogs. *J Obes Weight Loss Ther* 2.

(*294*). Chavez-Santoscoy, R. A., J. A. Gutierrez-Uribe, and S. O. Serna-Saldivar. **2013**. Effect of flavonoids and saponins extracted from black bean (Phaseolus vulgaris L.) seed coats as cholesterol micelle disruptors. *Plant Foods Hum Nutr* 68: 416-423.

(*295*). Sidhu, G. S., and D. G. Oakenfull. **1986**. A mechanism for the hypocholesterolaemic activity of saponins. *The British journal of nutrition* 55: 643-649.

(*296*). Costa, N. M., A. G. Low, A. F. Walker, R. W. Owen, and H. N. Englyst. **1994**. Effect of baked beans (Phaseolus vulgaris) on steroid metabolism and non-starch polysaccharide output of hypercholesterolaemic pigs with or without an ileo-rectal anastomosis. *The British journal of nutrition* 71: 871-886.

(*297*). Rosa, C. O., N. M. Costa, P. F. Leal, and T. T. Oliveira. **1998**. The cholesterol-lowering effect of black beans (Phaseolus vulgaris, L.) without hulls in hypercholesterolemic rats. *Archivos latinoamericanos de nutricion* 48: 299-305.

(*298*). Chavez-Santoscoy, R. A., J. A. Gutierrez-Uribe, O. Granados, I. Torre-Villalvazo, S. O. Serna-Saldivar, N. Torres, B. Palacios-Gonzalez, and A. R. Tovar. **2014**. Flavonoids and saponins extracted from black bean (Phaseolus vulgaris L.) seed coats modulate lipid metabolism and biliary cholesterol secretion in C57BL/6 mice. *The British journal of nutrition* 112: 886-899.

(*299*). Cohn, J. S., A. Kamili, E. Wat, R. W. Chung, and S. Tandy. **2010**. Reduction in intestinal cholesterol absorption by various food components: mechanisms and implications. *Atherosclerosis. Supplements* 11: 45-48.

(*300*). Marzolo, M. P., L. Amigo, and F. Nervi. **1993**. Hepatic production of very low density lipoprotein, catabolism of low density lipoprotein, biliary lipid secretion, and bile salt synthesis in rats fed a bean (Phaseolus vulgaris) diet. *J Lipid Res* 34: 807-814.

(*301*). Anderson, J. W., L. Story, B. Sieling, W. J. Chen, M. S. Petro, and J. Story. **1984**. Hypocholesterolemic effects of oat-bran or bean intake for hypercholesterolemic men. *The American journal of clinical nutrition* 40: 1146-1155.

(*302*). Ishimwe, N., E. B. Daliri, B. H. Lee, F. Fang, and G. Du. **2015**. The perspective on cholesterol-lowering mechanisms of probiotics. *Mol Nutr Food Res* 59: 94-105.

(*303*). Leeming, R., A. Ball, N. Ashbolt, and P. Nichols. **1996**. Using faecal sterols from humans and animals to distinguish faecal pollution in receiving waters. *Water Research* 30: 2893-2900.

(*304*). Flegal, K. M., M. D. Carroll, B. K. Kit, and C. L. Ogden. **2012**. Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999-2010. *JAMA* 307: 491-497.

(*305*). Jia, H. M., and E. I. Lubetkin. **2010**. Trends in Quality-Adjusted Life-Years Lost Contributed by Smoking and Obesity. *Am J Prev Med* 38: 138-144.

(*306*). German, A. **2010**. Obesity in companion animals. *In Pract* 32: 42-+.

(*307*). Kopelman, P. G. **2000**. Obesity as a medical problem. *Nature* 404: 635-643.

(*308*). Cignarelli, M., and O. Lamacchia. **2007**. Obesity and kidney disease. *Nutr Metab Cardiovasc Dis* 17: 757-762.

(*309*). Howard, B. V., G. Ruotolo, and D. C. Robbins. **2003**. Obesity and dyslipidemia. *Endocrinol Metab Clin North Am* 32: 855-+.

(*310*). Thamm, D., and S. Dow. **2009**. How companion animals contribute to the fight against cancer in humans. *Veterinaria Italiana* 45: 111-120.

(*311*). Pinho, S., S. Carvalho, J. Cabral, C. Reis, and F. Gartner. **2012**. Canine tumors: a spontaneous animal model of human carcinogenesis. *Transl Res* 159: 165-172.

(*312*). Baehr, W., and J. M. Frederick. **2009**. Naturally occurring animal models with outer retina phenotypes. *Vision Res* 49: 2636-2652.

(*313*). Schotanus, B. A., T. van den Ingh, L. C. Penning, J. Rothuizen, T. A. Roskams, and B. Spee. **2009**. Cross-species immunohistochemical investigation of the activation of the liver progenitor cell niche in different types of liver disease. *Liver Int* 29: 1241-1252.

(*314*). Murphy, M. P., J. Morales, T. L. Beckett, G. Astarita, D. Piomelli, A. Weidner, C. M. Studzinski, A. L. S. Dowling, X. H. Wang, H. LeVine, R. J. Kryscio, Y. S. Lin, E. Barrett, and E. Head. **2010**. Changes in Cognition and Amyloid-beta Processing with Long Term Cholesterol Reduction using Atorvastatin in Aged Dogs. *J Alzheimers Dis* 22: 135-150.

(*315*). Vegesna, A., A. Korimilli, R. Besetty, L. Bright, A. Milton, A. Agelan, K. McIntyre, A. Malik, and L. Miller. **2010**. Endoscopic pyloric suturing to facilitate weight loss: a canine model. *Gastrointest Endosc* 72: 427-431.

(*316*). Guo, X. M., H. Zheng, S. G. Mattar, X. Lu, G. Sandusky, J. A. Navia, and G. Kassab. **2011**. Reversible Gastric Restriction Implant: Safety and Efficacy in a Canine Model. *Obes Surg* 21: 1444-1450.

(*317*). Rao, R. S., V. Rao, and S. Kini. **2010**. Animal Models in Bariatric Surgery-A Review of the Surgical Techniques and Postsurgical Physiology. *Obes Surg* 20: 1293-1305.

