EVALUATION OF HIGH-PROTEIN DIETS DIFFERING IN PROTEIN SOURCE IN HEALTHY ADULT DOGS

BY

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THESIS

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ABSTRACT

Given the dynamic market for protein-based ingredients in the pet food industry, demand continues to increase for both plant- and animal-based options. Animal and plant protein sources contain different amino acid profiles and vary in digestibility, which can affect the protein quality provided to the animal. The objective of this study was to evaluate the apparent total tract digestibility of canine diets differing in protein source and test their effects on serum metabolites, whole blood gene expression, and fecal characteristics, metabolites, and microbiota of healthy adult dogs consuming them. Four isocaloric and isonitrogenous extruded diets were formulated to meet all Association of American Feed Control Officials (AAFCO) nutrient profiles for adult dogs at maintenance, with the primary difference being protein source: 1) chicken by-product meal (CBPM), 2) deboned chicken, dried chicken, and spray dried chicken (DC), 3) corn gluten meal (CGM), or 4) wheat gluten meal (WGM). Twelve adult spayed female beagles (BW = 9.9± 1.0 kg; age = 6.3 ±1.1 yr) were used in a replicated 4×4 Latin square design (n=12/treatment). Each period consisted of a 22-d adaptation phase, 5 d for total and fresh fecal collection, and 1 d for blood collection. Fecal microbiota data were analyzed using QIIME 2.2020.8. All other data were analyzed using the Mixed Models procedure of SAS version 9.4. Fecal scores were higher ($p<0.05$; looser stools) in dogs fed DC or CBPM than those fed WGM or CGM, but all remained within an appropriate range. Apparent dry matter digestibility was lower ($p<0.05$) in dogs fed CBPM or CGM than those fed DC or WGM. Apparent crude protein digestibility was also lower ($p<0.05$) in dogs fed DC or CGM than those fed WGM. Dogs fed CBPM had lower ($p<0.05$) apparent organic matter, crude protein, and energy digestibilities than those fed the other 3 diets. Fecal indole concentrations were higher ($p<0.05$) in dogs fed CBPM than those fed WGM, but phenol and total phenol and indole concentrations were not different. Fecal total short-chain fatty acid (SCFA) concentrations were higher ($p<0.05$) in dogs fed DC than those fed CGM, but

individual SCFA (i.e., acetate; propionate; butyrate) were not different. Fecal total branched-chain fatty acid concentrations were higher ($p<0.05$) in dogs fed DC or CBPM than those fed WGM. Fecal ammonia concentrations were higher ($p<0.05$) in dogs fed the animal-based protein diets than those fed the plant-based protein diets. Gene expression was not affected by diet. The relative abundance of 3 bacterial phyla and 9 bacterial genera were significantly shifted among treatment groups ($p<0.05$). Considering AA profiles and digestibility data together, the protein sources of the DC diet provided the most and highest quality protein without AA supplementation of all diets tested. However, the animal-based protein diets resulted in higher concentrations of proteolytic fermentative end-products. Further studies evaluating moderate dietary protein concentrations are needed to better compare plant- and animal-based protein sources.

To Beth,

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CHAPTER 1: INTRODUCTION

Many pet owners think of their pets as part of the family, and are concerned about their health and longevity. Owners are becoming more aware and selective of the foods they are choosing to purchase for their pets. Nutrition is seen as a way to safeguard their animals' health and welfare. The most frequent consideration of consumers and pet food manufacturers is protein source and concentration (Oberbauer and Larsen, 2021). Well formulated diets provide protein and amino acids in dietary concentrations that meet the needs of the target dog population. Diets must ensure nutritional adequacy for the dogs. If not, health can become compromised.

Recently, some owners have moved away from traditional pet food protein sources (i.e., animal by-products) to different choices, such as fresh meat or a more sustainable plant-based protein option. Many diet choices for companion dogs have begun to reflect the personal preferences of their owners, with different social and cultural factors influencing the decision-making process (Vinassa et al., 2020). Different pet owners will consider different and specific criteria from their food choices, creating more diverse protein source needs that the pet food industry must fulfill.

There is scant scientific research comparing the nutritional value of dog foods containing high concentrations of animal-based and plant-based proteins. Therefore, the objective of this study was to evaluate the apparent total tract digestibility of canine diets differing in protein source and test their effects on serum metabolites, whole blood gene expression, and fecal characteristics, metabolites, and microbiota of healthy adult dogs consuming them. We hypothesized that the plant-based protein diets would be less digestible, increase fecal metabolite

concentrations coming from protein fermentation (branched-chain fatty acids; phenols and indoles; ammonia), and negatively impact fecal microbiota populations.

Literature Cited

Oberbauer, A. M., and J. A. Larsen. 2021. Amino acids in dog nutrition and health. Adv. Exp. Med. Biol. 1285:199–216. doi:10.1007/978-3-030-54462-1_10

Vinassa, M., D. Vergnano, E. Valle, M. Giribaldi, J. Nery, L. Prola, D. Bergero, and A. Schiavone. 2020. Profiling Italian cat and dog owners' perceptions of pet food quality traits. BMC Vet. Res. 16:131. doi:10.1186/s12917-020-02357-9

CHAPTER 2: LITERATURE REVIEW

Pet Food Market

It is theorized that initially, dogs became domesticated when humans were able to share excess lean protein with incipient dogs during the ice age (Lahtinen, 2021). The basis of domesticating pets began when people were able to feed leftover food to dogs to create a harmonious relationship between both. At first, domestic dogs served in a utilitarian service of guarding and protecting families and livestock, but have evolved for many other purposes over time (Smith, 2019). Canine companionship provides many physical and psychological benefits in modern life. Dogs are used by owners for leisure activities such as walking, dog sledding, dog training, and other sports. Dogs also often help humans as working dogs, functioning as service animals, for bio-detection, herding livestock, military support, and more. Regardless of the role or relationship, dogs are now primarily considered to be members of the family. In 2020, 45% of households owned a dog, an increase from 38% in 2016. The COVID-19 pandemic has played a role in the increased number of households with pets, as people who worked remotely were eight times as likely to obtain a new pet during this time (Larkin, 2021). As pet populations and ownership have increased, it has created more opportunities for the pet food industry to diversify products.

The close bonds and relationships formed between pets and pet owners place anthropomorphic views or humanization on pets. The growing humanization of pets has impacted the pet food industry, with new pet food products, brands, and companies tailored to consumer views. Consumer views vary widely, creating a diverse product offering in today's market. There is a greater emphasis on premium diets, such as those that are natural or organic, have high meat inclusion levels, are vegetarian/vegan, and/or contain novel ingredients. Some pet owners are

increasingly interested in plant-based diets for themselves and their pets due to concerns about production animal welfare, the environment, and health (Knight and Leitsberger, 2016). The natural trend is focused on avoiding ingredients that are heavily processed, such as refined grains, fiber sources, and byproducts, and feeding similarly to ancestral nutritional philosophies (Buff et al., 2014). Others prefer the trend of feeding high amounts of fresh raw muscle meat, offal, and bones, with relatively small amounts of plant ingredients. This feeding style has been referred as Bones and Raw Food or "BARF". This feeding strategy is promoted to replicate the food selection of wolves (Schmidt et al., 2018). In many of these feeding philosophies, the ingredients are not processed secondary products of the human food industry, but are principal products that compete directly with the human food system. Whatever the trend is, when novel consumer preferences and marketing strategies are implemented, it is necessary to ensure the nutritional adequacy of the newly created products. All complete and balanced diets must meet the nutritional needs of the dog.

Common Protein Sources for Pet Foods

In regard to protein source, there is not a "one-size-fits-all" ingredient or strategy due to the differing consumer and pet preferences. Protein plays a role in promoting optimal health and protein selection is a key consideration for both consumers and pet food manufacturers. Although the majority of commercial diets available use a combination of protein sources, the market spans the spectrum from vegan to high meat protein inclusion. Protein quality has been defined as the ability of dietary protein to meet the needs for regular metabolism and maintenance of body issues (Millward et al., 2008). If an ingredient meets all amino acid (AA) requirements, it is considered a complete, high-quality protein source. However, the use of complementary proteins, by combining two or more sources to meet the necessary AA requirements, is also possible. Because

the body requires specific AA, dietary AA concentrations are more important than the crude protein (CP) concentration per se (Knight and Leitsberger, 2016). Having the knowledge of how different protein sources can impact the nutritional status of dogs and cats and how they may be applied to the pet food industry may optimize the use of different products to fit unique needs and goals.