(*318*). Carpenter, K. J. **1991**. CONTRIBUTION OF THE DOG TO THE SCIENCE OF NUTRITION. *Journal of Nutrition* 121: S1-S7.

(*319*). Snigdha, S., N. Berchtold, G. Astarita, T. Saing, D. Piomelli, and C. W. Cotman. **2011**. Dietary and Behavioral Interventions Protect against Age Related Activation of Caspase Cascades in the Canine Brain. *PLoS One* 6.

(*320*). German, A. J., S. L. Holden, P. J. Morris, and V. Biourge. **2012**. Long-term follow-up after weight management in obese dogs: The role of diet in preventing regain. *Veterinary Journal* 192: 65-70.

(*321*). Schmidt, P. L. **2009**. Companion Animals as Sentinels for Public Health. *Vet Clin North Am Small Anim Pract* 39: 241-+.

(*322*). Reif, J. S. **2011**. Animal Sentinels for Environmental and Public Health. *Public Health Reports* 126: 50-57.

(*323*). Jeusette, I. C., E. T. Lhoest, L. P. Istasse, and M. O. Diez. **2005**. Influence of obesity on plasma lipid and lipoprotein concentrations in dogs. *American Journal of Veterinary Research* 66: 81-86.

(*324*). Jericó, M. M., F. De Camargo Chiquito, K. Kajihara, M. A. Moreira, R. Gonzales, F. L. Machado, V. S. Nunes, S. Catanozi, and E. R. Nakandakare. **2009**. Chromatographic analysis of lipid fractions in healthy dogs and dogs with obesity or hyperadrenocorticism. *J Vet Diagn Invest* 21: 203-207.

(*325*). Abete, I., E. Goyenechea, M. A. Zulet, and J. A. Martinez. **2011**. Obesity and metabolic syndrome: Potential benefit from specific nutritional components. *Nutr Metab Cardiovasc Dis* 21: B1-B15.

(*326*). Bazzano, L. A., J. He, L. G. Ogden, C. Loria, S. Vupputuri, L. Myers, and P. K. Whelton. **2001**. Legume consumption and risk of coronary heart disease in US men and women. *Arch Intern Med* 161: 2573-2578.

(*327*). Winham, D. M., A. M. Hutchins, and C. S. Johnston. **2007**. Pinto bean consumption reduces biomarkers for heart disease risk. *J Am Coll Nutr* 26: 243-249.

(*328*). Lanza, E., T. J. Hartman, P. S. Albert, R. Shields, M. Slattery, B. Caan, E. Paskett, F. Iber, J. W. Kikendall, P. Lance, C. Daston, and A. Schatzkin. **2006**. High dry bean intake and reduced risk of advanced colorectal adenoma recurrence among participants in the polyp prevention trial. *J Nutr* 136: 1896-1903.

(*329*). Aune, D., E. De Stefani, A. Ronco, P. Boffetta, H. Deneo-Pellegrini, G. Acosta, and M. Mendilaharsu. **2009**. Legume intake and the risk of cancer: a multisite case-control study in Uruguay. *Cancer Causes Control* 20: 1605-1615.

(*330*). Forster, G. M., D. Hill, G. Gregory, K. M. Weishaar, S. Lana, J. E. Bauer, and E. P. Ryan. **2012**. Effects of cooked navy bean powder on apparent total tract nutrient digestibility and safety in healthy adult dogs. *J Anim Sci* 90: 2631-2638.

(*331*). German, A. J., S. L. Holden, T. Bissot, R. M. Hackett, and V. Biourge. **2007**. Dietary energy restriction and successful weight loss in obese client-owned dogs *J Vet Intern Med* 21: 1174-1180.

(*332*). Daminet, S., M. Paradis, K. R. Refsal, and C. Price. **1999**. Short term influence of prednisone and phenobarbital on thyroid function in euthyroid dogs. *Can Vet J* 40: 411-415.

(*333*). Friedewald, W. T., R. I. Levy, and D. S. Fredrickson. **1972**. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 18: 499-&.

(*334*). McCrory, M. A., B. R. Hamaker, J. C. Lovejoy, and P. E. Eichelsdoerfer. **2010**. Pulse consumption, satiety, and weight management. *Adv Nutr* 1: 17-30.

(*335*). McCrory, M. A., J. C. Lovejoy, P. A. Palmer, P. E. Eichelsdoerfer, M. Gehrke, M, I. T. Kavanaugh, S. A. Buesing, and T. L. Rose. **2008**. Effectiveness of legume consumption for facilitating weight loss: a randomized trial. *FASEB J* 22: 1084.1084.

(*336*). Weigle, D. S., P. A. Breen, C. C. Matthys, H. S. Callahan, K. E. Meeuws, V. R. Burden, and J. Q. Purnell. **2005**. A high-protein diet induces sustained reductions in appetite, ad libitum caloric intake, and body weight despite compensatory changes in diurnal plasma leptin and ghrelin concentrations. *Am J Clin Nutr* 82: 41-48.

(*337*). Keller, U. **2011**. Dietary Proteins in Obesity and in Diabetes. *Int J Vitam Nutr Res* 81: 125-133.

(*338*). Little, T. J., M. Horowitz, and C. Feinle-Bisset. **2005**. Role of cholecystokinin in appetite control and body weight regulation. *Obes Rev* 6: 297-306.

(*339*). Andersson, S. W., J. Skinner, L. Ellegard, A. A. Welch, S. Bingham, A. Mulligan, H. Andersson, and K. T. Khaw. **2004**. Intake of dietary plant sterols is inversely related to serum cholesterol concentration in men and women in the EPIC Norfolk population: a cross-sectional study. *Eur J Clin Nutr* 58: 1378-1385.

(*340*). Mangge, H., G. Almer, M. Truschnig-Wilders, A. Schmidt, R. Gasser, and D. Fuchs. **2010**. Inflammation, Adiponectin, Obesity and Cardiovascular Risk. *Curr Med Chem* 17: 4511-4520.

(*341*). Yamka, R. M., K. G. Friesen, and N. Z. Frantz. **2006**. Identification of canine markers related to obesity and the effects of weight loss on the markers of interest. *The Journal of Applied Research in Veterinary Medicine* 4: 282-292.

(*342*). Fernandez, M. L., S. Metghalchi, S. Vega-Lopez, K. Conde-Knape, T. G. Lohman, and Z. R. Cordene-MacIntyre. **2004**. Beneficial effects of weight loss on plasma apolipoproteins in postmenopausal women. *J Nutr Biochem* 15: 717-721.

(*343*). Jenkins, D. J. A., C. W. C. Kendall, D. G. Popovich, E. Vidgen, C. C. Mehling, V. Vuksan, T. P. P. Ransom, A. V. Rao, R. Rosenberg-Zand, N. Tariq, P. Corey, P. J. H. Jones, M. Raeini, J. A. Story, E. J. Furumoto, D. R. Illingworth, A. S. Pappu, and P. W. Connelly. **2001**. Effect of a very-high-fiber vegetable, fruit, and nut diet on serum lipids and colonic function. *Metabolism* 50: 494-503.

(*344*). Yamka, R. M., N. Z. Frantz, and K. G. Friesen. **2007**. Effects of 3 canine weight loss foods on body composition and obesity markers. *Int J Appl Res Vet M* 5: 125-132.

(*345*). German, A. J., A. Hervera, L. Hunter, S. L. Holden, P. J. Morris, V. Biourge, and P. Trayhurn. **2009**. Improvement in insulin resistance and reduction in plasma inflammatory adipokines after weight loss in obese dogs. *Domestic Animal Endocrinology* 37: 214-226.

(*346*). Diez, M., C. Michaux, I. Jeusette, P. Baldwin, L. Istasse, and V. Biourge. **2004**. Evolution of blood parameters during weight loss in experimental obese Beagle dogs. *J Anim Physiol Anim Nutr* 88: 166-171.

(*347*). Anderson, J. W., and A. W. Major. **2002**. Pulses and lipaemia, short- and long-term effect: Potential in the prevention of cardiovascular disease. *Br J Nutr* 88: S263-S271.

(*348*). Smit, E., F. J. Nieto, and C. J. Crespo. **1999**. Blood cholesterol and apolipoprotein B levels in relation to intakes of animal and plant proteins in US adults. *Br J Nutr* 82: 193-201.

(*349*). Lairon, D. **2004**. Dietary fibre, lipid metabolism and cardiovascular disease.

(*350*). Brown, L., B. Rosner, W. W. Willett, and F. M. Sacks. **1999**. Cholesterol-lowering effects of dietary fiber: a meta-analysis. *Am J Clin Nutr* 69: 30-42.

(*351*). Anderson, J. W., N. J. Gustafson, D. B. Spencer, J. Tietyen, and C. A. Bryant. **1990**. Serum-lipid response of hypercholesterolemic men to single and divided doses of canned beans. *Am J Clin Nutr* 51: 1013-1019.

(*352*). Kahlon, T. S., G. E. Smith, and O. Shao. **2005**. In vitro binding of bile acids by kidney bean (Phaseolus vulgaris), black gram (Vigna mungo), bengal gram (Cicer arietinum) and moth bean (Phaseolus aconitifolins). *Food Chem* 90: 241-246.

(*353*). Marzolo, M. P., L. Amigo, and F. Nervi. **1993**. Hepatic production fo very-low-density lipoprotein, catabolism of low-density lipoprotein, biliary lipid secretion, and bile-salt synthesis in rats fed a bean (*Phaseolus vulgaris*) diet. *J Lipid Res* 34: 807-814.

(*354*). Amigo, L., M. P. Marzolo, J. M. Aguilera, A. Hohlberg, M. Cortes, and F. Nervi. **1992**. Influence of different dietary constituents of beans (*Phaseolus vulgaris*) on serum and biliary lipids in the rat. *J Nutr Biochem* 3: 486-490.

(*355*). Han, K. H., M. Iijuka, K. Shimada, M. Sekikawa, K. Kuramochi, K. Ohba, L. Ruvini, H. Chiji, and M. Fukushima. **2005**. Adzuki resistant starch lowered serum cholesterol and hepatic 3-hydroxy-3-methylglutaryl-CoA mRNA levels and increased hepatic LDL-receptor and cholesterol 7 alpha-hydroxylase mRNA levels in rats fed a cholesterol diet. *Br J Nutr* 94: 902-908.

(*356*). Mensack, M. M., V. K. Fitzgerald, M. R. Lewis, and H. J. Thompson. **2010**. Characterization of low molecular weight chemical fractions of dry bean (Phaseolus vulgaris) for bioactivity using Caenorhabditis elegans longevity and metabolite fingerprinting. *J Agric Food Chem* 58: 6697-6705.

(*357*). Tvarijonaviciute, A., J. J. Ceron, S. L. Holden, P. J. Morris, V. Biourge, and A. J. German. **2012**. Effects of weight loss in obese dogs on a range of renal biomarkers. *J Vet Intern Med* 26: 809-809.

(*358*). Wyss, M., and R. Kaddurah-Daouk. **2000**. Creatine and creatinine metabolism. *Physiol Rev* 80: 1107-1213.

(*359*). Choi, J. W. **2003**. Association between elevated serum hepatic enzyme activity and total body fat in obese humans. *Ann Clin Lab Sci* 33: 257-264.

(*360*). Peña, C., L. Suárez, I. Bautista, J. A. Montoya, and M. C. Juste. **2008**. Relationship between analytic values and canine obesity. *J Anim Physiol Anim Nutr* 92: 324-325.

(*361*). Hangen, L., and M. R. Bennink. **2002**. Consumption of black beans and, navy beans (Phaseolus vulgaris) reduced azoxymethane-induced colon cancer in rats. *Nutr Cancer* 44: 60-65.

(*362*). Hughes, J. S., C. Ganthavorn, and S. Wilson-Sanders. **1997**. Dry beans inhibit azoxymethane-induced colon carcinogenesis in F344 rats. *J Nutr* 127: 2328-2333.

(*363*). Key, T. J. A., P. B. Silcocks, G. K. Davey, P. N. Appleby, and D. T. Bishop. **1997**. A case-control study of diet and prostate cancer. *Br J Cancer* 76: 678-687.