Protein is the most expensive nutrient to provide in pet foods, both economically and environmentally speaking. Animal co-products provide the majority of protein used in commercial pet foods today. Society uses much of the leftover human food system waste, including protein-rich ingredients, to feed pets and livestock to create a sustainable food cycle. Sustainability can be broadly defined as practices that meet current needs without compromising future generations to meet theirs (Swanson et al., 2013). A key component of producing usable commodities from the human food system is the rendering of poultry and other animal products. Rendering is the process of both physical and chemical transformation using a variety of equipment and processes on animal materials that includes the application of heat, extraction of moisture, and separation of fat (Meeker and Meisinger, 2015). This process transforms parts of the animal that otherwise would be considered waste.

Rendering is one of the oldest forms of recycling and makes animal agriculture a sustainable practice (Meeker and Meisinger, 2015). The Food and Agriculture Organization of the United Nations (FAO) reports that poultry meat consumption has increased five-fold, and consumption of eggs has almost doubled since the early 1960's. With poultry being the primary source of animal protein in the world, the rendering industry is important because it makes use of the human wastes, which is critical for the food system. About 37% of the live weight of broilers is not consumed by humans. The leftover co-products to be processed include heads, legs, bones, viscera, skin, feathers, and whole chicken carcasses that were dead on arrival to the processing

plant. Without the rendering industry, there would be an accumulation of these wastes that would impede the poultry industry, add costs to the human food system, and pose a potential hazard to both animals and humans.

In addition to being a potential health hazard, unprocessed or wasted animal co-products pose serious environmental issues if they are not converted to usable products. Decomposition of food waste that is thrown away would generate carbon dioxide and other greenhouse gases and landfills would quickly become full, but rendering offers efficient and environmentally friendly alternatives. Rendering is an important greenhouse gas avoidance technology that recycles carbon and nitrogen wastes into usable materials, and is a very important sector of the animal agriculture industry. Rendering contributes to a significant amount of the profitability of the animal industry, as animal co-products produce 10-15% of the profits in the poultry, livestock, and aquaculture industries (Irshad and Sharma, 2015). Not only does that provide more profit to those raising poultry, but reduces the cost for consumers purchasing meat and eggs.

There are around 300 rendering plants found in North America, making co-products that account for almost half of the total volume produced by animal agriculture. Poultry co-products are often fed as ingredients to livestock, poultry, aquaculture, and pets (Meeker and Hamilton, 2006). According to the North American Renders Association (NARA), the US rendering industry accounts for $10 billion in annual economic output across the country, including many parts of rural America. This means that the rendering industry, which is just part of the poultry industry, provides many people with valuable jobs in a skilled workforce. NARA also reports that in 2020, 62 billion pounds of renderable raw materials were produced and 16 million tons of rendered products came from it. From that, an incredible amount of wasted human food was made into valuable ingredients and products.

Rendered products are very valuable to the pet food industry, as these co-products are typically the main source of protein and fat in pet diets. Approximately 31% of rendered proteins and 15% of rendered fats, or about 10 million tons of product, are used as pet food ingredients in the US and Canada each year. Using co-products from rendering allows the pet food industry to reduce the need for virgin-use ingredients (Meeker and Meisinger, 2015). If human-grade meat is used instead of co-products, then higher meat production is needed and more waste is generated. Also, additional water, land, fertilizer, and other resources are needed for pet foods if rendered products are not used. While the environmental components are typically what first come to mind in regard to sustainability, a truly sustainable system is comprised of environmental, social, and economic components (Swanson et al., 2013). Other considerations of food safety, food quality, health, and nutrition are very important to ensure a high quality of life to be maintained over the long term.

Rendered products coming from the poultry industry include poultry by-product meal, poultry fat, hydrolyzed poultry feather meal, and blood meal. Rendering produces valuable fats and proteins that contribute to the nutrition of pets, livestock, poultry, and fish. The raw materials of the co-products for rendering can vary in composition, but typically contain an average of 60% water, 20% protein and mineral, and 20% fat (Meeker and Hamilton, 2006). The Association of American Feed Control Officials (AAFCO) has specifications and definitions for rendered animal products. In the AAFCO ingredient manual, there are about 125 animal co-product definitions that the rendered ingredients may fall under. These definitions describe the certain parts of the carcass that may be included as well as the end-product nutrient requirements. For instance, poultry by-product meal consists of “ground, rendered, clean parts of the slaughtered poultry such as necks, feet, undeveloped eggs, and intestines, exclusive of feathers except in amounts that might occur unavoidably in good processing practices.” The poultry by-product meal label must guarantee

minimum CP, minimum crude fiber, minimum phosphorus, and minimum and maximum calcium. The calcium concentration also cannot exceed the amount of phosphorus by 2.2 times. While all "meals" fall under AAFCO definitions, the variation in co-products can differ due to processing and/or the variation in raw material composition. Factors such as the presence of connective tissue, ash content, and the processing temperature used to prepare the co-product can decrease digestibility (Kies, 1981; Friedman, 1996; Parsons, 2002). Temperatures far in excess of the required thermal kill time of pathogens can also lower nutritional values and digestibilities of the final co-product (Johnson et al. 1998; Meeker and Meisinger, 2015). In addition, it must be considered that these co-products will undergo a further high heat step during extrusion – the process used to produce commercial kibble diets.

Despite their benefits, co-products have been painted in a negative light by some pet food companies and owners, possibly for the unknown identity of the meat and because it is considered to be a low-quality meat product. This is especially true of ingredients with "by-product" in the term. Pet owners may also be aware of recent pet food recalls related to pentobarbital residue contamination. Pentobarbital sodium is a drug that is routinely used to euthanize animals. Therefore, when used in food-producing animals, it could end up in pet food by way of rendered products. Pentobarbital is not nullified during the high heat rendering process, despite prior perceptions of processing making tissues safe (Wells, 2020). The US Food and Drug Administration (FDA) has a zero-tolerance policy for pentobarbital, therefore, making any pet food with a trace of the drug adulterated. The presence of pentobarbital residues in pet food are often isolated incidents, but are a threat to companion animals. Continued development of safety standards pertaining to the rendering of euthanized animal carcasses to prevent the incorporation of these rendered co-products into the pet food supply are needed. While pentobarbital may not be

the primary way to euthanize chickens, pet owners may perceive all animal co-products to have the same negative connotations and avoid any pet food that contains co-products.

Consumer uncertainty about what "by-products" are makes the case for fresh meat, which is perceived to be a more desirable ingredient. Fresh mechanically deboned meat refers to meat that has not undergone any treatment except to maintain cold temperature during processing (Meineri et al., 2021). Deboned meat is obtained by forcing pureed or ground meat under high pressure through a sieve to separate the bone from the edible meat tissue. Mechanically deboned chicken meat inclusion has been reported to decrease bitterness, and fishy flavor in pet foods (Koppel et al., 2014). Mechanically deboned meat is typically listed as the first ingredient on food labels due to its high water content, which also can influence consumer selection. Due to processing limitations, it is often necessary to use dried proteins (i.e., meals) along with fresh meats in order to form dry extruded kibbles.

Previous research evaluated four chicken-based ingredients of different heat processing treatments, including raw chicken, steamed chicken, retorted chicken, and chicken meal using the precision-fed cecectomized rooster assay. Raw chicken and steamed chicken were minimally processed, while retorted and chicken meal were similar to forms traditionally found in pet foods. The dry matter (DM) and organic matter (OM) digestibility was similar among raw chicken (75.9% DM; 80.5% OM), steamed chicken (76.5% DM; 80.6% OM), and retorted chicken (73.49% DM; 77.78% OM), and greater than that of chicken meal (60% DM; 65.9% OM). The steamed chicken had the highest indispensable AA digestibilities, with all having a true digestibility greater than 88% and most being over 90%. According to the digestible indispensable AA scores (DIAAS)-like values calculated by using the AAFCO recommended allowances for adult dogs, chicken meal was the only protein source that did not have a value exceeding 100 for all AA (e.g., methionine, tryptophan, and threonine). Using the National Research Council (NRC) recommended allowances

for adult dogs, all protein sources had some DIAAS-like values lower than 100%. Steamed chicken had the most DIAAS-like values for indispensable AA over 100 (arginine, histidine, isoleucine, leucine, and lysine), followed by raw chicken (arginine, histidine, isoleucine, and lysine), retorted chicken (arginine, histidine, and lysine), and chicken meal (arginine and lysine). Although animal proteins are often considered to be complete proteins, DIAAS-like values lower than 100 suggest that individual chicken ingredients may not provide all indispensable AA when included at levels to meet minimal CP recommendations (Oba et al., 2019). Prior processing during the rendering process may contribute to the lower DIAAS-like score of chicken meal.

The number of people choosing not to eat animal-based products is steadily increasing, as plant-based diets are perceived to be healthier, have less impact on the environment, and reduce animal welfare concerns. It has been suggested that pet owners that feed plant-based diets are more likely to be vegan or vegetarian themselves (Dodd et al., 2018). Some people may face a moral dilemma when it comes to feeding their pets. While they avoid animal products in their own diet, they live with pets and may feed them a diet containing animal products. There is growing interest in the availability of plant-based diets in the North American pet food market. It may be feasible to create a high-quality plant protein-based pet diet that provides all of the indispensable AA, however, it is necessary to evaluate all nutrients, especially micronutrients that may be at risk when removing animal-sourced proteins from the formula. Nutrients that often are insufficient in plant-based diets include sulfur-containing AA, taurine, arachidonic acid, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), calcium, phosphorus, and vitamin D (Dodd et al., 2021). These nutrients are typically found in animal tissues, but non-animal sourcing is also available. Sources for some of the nutrients may be limited, however, so careful formulation and potential supplementation of animal-free pet products is needed.

Cereal grains are important staple foods for people around the world, providing secondary products that are readily available and economically viable as pet food ingredients. Corn has been an ingredient used in pet foods for decades due to its structure-forming properties, economics, and history of nutritional utility. Corn is a major crop in the US that yielded 15.6 billion bushels last year (USDA ERS, 2022). Corn is typically processed by one of three methods, including wet milling, dry milling, and dry grinding, that creates different primary and secondary co-products. The raw ingredient source and processing methods impact the final nutrient composition. Starch from corn can be converted into a renewable energy source that is used in fuel ethanol production. Wet milling processing of corn to produce ethanol as the primary product also produces corn co-products that include corn gluten meal, corn germ meal, corn gluten feed, and corn fiber. Corn gluten meal is a high-protein residue with low amounts of ash and total dietary fiber (TDF), and moderate nitrogen-free extract (NFE) concentrations following the removal of the majority of starch, germ, and bran portions (de Godoy et al., 2009). Corn gluten meal is usually used in combination with another protein source such as soy protein to overcome deficiencies in indispensable AA such as lysine and tryptophan. A previous study utilizing cecectomized roosters to measure AA digestibilities reported that corn gluten meal had indispensable AA and dispensable AA mean value digestibilities of 94.3% and 97.0%, respectively (de Godoy et al., 2009). In that study, chicks were also fed corn gluten meal to determine a protein efficiency ratio (PER; g weight change/g protein consumed) of 0.76. The PER experiment indicated that corn gluten meal alone is a poor-quality protein due to low lysine concentrations.

Wheat gluten meal is a plant-based protein concentrate co-product of the wheat starch industry and is used in a wide variety of dog and cat foods. Wheat gluten is high in total protein, but when its AA profile is compared with other plant-based proteins, it is unfavorable because it is low in methionine, tryptophan, and lysine. A previous study evaluated beagle puppies aged 9-

12 weeks of age fed a semi-purified diet containing 10% CP from either casein or wheat as the sole protein source (Burns et al., 1982). The PER calculated in that study was 2.3 for casein and -0.4 for wheat gluten. When compared with the minimum AA requirements of puppies, the wheat gluten diet was severely deficient in lysine, causing diminished growth.

Nery et al. (2010) evaluated the influence of varying dietary protein content (22, 29, and 39% CP on a DM basis for low, medium, and high, respectively) and primary source (wheat gluten, poultry meal, or 50:50 mixture of both) on fecal quality and nutrient digestibility in adult dogs differing in body size. Different dog sizes were used because large-breed dogs, particularly German Shepherds, are prone to digestive intolerance and poor fecal quality (Zentek et al., 2002). Given the AA deficiencies of wheat gluten, proteins were combined with corn gluten meal. Considering all dogs, the apparent CP digestibility was greater for the wheat gluten diet (89.9%) than the poultry meal diet (82.9%). Better fecal quality (e.g., fecal scores; lower fecal moisture) was observed in dogs fed the wheat gluten diet than those fed the poultry meal diet. Wheat gluten also helped modulate the fecal quality of dogs, particularly in the sensitive breed German Shepherds (Nery et al., 2010). Altogether, it is clear that wheat gluten alone is a poor-quality protein, but can be supplemented with the necessary indispensable AA to be suitable protein source.

Pet foods often follow human food trends, as people want to feed their pets what they believe would be healthy for themselves. While some have moved to plant-based proteins, others have begun to exclude certain plant-based ingredients from their pets' diet. This movement has been exemplified by grain-free pet diets, which have become popular for a few reasons, including the fact that they replicate the human food trend of gluten-free foods. Gluten-free diets are a necessity for those with major wheat sensitivities, including celiac disease, but others have reported benefits such as weight loss from reducing excess refined carbohydrates in their diets.

Gluten sensitivity enteropathy is common in Irish setter and Border terrier dog breeds (Hall and Batt, 1992; Lowrie et al., 2018). Paroxysmal gluten-sensitive dyskinesia in border terriers results from an immunologic response directed against transglutaminase and gliadin. Symptoms of paroxysmal gluten-sensitive dyskinesia consists of episodes of difficulty walking, ranging from ataxia to complete inability to stand, and tremors that last for minutes or hours. This is an inherited disease, but owners of predisposed breeds may avoid gluten in pet foods, especially for puppies that have unknown sensitivities. The demand for and supply of grain-free dog foods on the market today is much larger than that needed to address gluten hypersensitivity, however, so there are other factors involved.

Recent marketing claims have negatively labeled grains as “fillers” (Alvarenga et al., 2021), suggesting that they have no purpose in the foods. This is untrue, of course, as grains provide a readily available energy source in the form of starch, contain many essential micronutrients, and are useful from a pet food processing perspective. Some pet owners may have aversions to the use of genetically modified (GM) grains such as corn. Despite the concerns, there is no evidence of negative health effects and there may actually be benefits to GM corn compared with traditional corn. In fact, the most common GM corn in the US has genes encoded to control lepidopteran insect infestations and had lower incidences of mycotoxin contamination (Alvarenga et al., 2021). Despite GM corn being a controversial topic, it can produce high and safer crop yields.