(*364*). Helmstadter, A. **2010**. Beans and Diabetes: Phaseolus vulgaris Preparations as Antihyperglycemic Agents. *J Med Food* 13: 251-254.

(*365*). Jimenez-Cruz, A., V. M. Loustaunau-Lopez, and M. Bacardi-Gascon. **2006**. The use of low glycemic and high satiety index food dishes in Mexico: a low cost approach to prevent and control obesity and diabetes. *Nutr Hosp* 21: 353-356.

(*366*). Hutchins, A. M., D. M. Winham, and S. V. Thompson. **2012**. Phaseolus beans: impact on glycaemic response and chronic disease risk in human subjects. *British Journal of Nutrition* 108: S52-S65.

(*367*). Lanza, E., T. J. Hartman, P. S. Albert, R. Shields, M. Slattery, B. Caan, E. Paskett, F. Iber, J. W. Kikendall, P. Lance, C. Daston, and A. Schatzkin. **2006**. High dry bean intake and

reduced risk of advanced colorectal adenoma recurrence among participants in the polyp prevention trial. *The Journal of nutrition* 136: 1896-1903.

(*368*). Tonstad, S., N. Malik, and E. Haddad. **2014**. A high-fibre bean-rich diet versus a low-carbohydrate diet for obesity. *Journal of Human Nutrition and Dietetics* 27: 109-116.

(*369*). Mollard, R. C., B. L. Luhovyy, S. Panahi, M. Nunez, A. Hanley, and G. H. Anderson. **2012**. Regular consumption of pulses for 8 weeks reduces metabolic syndrome risk factors in overweight and obese adults. *British Journal of Nutrition* 108: S111-S122.

(*370*). Zoran, D. L. **2010**. Obesity in Dogs and Cats: A Metabolic and Endocrine Disorder. *Vet Clin N Am-Small* 40: 221-+.

(*371*). German, A. J., M. Hervera, L. Hunter, S. L. Holden, P. J. Morris, V. Biourge, and P. Trayhurn. **2009**. Improvement in insulin resistance and reduction in plasma inflammatory adipokines after weight loss in obese dogs. *Domestic animal endocrinology* 37: 214-226.

(*372*). RC de Godoy, M. **2015**. Plasma Metabolite Profiling and Search for Biomarkers of Metabolic Dysfunction in Dogs undergoing Rapid Weight Gain. *Current Metabolomics* 3: 102-121.

(*373*). Osto, M., and T. A. Lutz. **2015**. Translational value of animal models of obesity-Focus on dogs and cats. *European journal of pharmacology* 759: 240-252.

(*374*). Bastien, B. C., A. Patil, and E. Satyaraj. **2015**. The impact of weight loss on circulating cytokines in Beagle dogs. *Veterinary immunology and immunopathology* 163: 174-182.

(*375*). Bouthegourd, J. C., M. Kelly, N. Clety, S. Tardif, and D. Smeets. **2009**. Effects of Weight Loss on Heart Rate Normalization and Increase in Spontaneous Activity in Moderately Exercised Overweight Dogs. *Int J Appl Res Vet M* 7: 153-164.

(*376*). German, A. J., S. L. Holden, M. L. Wiseman-Orr, J. Reid, A. M. Nolan, V. Biourge, P. J. Morris, and E. M. Scott. **2012**. Quality of life is reduced in obese dogs but improves after successful weight loss. *Veterinary Journal* 192: 428-434.

(*377*). Manens, J., R. Ricci, C. Damoiseaux, S. Gault, B. Contiero, M. Diez, and C. Clercx. **2014**. Effect of Body Weight Loss on Cardiopulmonary Function Assessed by 6-Minute Walk Test and Arterial Blood Gas Analysis in Obese Dogs. *J Vet Intern Med* 28: 371-378.

(*378*). Marshall, W. G., B. A. Bockstahler, D. A. Hulse, and S. Carmichael. **2009**. A review of osteoarthritis and obesity: current understanding of the relationship and benefit of obesity treatment and prevention in the dog. *Vet Comp Orthopaed* 22: 339-345.

(*379*). Tvarijonaviciute, A., J. J. Ceron, S. L. Holden, V. Biourge, P. J. Morris, and A. J. German. **2013**. Effect of Weight Loss in Obese Dogs on Indicators of Renal Function or Disease. *J Vet Intern Med* 27: 31-38.

(*380*). Tvarijonaviciute, A., A. M. Gutierrez, I. Miller, E. Razzazi-Fazeli, F. Tecles, and J. J. Ceron. **2012**. A proteomic analysis of serum from dogs before and after a controlled weight-loss program. *Domestic Animal Endocrinology* 43: 271-277.

(*381*). Weir, T. L., D. K. Manter, A. M. Sheflin, B. A. Barnett, A. L. Heuberger, and E. P. Ryan. **2013**. Stool Microbiome and Metabolome Differences between Colorectal Cancer Patients and Healthy Adults. *PloS one* 8.

(*382*). Spegel, P., A. Lindqvist, M. Sandberg, and N. Wierup. **2014**. Glucose-dependent insulinotropic polypeptide lowers branched chain amino acids in hyperglycemic rats. *Regul Peptides* 189: 11-16.

(*383*). Nasteska, D., N. Harada, K. Suzuki, S. Yamane, A. Hamasaki, E. Joo, K. Iwasaki, K. Shibue, T. Harada, and N. Inagaki. **2014**. Chronic Reduction of GIP Secretion Alleviates Obesity and Insulin Resistance Under High-Fat Diet Conditions. *Diabetes* 63: 2332-2343.

(*384*). Xu, B. J., and S. K. C. Chang. **2012**. Comparative study on antiproliferation properties and cellular antioxidant activities of commonly consumed food legumes against nine human cancer cell lines. *Food Chemistry* 134: 1287-1296.

(*385*). Spanou, C., D. Stagos, N. Aligiannis, and D. Kouretas. **2010**. Influence of potent antioxidant Leguminosae family plant extracts on growth and antioxidant defense system of Hep2 cancer cell line. *Journal of Medicinal Food* 13: 149-155.