Other pet parent opinions and preferences may have contributed to the success of the grain-free diet trend, including the theory that by reducing grain intake, the potential exposure to mycotoxins is reduced (Tegzes et al., 2019). Cereal grains are prone to fungal growth that can produce mycotoxins. The most problematic mycotoxins in the pet food industry are aflatoxin, fumonisin, and deoxynivalenol (Atungulu et al., 2018). Aflatoxins are toxins produced by the mold

Aspergillus flavus, commonly found in corn. At high concentrations, aflatoxins can cause illness and death in pets and the FDA considers pet food containing more than 20 parts per billion of aflatoxin to be adulterated. A recent study evaluated 60 samples of grain-free and grain-based (dry and wet) dog foods produced by five major manufacturers within the US for mycotoxin concentrations. *Fusarium* mycotoxins were present in 9 of 12 commercial dry grain-based diets, but concentrations were below the FDA threshold. Only grain-based dry dog foods had detectable mycotoxin contamination (Tegzes et al., 2019). Contamination does occur from time to time, however. In August 2021, the FDA issued a warning letter to Midwestern Pet Foods, Inc. after finding that their dry dog diets contained aflatoxin concentrations as high as 558 parts per billion (FDA, 2021). There were more than 130 pet deaths and 220 pet illnesses that were potentially linked to this single recall. While pet food recalls due to mycotoxin contamination are rare, there is always the risk, especially if poor ingredient sourcing practices are used. To mitigate this risk, it is suggested that pet food manufacturers incorporate grains that are categorized as US No. 1 by the USDA and are therefore less susceptible to mycotoxin formation (Tegzes et al., 2019). Safety measures and control points can be implemented pre- and post-harvest to prevent fungal growth. Mycotoxin risks can also be controlled by pet food and ingredient supplies through testing to comply with the FDA and Food Safety Modernization Act (FSMA) regulations.

Protein Digestion, Metabolism, and Feeding Recommendations for Adult Dogs

Proteins provide AA that play diverse and critical roles in meeting the physiological needs of dogs. Although they are in the phylogenic Order of Carnivora, dogs are more appropriately classified as omnivores metabolically (Oberbauer and Larsen, 2021). Compared with their wolf ancestors, dogs can consume and utilize a wider range of food sources, possibly a reflection of their long evolutionary relationship with people (Axelsson et al., 2013). Protein is required for

supplying indispensable (i.e., essential) AA and to supply nitrogen for the synthesis of dispensable AA. Dogs have a non-ruminant digestive system that is comprised of a relatively large stomach and short intestinal tract. Dietary proteins are broken down by different proteolytic enzymes in the stomach and small intestine. Pepsin, secreted by chief cells in the fundic region, breaks down long-chain peptides, while other proteases cleave peptide bonds into tri- or di-peptides or free AA that can be taken up by enterocytes. The fate of AA after absorption can be divided into three categories: tissue protein synthesis; synthesis of enzymes, hormones, and other metabolites; and deamination or transamination as an energy source (Pond et al., 2005).

Animals do not have a dietary requirement for protein in itself, but require AA and a certain level of nitrogen to synthesize dispensable AA (Case et al., 2000). There are several hundred AA in nature, with 30-50 being present in the animal body, 23-25 present in body proteins, and 20 used for protein synthesis. Ten AA have been categorized as indispensable for dogs and are required to be consumed through the diet. The indispensable AA include arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (NRC, 2006). If indispensable AA requirements are not met, protein synthesis will be compromised, and homeostasis is not possible. All AA need to be available at the same time for protein synthesis to occur, offering the concept of the first limiting AA. The first limiting AA, often methionine, tryptophan, or lysine, will determine the level of protein production. The branched-chain AA, including leucine, isoleucine, and valine, represent the majority of AA present in muscle proteins. They also promote muscle protein synthesis through the mechanistic target of the rapamycin (mTOR) pathway and translational activation, while reducing protein catabolism. The mTOR pathway is involved in many physiological processes, including protein and lipid synthesis and energy metabolism (Oberbauer and Larsen, 2021). The body is unable to store surplus AA, therefore, all are metabolized once consumed.

The AAFCO recommends a dietary minimum of 18% CP for adult dogs at maintenance. The NRC lists the minimal CP requirement for adult dogs at 8%, but the recommended allowance is 20% CP for that life stage (NRC, 2006). Commercial pet foods are typically formulated to meet or exceed the AAFCO nutrient profiles and/or the NRC recommended allowances rather than the minimal requirements. This is to compensate for possible nutrient losses during processing, variation in bioavailability of ingredient sources, and variation among individual animals.

Some diets provide protein at concentrations that go well beyond these recommendations. High-protein diets, for example, may be beneficial during weight loss or endurance exercise in dogs to minimize lean muscle mass loss Reynolds et al., 1999; (Phungviwatnikul et al., 2022). For intense interval exercise work, a diet containing 35% of energy as protein has been shown to enhance performance by promoting an increase in plasma volume, as has been shown in sled dogs undergoing rigorous training (Reynolds et al., 1999). Puppies have a greater demand for protein than adults, as a large amount of muscle is deposited during rapid growth. No maximum dietary protein concentrations have been established, however, excess dietary protein may be damaging to pets with liver or kidney problems. Protein in excess of an animal's requirement is metabolized for energy, producing urea and other end-products that are excreted by the kidneys (Case et al., 2000). For pets with renal disease, protein restriction may be beneficial and is recommended for managing clinical signs of the disease. High concentrations of blood urea, a nitrogenous end-product of protein and AA metabolism, often leads to clinical signs such as nausea, vomiting, and osmotic diuresis. Restricting protein to normalize urea concentrations contributes to the return of appetite, weight gain, and diminishment of clinical signs (Case et al., 2000).

Fecal Metabolites and Microbiota

Nowadays, many pets live indoors and closely by their owners. While this close proximity may be desirable for the pet and human, one problem people may deal with is the pet's flatulence and odor. Flatulence is caused by the formation of gases via fermentation by microbiota in the gastrointestinal tract. For healthy animals, this is not a significant matter, but it is a common complaint of the humans living with the pet (Urrego et al., 2021). Diet is certainly a factor, but the breed of dog may also influence the predisposition to flatulence, as is commonly observed in dogs such as French bulldogs (Urrego et al., 2021). Strong fecal malodor is a concern of pet owners so the problem cannot be ignored.

Odorous compounds from the gastrointestinal tract are comprised of more than 230 materials, but can be grouped into four major categories: sulfur-containing compounds, phenolic and indolic compounds, volatile fatty acid compounds, and ammonia and amines. Endogenous protein sources, including intestinal epithelial cells, enzymes, and dead bacteria can contribute to the generation of malodorous gas production (Cho et al., 2015). Exogenous (dietary) sources of protein may also contribute to the generation of these compounds. High dietary protein concentrations and less digestible protein have been associated with higher concentrations of fecal odor compounds in dogs (Hesta et al., 2003).

For proteins that were not digested and absorbed in the stomach and small intestine, the leftover protein is available for microbial fermentation, which predominantly occurs in the distal colon (Tiwari et al., 2019). Different protein sources will not only provide different AA, but other micro- and macronutrients that may influence nutrient digestibility and the gastrointestinal microbiome (Wernimont et al. 2020). If an abundant amount of poorly digestible protein is fed, less will be digested, leading to more dietary protein undergoing putrefaction that may cause inflammation and fecal odor. Putrefaction is the process of gut microbiota decomposing AA into

by-products, such as branched-chain fatty acids (BCFA), ammonia, phenols, and indoles. Ammonia, a potentially harmful metabolite, is produced from the deamination of AA and hydrolysis of urea (Tiwari et al., 2019). BCFA are produced from the fermentation of branched-chain AA; isobutyrate, isovalerate, and 2-methybultrate are products of valine, leucine, and isoleucine, respectively (Windey et al., 2011). Phenols and indoles are produced during bacterial metabolism of tyrosine and tryptophan, respectively. Degradation of undigested protein will not only produce a strong fecal odor, but is also toxic to the host at high concentrations (Ramakrishna et. al., 1991). A recent study in dogs reported that some plant protein-based diets such as those containing peanut flour (16.0 μmol/g DMB) and green lentils (16.1 μmol/g DMB) led to lower total fecal BCFA concentrations than a diet based on poultry by-product meal (20.1 μmol/g DMB) (Reilly et al. 2021). Feeding highly digestible protein sources can reduce dietary protein flow into the colon, minimizing protein fermentation and production of odor compounds.