(*386*). Guajardo-Flores, D., S. O. Serna-Saldivar, and J. A. Gutierrez-Uribe. **2013**. Evaluation of the antioxidant and antiproliferative activities of extracted saponins and flavonols from germinated black beans (Phaseolus vulgaris L.). *Food Chemistry* 141: 1497-1503.

(*387*). Dong, M., X. He, and R. H. Liu. **2007**. Phytochemicals of black bean seed coats: isolation, structure elucidation, and their antiproliferative and antioxidative activities. *Journal of agricultural and food chemistry* 55: 6044-6051.

(*388*). Cruz-Bravo, R. K., R. G. Guevara-Gonzalez, M. Ramos-Gomez, B. D. Oomah, P. Wiersma, R. Campos-Vega, and G. Loarca-Pina. **2014**. The fermented non-digestible fraction of common bean (Phaseolus vulgaris L.) triggers cell cycle arrest and apoptosis in human colon adenocarcinoma cells. *Genes Nutr* 9: 359.

(*389*). Campos-Vega, R., R. G. Guevara-Gonzalez, B. L. Guevara-Olvera, B. D. Oomah, and G. Loarca-Pina. **2010**. Bean (Phaseolus vulgaris L.) polysaccharides modulate gene expression in human colon cancer cells (HT-29). *Food Research International* 43: 1057-1064.

(*390*). Aparicio-Fernandez, X., T. Garcia-Gasca, G. G. Yousef, M. A. Lila, E. G. De Mejia, and G. Loarca-Pina. **2006**. Chemopreventive activity of polyphenolics from black Jamapa bean (Phaseolus vulgaris L.) on HeLa and HaCaT cells. *Journal of Agricultural and Food Chemistry* 54: 2116-2122.

(*391*). Ang, A. S., R. C. Cheung, X. Dan, Y. S. Chan, W. Pan, and T. B. Ng. **2014**. Purification and characterization of a glucosamine-binding antifungal lectin from Phaseolus vulgaris cv. Chinese pinto beans with antiproliferative activity towards nasopharyngeal carcinoma cells. *Appl Biochem Biotechnol* 172: 672-686.

(*392*). De Silva, A., and S. R. Bloom. **2012**. Gut Hormones and Appetite Control: A Focus on PYY and GLP-1 as Therapeutic Targets in Obesity. *Gut and Liver* 6: 10-20.

(*393*). Wakshlag, J. J., A. M. Struble, C. B. Levine, J. J. Bushey, D. P. Laflamme, and G. M. Long. **2011**. The effects of weight loss on adipokines and markers of inflammation in dogs. *British Journal of Nutrition* 106: S11-S14.

(*394*). Tvarijonaviciute, A., F. Tecles, S. Martinez-Subiela, and J. J. Ceron. **2012**. Effect of weight loss on inflammatory biomarkers in obese dogs. *Veterinary Journal* 193: 570-572.

(*395*). German, A. J. **2012**. Barking up the wrong tree: What's the deal with obesity, adiponectin and inflammation in dogs? *Veterinary Journal* 194: 272-273.

(*396*). Forster, G. M., C. A. Ollila, J. H. Burton, A. L. Heuberger, C. D. Broeckling, D. Hill, J. E. Bauer, A. M. Hess, and E. P. Ryan. 2013. **Dry bean consumption modulates metabolic byproducts in overweight dogs undergoing weight reduction** In: CVMBS Research Day, Fort Collins, CO

(*397*). Perez-Cornago, A., L. Brennan, I. Ibero-Baraibar, H. H. Hermsdorff, A. O'Gorman, M. A. Zulet, and J. A. Martinez. **2014**. Metabolomics identifies changes in fatty acid and amino acid profiles in serum of overweight older adults following a weight loss intervention. *J Physiol Biochem* 70: 593-602.

(*398*). Piccolo, B. D., N. L. Keim, O. Fiehn, S. H. Adams, M. D. Van Loan, and J. W. Newman. **2015**. Habitual Physical Activity and Plasma Metabolomic Patterns Distinguish Individuals with Low vs. High Weight Loss during Controlled Energy Restriction. *The Journal of Nutrition* 145: 681-690.

(*399*). Allouche, Y., G. Beltran, J. J. Gaforio, M. Uceda, and M. D. Mesa. **2010**. Antioxidant and antiatherogenic activities of pentacyclic triterpenic diols and acids. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 48: 2885-2890.

(*400*). Sanchez-Quesada, C., A. Lopez-Biedma, F. Warleta, M. Campos, G. Beltran, and J. J. Gaforio. **2013**. Bioactive properties of the main triterpenes found in olives, virgin olive oil, and leaves of Olea europaea. *Journal of agricultural and food chemistry* 61: 12173-12182.

(*401*). Koren, M. S., J. Q. Purnell, P. A. Breen, C. C. Matthys, H. S. Callahan, K. E. Meeuws, V. R. Burden, and D. S. Weigle. **2007**. Changes in plasma amino Acid levels do not predict satiety and weight loss on diets with modified macronutrient composition. *Ann Nutr Metab* 51: 182-187.

(*402*). Rondanelli, M., C. Klersy, P. Iadarola, F. Monteferrario, and A. Opizzi. **2009**. Satiety and amino-acid profile in overweight women after a new treatment using a natural plant extract sublingual spray formulation. *International journal of obesity (2005)* 33: 1174-1182.

(*403*). Mangge, H., K. L. Summers, A. Meinitzer, S. Zelzer, G. Almer, R. Prassl, W. J. Schnedl, E. Reininghaus, K. Paulmichl, D. Weghuber, and D. Fuchs. **2014**. Obesity-related dysregulation of the tryptophan-kynurenine metabolism: role of age and parameters of the metabolic syndrome. *Obesity (Silver Spring, Md.)* 22: 195-201.

(*404*). Rioli, G., G. Mattei, and S. Ferrari. **2014**. EPA-0966 – The kynurenine pathway: a possible link between the depression and anxiety spectrum, metabolic syndrome and eating disorders. *European Psychiatry* 29, Supplement 1: 1.