Several strategies may be used to alter odor compound production. One method may be to reduce the availability of precursors (e.g., protein) by reducing it in the diet or by increasing its digestibility. Another method may be to alter the pH of the digestive tract such as modulating ammonia concentrations that can influence microenvironment pH, potentially modulating host cell metabolism as well as other microorganisms (Polansky et al., 2016). Lastly, another substrate that is preferentially fermented may be included in the diet, shifting microbial metabolism and the type of compounds produced. Dietary fibers may aid in the latter two strategies. Fiber fermentation products are predominantly short-chain fatty acids (SCFA), including acetate, propionate, and butyrate (Macfarlane and Macfarlane, 2012). SCFA production is considered beneficial for intestinal health because epithelial cells derive 60-70% of their energy from these bacterial end-products, and greater SCFA will lower fecal pH (Cummings, 1981). SCFA can also support immune function and homeostasis (Correa-Oliveira et al., 2016). Butyrate is almost entirely used

by colonocytes as an energy source, whereas acetate and propionate are transported to the liver through the portal vein (Haenen et al., 2013) and converted to energy substrates. Butyrate is produced from several gastrointestinal microbiota, including *Roseburia*, *Faecalibacterium*, *Clostridium*, and *Fusobacterium*, while propionate can be produced by *Bacteroides*, *Propionibacterium*, and *Veillonella* (Macfarlane and Macfarlane, 2012). While dogs do not rely on microbiota for energy, given the microbiome's plasticity, it has the potential as a therapeutic target due to the involvement of the gut microbiome in multiple diseases (Deng and Swanson, 2015; Suez and Elinav, 2017).

Thesis Objective

The objective of this thesis was to determine the apparent total tract macronutrient digestibility of canine diets differing in protein source and to determine the whole blood gene expression, and fecal characteristics, metabolites, and microbiota of healthy adult dogs consuming them. We hypothesized that the plant-based protein diets would be less digestible, increase fecal metabolite concentrations coming from protein fermentation (BCFA; phenols and indoles; ammonia), and negatively impact fecal microbiota populations.

Literature Cited

AAFCO. 2020. Official Publication 2020. Association of American Feed Control Officials. Oxford, IN.

Axelsson, E., A. Ratnakumar, M. L. Arendt, K. Maqbool, M. T. Webster, M. Perloski, O. Liberg, J. M. Arnemo, A. Hedhammar, and K. Lindblad-Toh. 2013. The genomic signature of dog domestication reveals adaptation to a starch-rich diet. Nature 495:360–364. doi:10.1038/nature11837.

Buff, P. R., R. A. Carter, J. E. Bauer, and J. H. Kersey. 2014. Natural pet food: A review of natural diets and their impact on canine and feline physiology. J. Anim. Sci. 92:3781-3791. doi:10.2527/jas.2014-7789.

Burns, R. A., M. H. LeFaivre, and J. A. Milner. 1982. Effects of dietary protein quantity and quality on the growth of dogs and rats. J. Nutr. 112:1843–1853. doi:10.1093/jn/112.10.1843

Case, L. P., D. P. Carey, D. A. Hirakawa, and L. Daristotle. 2000. Chronic Renal Failure. Canine and Feline Nutrition, p. 451-476. Mosby Year Book Inc., St. Louis, MO.

Cho, S., O. Hwang, and S. Park. 2015. Effect of dietary protein levels on composition of odorous compounds and bacterial ecology in pig manure. As. Aust. J. Anim. Sci. 28:1362–1370. doi:10.5713/ajas.15.0078

Correa-Oliveira, R., J. L. Fachi, A. Vieira, F. T. Sato, and M. A. R. Vinolo. 2016. Regulation of immune cell function by short-chain fatty acids. Clin. Trans. Immun. 5:73. doi:10.1038/cti.2016.17

Corsato Alvarenga, I., A. N. Dainton, and C. G. Aldrich. 2021. A review: Nutrition and process attributes of corn in pet foods. Crit. Rev. Food. Sci. Nutr. 1–10. doi:10.1080/10408398.2021.1931020

Cummings, J. H. 1981. Short chain fatty acids in the human colon. Gut 22:763–779. doi:10.1136/gut.22.9.763

de Godoy, M. R., L. L. Bauer, C. M. Parsons, and G. C. Fahey. 2009. Select corn coproducts from the ethanol industry and their potential as ingredients in pet foods. J. Anim. Sci. 87:189–199. doi:10.2527/jas.2007-0596

Deng, P., and K. S. Swanson. 2015. Gut microbiota of humans, dogs and cats: current knowledge and future opportunities and challenges. Br. J. Nutr. 113:S6–S17 doi:10.1017/S0007114514002943

Dodd, S., J. L. Adolphe, and A. Verbrugghe. 2018. Plant-based diets for dogs. J .Anim. Vet. Med. Assoc. doi:10.2460/javma.253.11.1425

Dodd, S., A. K. Shoveller, A. J. Fascetti, Z. Z. Yu, D. Ma, and A. Verbrugghe. 2021. A comparison of key essential nutrients in commercial plant-based pet foods sold in Canada to American and European canine and feline dietary recommendations. Animals 11:2348. doi:10.3390/ani11082348

EPA. Food Recovery Hierarchy. https://www.epa.gov/sustainable-management-food/reduce-wasted-food-feeding-animals

Friedman, M. 1996. Nutritional value of proteins from different food sources: A review. J. Agric. Food Chem. 44:6–29. doi:10.1021/jf9400167

Food and Agriculture Organization of the United Nations. FAO. Retrieved from http://www.fao.org/poultry-production-products/products-processing/en/

Haenen, D., J. Zhang, C. S. da Silva, G. Bosch, I. M. van der Meer, J. van Arkel, J. J. G. C. van den Borne, O. P. Gutierrez, H. Smidt, and B. Kemp. 2013. A diet high in resistant starch modulates microbiota composition, SCFA concentrations, and gene expression in pig intestine. J. Nutr. 143:274–283. doi:10.3945/jn.112.169672

Hall, E. J., and R. M. Batt 1992. Dietary modulation of gluten sensitivity in a naturally occurring enteropathy of Irish setter dogs. Gut 33:198–205. doi:10.1136/gut.33.2.198

Hesta, M., G. Janssens, S. Millet, and R. De Wilde. 2003. Prebiotics affect nutrient digestibility but not faecal ammonia in dogs fed increased dietary protein levels. Br. J. Nutr. 90:1007-1014. doi:10.1079/BJN2003988

Irshad, A., and B. D. Sharma. 2015. Slaughter house by-product utilization for sustainable meat industry-a review. J. Anim. Pro. Adv. 5:681-696. doi:10.5455/japa.20150626043918

Johnson, M. L., C. M. Parsons, G. C. Fahey, N. R. Merchen, and C. G. Aldrich. 1998. Effects of species raw material source, ash content, and processing temperature on amino acid digestibility of animal by-product meals by cecectomized roosters and ileally cannulated dogs. J. Anim. Sci. 76:1112–1122. doi:10.2527/1998.7641112x

Kies, C. 1981. Bioavailability: A factor in protein quality. J. Agric. Food Chem. 29:435–440. doi:10.1021/jf00105a002

Knight, A., and M. Leitsberger, 2016. Vegetarian versus meat-based diets for companion animals. Animals 6:57. doi:10.3390/ani6090057

Koppel, K., M. Gibson, S. Alavi, and G. Aldrich. 2014. The effects of cooking process and meat inclusion on pet food flavor and texture characteristics. Animals 4:254–271. doi:10.3390/ani4020254

Lahtinen, M., D. Clinnick, K. Mannermaa, J. S. Salonen, and S. Viranta. 2021. Excess protein enabled dog domestication during severe Ice Age winters. Sci. Rep. 11:7. doi:10.1038/s41598-020-78214-4

Larkin, L. 2021. Pet population still on the rise, with fewer pets per household. American Veterinary Medical Association. Retrieved from https://www.avma.org/javma-news/2021-12-01/pet-population-still-rise-fewer-pets-household.