(*405*). Park, H. J., S. E. Lee, J. H. Oh, K. W. Seo, and K. H. Song. **2014**. Leptin, adiponectin and serotonin levels in lean and obese dogs. *BMC veterinary research* 10: 113.

(*406*). Pedroso, J. A., T. T. Zampieri, and J. Donato, Jr. **2015**. Reviewing the Effects of L-Leucine Supplementation in the Regulation of Food Intake, Energy Balance, and Glucose Homeostasis. *Nutrients* 7: 3914-3937.

(*407*). Qin, L. Q., P. Xun, D. Bujnowski, M. L. Daviglus, L. Van Horn, J. Stamler, and K. He. **2011**. Higher branched-chain amino acid intake is associated with a lower prevalence of being overweight or obese in middle-aged East Asian and Western adults. *The Journal of nutrition* 141: 249-254.

(*408*). Fromentin, G., N. Darcel, C. Chaumontet, A. Marsset-Baglieri, N. Nadkarni, and D. Tome. **2012**. Peripheral and central mechanisms involved in the control of food intake by dietary amino acids and proteins. *Nutrition Research Reviews* 25: 29-39.

(*409*). Plaisance, E. P., F. L. Greenway, A. Boudreau, K. L. Hill, W. D. Johnson, R. A. Krajcik, C. E. Perrone, N. Orentreich, W. T. Cefalu, and T. W. Gettys. **2011**. Dietary methionine restriction increases fat oxidation in obese adults with metabolic syndrome. *The Journal of clinical endocrinology and metabolism* 96: E836-840.

(*410*). Belchior, T., V. A. Paschoal, J. Magdalon, P. Chimin, T. M. Farias, A. B. Chaves-Filho, R. Gorjao, P. St-Pierre, S. Miyamoto, J. X. Kang, Y. Deshaies, A. Marette, and W. Festuccia. **2015**. Omega-3 fatty acids protect from diet-induced obesity, glucose intolerance, and adipose tissue inflammation through PPARgamma-dependent and PPARgamma-independent actions. *Mol Nutr Food Res* 59: 957-967.

(*411*). Gray, B., F. Steyn, P. S. Davies, and L. Vitetta. **2013**. Omega-3 fatty acids: a review of the effects on adiponectin and leptin and potential implications for obesity management. *European journal of clinical nutrition* 67: 1234-1242.

(*412*). Lund, A. S., A. L. Hasselbalch, M. Gamborg, K. Skogstrand, D. M. Hougaard, B. L. Heitmann, K. O. Kyvik, T. I. Sorensen, and T. Jess. **2013**. N-3 polyunsaturated fatty acids, body fat and inflammation. *Obes Facts* 6: 369-379.

(*413*). Figueras, M., M. Olivan, S. Busquets, F. J. Lopez-Soriano, and J. M. Argiles. **2011**. Effects of eicosapentaenoic acid (EPA) treatment on insulin sensitivity in an animal model of diabetes: improvement of the inflammatory status. *Obesity (Silver Spring, Md.)* 19: 362-369.

(*414*). Lenox, C. E., and J. E. Bauer. **2013**. Potential adverse effects of omega-3 Fatty acids in dogs and cats. *Journal of veterinary internal medicine / American College of Veterinary Internal Medicine* 27: 217-226.

(*415*). Moreau, M., E. Troncy, J. R. Del Castillo, C. Bedard, D. Gauvin, and B. Lussier. **2012**. Effects of feeding a high omega-3 fatty acids diet in dogs with naturally occurring osteoarthritis. *Journal of animal physiology and animal nutrition* 10.1111/j.1439-0396.2012.01325.x.

(*416*). Bauer, J. E. **2007**. Responses of dogs to dietary omega-3 fatty acids. *Journal of the American Veterinary Medical Association* 231: 1657-1661.

(*417*). Messina, V. **2014**. Nutritional and health benefits of dried beans. *The American journal of clinical nutrition* 100: 437S-442S.

(*418*). Iggman, D., F. Rosqvist, A. Larsson, J. Arnlov, L. Beckman, M. Rudling, and U. Riserus. **2014**. Role of Dietary Fats in Modulating Cardiometabolic Risk During Moderate Weight Gain: A Randomized Double-Blind Overfeeding Trial (LIPOGAIN Study). *Journal of the American Heart Association* 3.

(*419*). Linder, D. E. Top 5 Clinical Consequences of Obesity. *Clinician's Brief*.

(*420*). Wurtz, P., Q. Wang, A. J. Kangas, R. C. Richmond, J. Skarp, M. Tiainen, T. Tynkkynen, P. Soininen, A. S. Havulinna, M. Kaakinen, J. S. Viikari, M. J. Savolainen, M. Kahonen, T. Lehtimaki, S. Mannisto, S. Blankenberg, T. Zeller, J. Laitinen, A. Pouta, P. Mantyselka, M. Vanhala, P. Elliott, K. H. Pietilainen, S. Ripatti, V. Salomaa, O. T. Raitakari, M. R. Jarvelin, G. D. Smith, and M. Ala-Korpela. **2014**. Metabolic Signatures of Adiposity in Young Adults: Mendelian Randomization Analysis and Effects of Weight Change. *Plos Med* 11.

(*421*). Moreno-Navarrete, J. M., M. Sabater, F. Ortega, W. Ricart, and J. M. Fernandez-Real. **2012**. Circulating zonulin, a marker of intestinal permeability, is increased in association with obesity-associated insulin resistance. *PloS one* 7: e37160.

(*422*). Yuan, H., J. Lu, J. Xiao, G. Upadhyay, R. Umans, B. Kallakury, Y. Yin, M. E. Fant, L. Kopelovich, and R. I. Glazer. **2013**. PPARδ Induces Estrogen Receptor-Positive Mammary Neoplasia through an Inflammatory and Metabolic Phenotype Linked to mTOR Activation. *Cancer research* 73: 4349-4361.

(*423*). Rezzi, S., F.-P. Martin, C. Alonso, M. Guilarte, M. a. Vicario, L. Ramos, C. Martínez, B. Lobo, E. Saperas, and J. R. Malagelada. **2009**. Metabotyping of biofluids reveals stress-based differences in gut permeability in healthy individuals. *Journal of proteome research* 8: 4799-4809.

(*424*). Xia, J., I. V. Sinelnikov, B. Han, and D. S. Wishart. **2015**. MetaboAnalyst 3.0—making metabolomics more meaningful. *Nucleic acids research*: gkv380.

APPENDICIES

Appendix 1: Baseline characteristics of 66 companion dogs that participated in one of three randomized, controlled dietary intervention clinical trials investigating the safety and digestibility of bean-based diets for weight maintenance and loss.

Dog ID	BCS	Weight Classification	Weight (kg)	Age (yrs)	Sex	Breed[1]
C11	4	Normal Weight	16.4	2	F/S	Mixed - Unknown
NB10	4	Normal Weight	19.7	5	F/S	Mixed - Unknown
NB2	4	Normal Weight	22.5	5	M/N	Wirehaired Pointer
NB9	4	Normal Weight	18.7	3	F/S	Rottweiler
C1	5	Normal Weight	30.9	2	M/N	Mixed - Unknown
C10	5	Normal Weight	25.8	2	M/N	Mixed - Unknown
C2	5	Normal Weight	19	3	F/S	Pit Bull
C3	5	Normal Weight	10.6	3	M/N	Mixed - Unknown
C5	5	Normal Weight	30.7	5	F/S	Golden Retriever
C6	5	Normal Weight	28.5	4	M/N	Pit Bull
C9	5	Normal Weight	33	3	M/I	Labrador Retriever
NB1	5	Normal Weight	32.1	3	M/N	Golden Retriever
NB3	5	Normal Weight	24.1	7	F/S	Dalmatian
NB4	5	Normal Weight	20	4	F/S	Mixed - Unknown
NB8	5	Normal Weight	20.5	3	F/S	Standard Poodle
C4	6	Overweight	39.2	2	M/N	Mixed - Unknown
C7	6	Overweight	50.4	4	F/S	Saint Bernard
NB5	6	Overweight	21.1	2	M/N	Australian Cattle Dog
NB6	6	Overweight	24.2	2	F/S	Australian Cattle Dog
O_BB2	6	Overweight	17.2	3	F/S	Basset Hound
O_C1	6	Overweight	27.4	6	F/S	Dalmatian
O_C6	6	Overweight	22.4	5	F/S	Australian Shepherd
O_NB7	6	Overweight	25.9	5	M/N	Australian Shepherd
C8	7	Overweight	25.6	5	F/S	Mixed - Unknown
NB7	7	Overweight	30.9	6	M/N	Mixed - Unknown
O_BB13	7	Overweight	28.5	3	F/S	Pit Bull
O_BB14	7	Overweight	46.8	3	F/S	Labrador Retriever
O_BB5	7	Overweight	14.2	2	F/S	Boston Terrier Mix
O_BB7	7	Overweight	31.8	5	F/S	Pit Bull
O_BB8	7	Overweight	30.6	5	M/N	Australian Cattle Dog
O_C11	7	Overweight	33.1	10	M/N	Cocker Spaniel
O_C14	7	Overweight	25.2	2	F/S	Labrador Retriever Mix
O_C2	7	Overweight	37.3	6	M/N	Labrador/Border Collie Mix
O_C3	7	Overweight	62.4	4	F/I	Saint Bernard
O_C4	7	Overweight	42.7	7	F/S	Labrador Retriever
O_C5	7	Overweight	14.7	7	M/N	Corgie
O_C7	7	Overweight	15.4	3	M/N	Mixed - Unknown
O_NB1	7	Overweight	32.6	6	M/N	Airdale Mix
O_NB12	7	Overweight	63.7	3	M/N	Border Collie/Corgi Mix
O_NB2	7	Overweight	36	2	M/N	Border Collie Mix
O_NB3	7	Overweight	17.8	4	F/S	Boston Terrier
O_NB4	7	Overweight	44.2	8	F/S	Labrador Retriever Mix

Dog ID	BCS	Weight Classification	Weight (kg)	Age (yrs)	Sex	Breed[1]
Appendix 1: Continued from previous page						
O_NB8	7	Overweight	21.2	6	F/S	Australian Shepherd
O_NB9	7	Overweight	34.3	2	F/S	Boxer
O_BB1	8	Obese	23.8	5	F/S	Keeshond
O_BB10	8	Obese	38.1	2	M/N	Australian Shepherd Mix
O_BB11	8	Obese	24.9	3	F/I	Labrador/Pitbull Mix
O_BB12	8	Obese	44.5	3	M/N	Labrador/Pitbull Mix
O_BB15	8	Obese	37.7	6	F/S	Labrador Retriever Mix
O_BB3	8	Obese	40.8	7	M/N	Australian Cattle Dog
O_BB9	8	Obese	32.8	6	F/S	Australian Cattle Dog
O_C10	8	Obese	31.6	7	F/S	Border Collie
O_C12	8	Obese	36.5	3	F/S	American Spaniel
O_C13	8	Obese	42.2	2	M/N	German Shepherd/Labrador Mix
O_C8	8	Obese	37.7	7	F/S	Labrador Reteriever Mix
O_NB10	8	Obese	44.4	5	M/N	Karelian Bear Dog Mix
O_NB13	8	Obese	39.5	6	M/N	Golden Retriever
O_NB14	8	Obese	38.3	6	F/S	Border Collie/NewFoundland Mix
O_NB5	8	Obese	10	4	M/N	Daschund
O_BB4	9	Obese	27	5	F/S	Border Collie/Australian Shepherd Mix
O_BB6	9	Obese	10.7	3	M/N	Shiz Shu
O_C15	9	Obese	38.5	7	F/S	Labrador Retriever
O_C9	9	Obese	38.6	6	F/S	Golden Retriever
O_NB11	9	Obese	16.2	7	M/N	Labrador Retriever
O_NB15	9	Obese	48.5	7	F/S	Labrador Retreiver
O_NB6	9	Obese	21.2	4	M/N	Daschund

[1]As reported by owner. BCS: Body Condition Score, 9 point scale [191]. Dog ID indicates diet group: C = Control, BB = Black Bean, NB = Navy Bean; O_ indicates dogs that participated in a weight loss study. The sex of each dog is reported as male (M) or female (F) and neutered (N) or intact (I).