Lowrie, M., M. Hadjivassiliou, D. S. Sanders, and O. A. Garden. 2016. A presumptive case of gluten sensitivity in a border terrier: A multisystem disorder? Vet. Rec. 179: 573. doi:10.1136/vr.103910

Macfarlane, G. T., and S. Macfarlane. 2012. Bacteria, colonic fermentation, and gastrointestinal health. J. AOAC Int. 95:50-60. doi:10.5740/jaoacint.sge_macfarlane

Meeker, D. L., and J. L. Meisinger. 2015. Rendered ingredients significantly influence sustainability, quality, and safety of pet food. J. Anim. Sci. 93:835–847. doi:10.2527/jas.2014-8524

Meeker, D., and C. R. Hamilton. 2006. An overview of the rendering industry. Essential Rendering: All about the Animal By-products Industry, p. 1-16.

Meineri, G., A. Candellone, S. Tassone, P. G. Peiretti, E. Longato, D. Pattono, N. Russo, E. Pagani, and L. Prola. 2021. Effects of "fresh mechanically deboned meat" inclusion on nutritional value, palatability, shelf-life microbiological risk and digestibility in dry dog food. PloS ONE 16:0250351. doi:10.1371/journal.pone.0250351

Millward, D. J., D. K. Layman, D. Tomé, and G. Schaafsma. 2008. Protein quality assessment: Impact of expanding understanding of protein and amino acid needs for optimal health. Amer. J. Clin. Nutr. 87:1576S–1581S. doi:10.1093/ajcn/87.5.1576S

NRC. 2006. Nutrient requirements of dogs and cats. Washington (DC): National Academies Press.

Nery, J., V. Biourge, C. Tournier, V. Leray, L. Martin, H. Dumon, and P. Nguyen. 2010. Influence of dietary protein content and source on fecal quality, electrolyte concentrations, and osmolarity, and digestibility in dogs differing in body size. J. Anim. Sci. 88:159–169. doi:10.2527/jas.2008-1666

Nery, J., R. Goudez, V. Biourge, C. Tournier, V. Leray, L. Martin, C. Thorin, P. Nguyen, and H. Dumon. 2012. Influence of dietary protein content and source on colonic fermentative activity in dogs differing in body size and digestive tolerance. J. Anim. Sci. 90:2570–2580. doi:10.2527/jas.2011-4112

North American Renderers Association. NARA. Retrieved from https://nara.org/about-us/facts/

Oba, P. M., P. L. Utterback, C. M. Parsons, M. R. C. de Godoy, and K. S. Swanson. 2019. Chemical composition, true nutrient digestibility, and true metabolizable energy of chicken-based ingredients differing by processing method using the precision-fed cecectomized rooster assay. J. Anim. Sci. 97:998-1009. doi:10.1093/jas/sky461

Oberbauer, A. M., and J. A. Larsen. 2021. Amino acids in dog nutrition and health. Adv. Exp. Med. Biol. 1285:199–216. doi:10.1007/978-3-030-54462-1_10

Parsons, C. M. 2002. Digestibility and bioavailability of protein and amino acids. Poultry Feedstuffs: Supply, Composition, and Nutritive Value. J. M. McNab and K. N. Boorman ed. 115–136. CABI Publ., Wallingford, UK.

Phungviwatnikul, T., A. H. Lee, S. E. Belchik, J. S. Suchodolski, and K. S. Swanson. 2022. Weight loss and high-protein, high-fiber diet consumption impact blood metabolite profiles, body composition, voluntary physical activity, fecal microbiota, and fecal metabolites of adult dogs. J. Anim. Sci. 100: skab379. doi:10.1093/jas/skab379

Pond, W. G., D. C. Church, K. R. Pond, and P. A. Schoknecht. 2005. Basic animal nutrition and feeding (5th ed.). Wiley, Hoboken, NJ.

Ramakrishna, B. S., I. C. Roberts-Thomas, P. R. Pannall, and W. E. W. Roediger. 1991. Impaired sulphation of phenol by the colonic mucosa in quiescent and active colitis. Gut 32:46–49. doi:10.1136/gut.32.1.46

Reilly, L. M., F. He, S. L. Rodriguez-Zas, B. R. Southey, J. M. Hoke, G. M. Davenport, and M. R. C. de Godoy. 2021. Use of legumes and yeast as novel dietary protein sources in extruded canine diets. Front. Vet. Sci. 8:667642. doi:10.3389/fvets.2021.667642

Reynolds, A. J., G. A. Reinhart, D. P. Carey, D. A. Simmerman, D. A. Frank, and F. A. Kallfelz. 1999. Effect of protein intake during training on biochemical and performance variables in sled dogs. Am. J. Vet. Res. 60:789–795.

Schmidt, M., S. Unterer, J. S. Suchodolski, J. B. Honneffer, B. C. Guard, J. A. Lidbury, J. M. Steiner, J. Fritz, and P. Kölle. 2018. The fecal microbiome and metabolome differs between dogs fed Bones and Raw Food (BARF) diets and dogs fed commercial diets. PloS ONE, 13:e0201279. doi:10.1371/journal.pone.0201279

Smith, B. P. 2019. The Domestic Dog: Its Evolution, Behavior and Interactions with People. Second edition, Anthrozoös 32: 719-722, doi:10.1080/08927936.2019.1645518

Suez, J., and E. Elinav. 2017. The path towards microbiome-based metabolite treatment. Nat. Microbiol. 2:17075. doi:10.1038/nmicrobiol.2017.75

Swanson, K. S., R. A. Carter, T. P. Yount, J. Aretz, and P. R. Buff. 2013. Nutritional sustainability of pet foods. Adv. Nutr. 4:141–150. doi:10.3945/an.112.003335

Tegzes, J. H., B. B. Oakley, and G. Brennan. 2019. Comparison of mycotoxin concentrations in grain versus grain-free dry and wet commercial dog foods. Tox. Comm. 3:61–66. doi:10.1080/24734306.2019.1648636.

United States Department of Agriculture Economic Research Service (USDA ERS). 2022. Feed Grains Database Custom Query. Retrieved from https://www.ers.usda.gov/data-products/feedgrains-database/.

United States Food and Drug Administration. FDA. Retrieved from https://www.fda.gov/news-events/press-announcements/fda-brief-fda-issues-corporate-wide-warning-letter-company-associated-contaminated-pet-food-hundreds

Urrego, M., R. S. Pedreira, K. M. Santos, M. C. Ernandes, J. Santos, T. Vendramini, M. N. Eberlin, J. Balieiro, C. Pontieri, and M. A. Brunetto. 2021. Dietary protein sources and their effects on faecal odour and the composition of volatile organic compounds in faeces of French Bulldogs. J. Anim. Phys. Anim. Nutr. 105:65–75. doi:10.1111/jpn.13605

Wells, K., A. Butterworth, and N. Richards. 2020. A review of secondary pentobarbital poisoning in scavenging wildlife, companion animals, and captive carnivores. J. Vet. Forensic Sci. 1:1. doi:10.32473/jvfs.v1i1.128307

Wernimont, S. M., J. Radosevich, M. I. Jackson, E. Ephraim, D. V. Badri, J. M. MacLeay, D. E. Jewell, and J. S. Suchodolski. 2020. The effects of nutrition on the gastrointestinal microbiome of cats and dogs: Impact on health and disease. Front. Microbiol. 11:1266. doi:10.3389/fmicb.2020.01266

Windey, K., V. De Preter, and K. Verbeke. 2012. Relevance of protein fermentation to gut health. Mol. Nutr. Food Res., 56:184–196. doi:10.1002/mnfr.201100542

Zentek J., D. Kaufmann, and T. Pietrzak. 2002. Digestibility and effects on fecal quality of mixed diets with various hydrocolloid and water contents in three breeds of dogs. J. Nutr. 132:1679S–1681S. doi:10.1093/jn/132.6.1679S