Appendix 2: Metabolite detected in navy bean and control dog foods and normal weight dog feces and urine with GC or LC-MS.

	Cluster	Annotation	Confidence	CID	SMILES
Diet Metabolites[a]	C4	2-Piperidinecarboxylic acid	2	849	C1CCNC(C1)C(=O)O
	C56	s-methyl cysteine	2	24417	CSCC(C(=O)O)N
	C186	5-hydroxynorvaline	2	95562	C(CC(C(=O)O)N)CO
	C1	Sucrose	2	5988	C(C1C(C(C(C(O1)OC2(C(C(C(O2)CO)O)O)CO)O)O)O)O
	C7	Citric acid	2	311	C(C(=O)O)C(CC(=O)O)(C(=O)O)O
	C490	γ-tocopherol	2	92729	CC1=C(C=C2CCC(OC2=C1C)(C)CCCC(C)CCCC(C)CCCC(C)C)O
	C84	β-sitosterol	2	222284	CCC(CCC(C)C1CCC2C1(CCC3C2CC=C4C3(CCC(C4)O)C)C)C(C)C
	C18	Taurine	2	1123	C(CS(=O)(=O)O)N
	C825	α-tocopherol acetate	2	86472	CC1=C2C(=C(C(=C1C)OC(=O)C)C)CCC(O2)(C)CCCC(C)CCCC(C)CCCC(C)C
	C91	Fructose	2	5984	C(C(C(C(C(=O)CO)O)O)O)O
	C30	Glycerol-3-Phosphate	2	754	C(C(COP(=O)(O)O)O)O
	C385	α-tocopherol	2	14985	CC1=C(C(=C2CCC(OC2=C1C)(C)CCCC(C)CCCC(C)CCCC(C)C)C)O
	C2	Deoxycholic acid	2	222528	CC(CCC(=O)O)C1CCC2C1(C(CC3C2CCC4C3(CCC(C4)O)C)O)C
	C5	Lithocholic acid	2	9903	CC(CCC(=O)O)C1CCC2C1(CCC3C2CCC4C3(CCC(C4)O)C)C
	C35	Palmitoleic acid	2	5282745	CCCCCCC=CCCCCCCCC(=O)O
	C269	L-Threitol	2	445969	C(C(C(CO)O)O)O
	C526	Hydroquinone	2	785	C1=CC(=CC=C1O)O
	C9	Glucose	2	79025	C(C1C(C(C(C(O1)O)O)O)O)O
	C10	D-Pinitol	2	164619	COC1C(C(C(C(C1O)O)O)O)O
	C19	Monosaccharide	3		
	C3	Trehalose	2	7427	C(C1C(C(C(C(O1)OC2C(C(C(C(O2)CO)O)O)O)O)O)O)O

Continued on next page

Appendix 2 Continued

	Cluster	Annotation	Confidence	CID	SMILES
Fecal Metabolites[a]	C40	α-Tocopherol	2	14985	CC1=C(C(=C2CCC(OC2=C1C)(C)CCCC(C)CCCC(C)CCCC(C)C)C)O
	C59	Lithocholic acid	2	9903	CC(CCC(=O)O)C1CCC2C1(CCC3C2CCC4C3(CCC(C4)O)C)C
	C246	Myristic Acid	2	11005	CCCCCCCCCCCCCC(=O)O
	C688	Margaric acid	2	10465	CCCCCCCCCCCCCCCCC(=O)O
	C190	Polycyclic Hydrocarbon	3		
	C691	Arachidic Alcohol	2	12404	CCCCCCCCCCCCCCCCCCCCO
	C428	Hydroquinone	2	785	C1=CC(=CC=C1O)O
	C292	Cycloartenol	2	382580	CC(CCC=C(C)C)C1CCC2(C1(CCC34C2CCC5C3(C4)CCC(C5(C)C)O)C)C
	C4	Deoxycholic acid	2	222528	CC(CCC(=O)O)C1CCC2C1(C(CC3C2CCC4C3(CCC(C4)O)C)O)C
	C232	α-Tocopherol acetate	2	86472	CC1=C2C(=C(C(=C1C)OC(=O)C)C)CCC(O2)(C)CCCC(C)CCCC(C)CCCC(C)C
	C1	Glucose	2	79025	C(C1C(C(C(C(O1)O)O)O)O)O
	C30	Steroid	3		
	C53	Steroid	3		
	C179	Steroid	3		

Continued on next page

	Cluster	Annotation	Confidence	CID	SMILES
Urine Metabolites[b]	C6	Estradiol	2	5757	CC12CCC3C(C1CCC2O)CCC4=C3C=CC(=C4)O
	C24	Peptide	3		
	C36	Peptide	3		
	C69	Peptide	3		
	C18	Suberic acid	2	10457	C(CCCC(=O)O)CCC(=O)O
	C5	Kynurenine	2	846	C1=CC=C(C(=C1)C(=O)CC(C(=O)O)N)N
	C63	Peptide	3		
	C180	Peptide	3		
	C625	Saccharide	3		
	C17	Peptide	3		
	C196	Trigonelline	2	5570	C[N+]1=CC=CC(=C1)C(=O)[O-]
	C909	Peptide	3		
	C156	homoeriodictyol chalcone	2	21117850	COC1=C(C=CC(=C1)C=CC(=O)C2=C(C=C(C=C2O)O)O)O
	C59	Dipeptide	3		
	C32	Kynurenic acid	2	3845	C1=CC=C2C(=C1)C(=O)C=C(N2)C(=O)O

Each annotated metabolite is reported with its corresponding "cluster" number (C), identification confidence level, PubChem Compound Identifier (CID), and Simplified Molecular Input Line Entry Specification (SMILES). Confidence levels were assigned based on criteria defined by Sumner et. al. (Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics* **2007,** *3* (3), 211-221.): 1 = metabolite annotation confirmed with authentic standards; 2 = annotation based on spectral similarities with in–house and public libraries; 3 = compound classification based on spectral similarities with in-house and public libraries; and 4 = unknown compounds. [a]Analyzed with GC-MS; [b]analyzed with LC-MS.