CHAPTER 3: EVALUATION OF HIGH-PROTEIN DIETS DIFFERING IN PROTEIN SOURCE IN HEALTHY ADULT DOGS

Abstract

Given the dynamic market for protein-based ingredients in the pet food industry, demand continues to increase for both plant- and animal-based options. Animal and plant protein sources contain different amino acid profiles and vary in digestibility, which can affect the protein quality provided to the animal. The objective of this study was to evaluate the apparent total tract digestibility of canine diets differing in protein source and test their effects on serum metabolites, whole blood gene expression, and fecal characteristics, metabolites, and microbiota of healthy adult dogs consuming them. Four isocaloric and isonitrogenous, extruded diets were formulated to meet all Association of American Feed Control Officials (AAFCO) nutrient profiles for adult dogs at maintenance, with the primary difference being protein source: 1) chicken by-product meal (CBPM), 2) deboned chicken, dried chicken, and spray dried chicken (DC), 3) corn gluten meal (CGM), or 4) wheat gluten meal (WGM). Twelve adult spayed female beagles (BW = 9.9± 1.0 kg; age = 6.3 ±1.1 yr) were used in a replicated 4×4 Latin square design (n=12/treatment). Each period consisted of a 22-d adaptation phase, 5 d for total and fresh fecal collection, and 1 d for blood collection. Fecal microbiota data were analyzed using QIIME 2.2020.8. All other data were analyzed using the Mixed Models procedure of SAS version 9.4. Fecal scores were higher ($p<0.05$; looser stools) in dogs fed DC or CBPM than those fed WGM or CGM, but all remained within an appropriate range. Apparent dry matter digestibility was lower ($p<0.05$) in dogs fed CBPM or CGM than those fed DC or WGM. Apparent crude protein digestibility was also lower ($p<0.05$) in dogs fed DC or CGM than those fed WGM. Dogs fed CBPM had lower ($p<0.05$) apparent organic matter, crude protein, and energy digestibilities than those fed the other 3 diets. Fecal

indole concentrations were higher ($p<0.05$) in dogs fed CBPM than those fed WGM, but phenol and total phenol and indole concentrations were not different. Fecal total short-chain fatty acid (SCFA) concentrations were higher ($p<0.05$) in dogs fed DC than those fed CGM, but individual SCFA (i.e., acetate; propionate; butyrate) were not different. Fecal total branched-chain fatty acid concentrations were higher ($p<0.05$) in dogs fed DC or CBPM than those fed WGM. Fecal ammonia concentrations were higher ($p<0.05$) in dogs fed the animal-based protein diets than those fed the plant-based protein diets. Gene expression was not affected by diet. The relative abundance of 3 bacterial phyla and 9 bacterial genera were significantly shifted among treatment groups ($p<0.05$). Considering AA profiles and digestibility data together, the protein sources in the DC diet provided the most and highest quality protein without additional AA supplementation of all diets tested. However, the animal-based protein diets resulted in higher concentrations of proteolytic fermentative end-products. Further studies evaluating moderate dietary protein concentrations are needed to better compare plant- and animal-based protein sources.

Introduction

Many pet owners think of their pets as part of the family, and are concerned about their health and longevity. Owners are becoming more aware and selective of the foods they are choosing to purchase for their pets. Nutrition is seen as a way to safeguard their animals' health and welfare. The most frequent consideration of consumers and pet food manufacturers is protein source and concentration (Oberbauer and Larsen, 2021). Well formulated diets provide adequate protein and amino acids in dietary concentrations that meet the needs of the target dog population. Diets must ensure nutritional adequacy for the dogs. If not, health can become compromised.

Recently, some owners have moved away from traditional pet food protein sources (i.e., animal by-products) to different choices, such as fresh meat or a more sustainable plant-based

protein option. Many diet choices for companion dogs have begun to reflect the personal preferences of their owners, with different social and cultural factors influencing the decision-making process (Vinassa et al., 2020). Different pet owners will consider specific criteria they desire from their food choice, creating more diverse protein source needs that the pet food industry must provide.

The objective of this study was determine the apparent total tract macronutrient digestibility of canine diets differing in protein source and to determine the whole blood gene expression and fecal characteristics, metabolites, and microbiota of healthy adult dogs consuming them. We hypothesized that the plant-based protein diets would be less digestible, increase fecal metabolite concentrations coming from protein fermentation [branched-chain fatty acids (BCFA); phenols and indoles], and negatively impact fecal microbiota populations.

Materials and Methods

All animal care procedures were approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee prior to animal experimentation.

Animals and housing:

Twelve adult spayed female beagles (BW = 9.9 ± 1.0 kg; age =6.3 ±1.1 yr) were used in this study. Dogs were housed individually in pens (1.22 m wide × 1.85 m long) in a humidity- and temperature-controlled room on a 14 h light: 10 h dark cycle. Dogs had access to fresh water ad libitum at all times and were fed once a day (8:00 am) to maintain body weight throughout the study. Dogs were weighed once a week in the morning before feeding. Leftover food was weighed every day to calculate intake. Dogs were weighed and body condition score was recorded weekly. Weighing was conducted in the morning before feeding. A 9-point body condition scoring system was used (Laflamme, 1997). Dogs had access to toys at all times and were socialized at least two

times per week where they were given other toys, further enrichment, and socialization with each other and humans.

Experimental timeline and diets:

Dogs consumed the four test diets using a replicated 4x4 Latin square design (n=12/group). The experiment consisted of four 28-d periods, with each period consisting of an adaptation phase (d 1-22), a 5-d fecal collection phase (d 23-27), and 1 d for blood collection (d 28). Four extruded kibble diets were formulated to meet all Association of American Feed Control Officials (AAFCO, 2020) nutrient profiles for adult dogs at maintenance. Diets differed primarily due to protein source, which included: 1) fresh (deboned), dried, and spray-dried chicken (DC), 2) chicken byproduct meal (CBPM), 3) wheat gluten meal (WGM), and 4) corn gluten meal (CGM) (Table 3.1; Table 3.2).

Fecal sample collection, scoring, and analysis:

From d 23 to d 27, total feces were collected from the pen floor, weighed and frozen at -20^{o}C until analyses. All fecal samples collected during the collection phase were scored according to the following scale: 1 = hard, dry pellets, small hard mass; 2 = hard, formed, dry stool; remains firm and soft; 3 = soft, formed, and moist stool, retains shape; 4 = soft, unformed stool, assumes shape of container; 5 = watery, liquid that can be poured. On the first day of the collection phase, one fresh fecal sample (within 15 min of defecation) was collected for measurement of pH, moisture content, microbiota populations, and metabolite concentrations. Fecal pH was measured immediately using an AP10 pH meter (Denver Instrument, Bohemia, NY) equipped with a Beckman Electrode (Beckman Instruments Inc., Fullerton, CA), and then aliquots were collected. Fecal aliquots for analysis of phenols and indoles were frozen at -20°C immediately after collection. One aliquot was collected and placed in 2 N hydrochloric acid for ammonia, short-chain fatty acid (SCFA), and BCFA analyses. An additional aliquot was used for fresh fecal DM

determination. Finally, 3-4 aliquots of fresh feces were collected for microbiota analysis. These samples were immediately transferred to sterile cryogenic vials (Nalgene, Rochester, NY), quickly frozen in dry ice, and stored at -80^{o}C until analysis.

Fecal and dietary chemical analysis:

Dietary treatments and fecal samples were dried at 55°C in a forced-air oven and ground in a Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ) through a 2-mm screen. Diet and fecal samples were analyzed for dry matter (DM) and ash according to AOAC (2006; methods 934.01 and 942.05), and organic matter calculated. Crude protein (CP) of the diet and feces was calculated from Leco (FP2000 and Tru-Mac) total nitrogen values according to AOAC (2006; method 992.15). Total lipid content (acid-hydrolyzed fat) of diet and fecal samples was determined according to the methods of the American Association of Cereal Chemists (1983) and Budde (1952). Total dietary fiber of the diets was determined according to AOAC 991.43. Gross energy of the diet and fecal samples was measured using an oxygen bomb calorimeter (model 6200, Parr Instruments, Moline, IL). Digestible energy was determined by subtracting the gross energy of feces from the gross energy of the food consumed. Fecal SCFA and BCFA concentrations were determined by gas chromatography according to Erwin et al. (1961) using a gas chromatograph (Hewlett-Packard 5890A series II, Palo Alto, CA) and a glass column (180 cm x 4 mm i.d.) packed with 10% SP-1200/1% H3PO4 on 80/100+ mesh Chromosorb WAW (Supelco Inc., Bellefonte, PA). Nitrogen was the carrier with a flow rate of 75 mL/min. Oven, detector, and injector temperatures were 125, 175, and 180°C, respectively. Fecal ammonia concentrations were determined according to the method of Chaney and Marbach (1962). Fecal phenol and indole concentrations were determined using gas chromatography according to the methods described by Flickinger et al. (2003) analysis.

Fecal DNA Extraction, MiSeq Illumina Sequencing of 16S Amplicons, and Microbiota Analysis:

Total DNA from fecal samples was extracted using Mo-Bio PowerSoil kits (MO BIO Laboratories, Inc., Carlsbad, CA). Concentrations of extracted DNA were quantified using a Qubit 3.0 Fluorometer (Life Technologies, Grand Island, NY). 16S rRNA gene amplicons were generated using a Fluidigm Access Array (Fluidigm Corporation, South San Francisco, CA) in combination with a Roche High Fidelity Fast Start Kit (Roche, Indianapolis, IN). The primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) that target a 252 bp-fragment of the V4 region of the 16S rRNA gene were used for amplification (primers synthesized by IDT Corp., Coralville, IA) (Caporaso et al., 2012). CS1 forward tag and CS2 reverse tag were added according to the Fluidigm protocol. Quality of the amplicons was assessed using a Fragment Analyzer (Advanced Analytics, Ames, IA) to confirm amplicon regions and sizes. A DNA pool was generated by combining equimolar amounts of the amplicons from each sample. The pooled samples were then size selected on a 2% agarose E-gel (Life technologies, Grand Island, NY) and extracted using a Qiagen gel purification kit (Qiagen, Valencia, CA). Cleaned size-selected pooled products were run on an Agilent Bioanalyzer to confirm appropriate profile and average size. Illumina sequencing was performed on a MiSeq using v3 reagents (Illumina Inc., San Diego, CA) at the W. M. Keck Center for Biotechnology at the University of Illinois.

Calculations:

Apparent total tract digestibility values were calculated using the equation as follows: [nutrient intake (g/d) – fecal output (g/d)/nutrient intake (g/d)] x 100.

Bioinformatics for Assessing Fecal Microbial Communities:

Forward reads were trimmed using the FASTX-Toolkit (version 0.0.14) and QIIME 2.2020.8 (Bolyen et al., 2019) was used to process the resulting sequence data. High-quality (quality value ≥ 20) sequence data derived from the sequencing process were demultiplexed. Data were then denoised and assembled into amplicon sequence variants (ASV) using DADA2 (Callahan et al., 2016).Sequences were clustered into operational taxonomic units (OTU) using UCLUST through an open-reference OTU picking strategy against the Silva 138 reference database (Quast et al., 2013) with a 99 % similarity threshold. An even sampling depth (41,369 sequences per sample) was used for assessing alpha- and beta-diversity measures. Beta-diversity was assessed using weighted and unweighted UniFrac (Lozupone and Knight, 2005) distance measures and presented using principal coordinates analysis (PCoA) plots.

Blood sample collection and analysis:

On the final day of each experimental period, 15 mL of fasted blood samples were collected via jugular puncture for serum chemistry, hematology, and gene expression analysis. Samples were immediately transferred to appropriate vacutainer tubes, with 2-3 mL going into #367841 BD Vacutainer Plus plastic whole blood tubes (Lavender with K_2EDTA additive), 2-3 mL going into #367974 BD Vacutainer Plus plastic serum tubes (red/grey with clot activator and gel for serum separation; BD, Franklin Lakes, NJ), and 7.5 mL of blood going into PAXgene Blood Tubes (#762165; Qiagen, Valencia, CA). The blood tube for serum isolation was centrifuged at 1,300 × g at 4^{o}C for 10 min (Beckman CS-6R centrifuge; Beckman Coulter Inc., Brea, CA). Serum was collected and transported to the University of Illinois Veterinary Medicine Diagnostics Laboratory for serum chemistry analysis. K_2EDTA tubes were cooled (but not frozen) and transported to the University of Illinois Veterinary Medicine Diagnostics Laboratory for hematology analyses.

Total RNA from blood cells was isolated using a PAXgene Blood RNA Kit (#762331; Qiagen, Valencia, CA, USA). RNA concentrations were determined using an ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). cDNA was synthesized using SuperScript IV reverse transcriptase (Invitrogen, Carlsbad, CA,). Gene expression was measured by real-time two-step RT-PCR using an Applied Biosystems 7900HT real-time PCR system (Applied Biosystems, Waltham, MA) and was carried out with SYBR Green chemistry (Bio-Rad Laboratories, Hercules, CA) in a QuantStudio 7 instrument (Thermo Fisher Scientific, Waltham, MA) using validated forward and reverse primers (Bio-Rad Laboratories, Hercules, CA). The genes of interest included mammalian target of rapamycin (*mTOR*, UniqueAssayID: qCfaCID0024417), insulin-like growth factor-1 (*IGF-1*, UniqueAssayID: qCfaCID0035607), matrix metallopeptidase-3 (*MMP-3*, UniqueAssayID: qCfaCED0026432), sterol regulatory element-binding transcription factor-1 (*SREBP-1*, UniqueAssayID: qCfaCED0038260), ribosomal protein S6 kinase A5 (*RPS6KA5*, UniqueAssayID: qCfaCID0024274), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC-1α*, UniqueAssayID: qCfaCED0028716), heat shock protein (HSP)-A1 (*HSP-A1*, UniqueAssayID: qCfaCED0035841), and heat shock protein-90AA1 (*HSP-90AA1*, UniqueAssayID: qCfaCED0026027). All gene expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method, represented as gene expression relative to the housekeeping gene (*RPS5*, UniqueAssayID: qCfaCED0028510).

Statistical analysis:

All data were analyzed using the Mixed Models procedure of SAS (version 9.4; SAS Institute, Cary, NC). Data normality was confirmed using the univariate procedure and Shapiro-Wilk statistic, with log transformation being used when normal distribution was lacking. If after the logarithmic transformation of the data, the data did not reach normality, the data were analyzed using the npar1way procedure and Wilcoxon statistic. $p<0.05$ was considered significant.

Results

Food intake (as-is and DM basis) was different ($p<0.001$) among diets (Table 3.3). Dogs fed the plant-based diets, WGM and CGM, had lower ($p<0.001$) food intake than those fed DC or CBPM. Fecal output (g/d, as-is and DM) was higher ($p<0.0001$) in dogs fed CBPM than those fed the other 3 diets. Fecal output (g/d, as-is) was also higher ($p<0.0001$) in dogs fed the DC or CGM diets than those fed the WGM diet. Apparent DM digestibility was lower ($p<0.0001$) in dogs fed CBPM or CGM than those fed DC or WGM. Dogs fed CBPM had lower ($p<0.0001$) apparent OM and energy digestibilities than those fed the other 3 diets. Apparent CP digestibility was lower ($p<0.0001$) in dogs fed CBPM than those fed the other 3 diets. Apparent CP digestibility was also lower ($p<0.0001$) in dogs fed DC or CGM than those fed WGM. AHF digestibility did not differ among treatments.

Fecal scores were higher ($p<0.01$; looser stools) in dogs fed DC or CBPM than those fed WGM or CGM (Table 3.4), but all remained within an appropriate range. Fecal pH was not different ($p = 0.07$) among diets, but fecal DM was higher ($p<0.0001$) in dogs fed WGM than those fed the other 3 diets. Fecal indole concentrations were higher ($p<0.05$) in dogs fed CBPM than those fed WGM, but phenol and total phenol and indole concentrations were not different. Fecal total SCFA concentrations were higher ($p<0.05$) in dogs fed DC than those fed CGM, but individual SCFA (i.e., acetate; propionate; butyrate) were not different. Fecal total BCFA concentrations were higher ($p=0.0002$) in dogs fed DC or CBPM than those fed WGM. Fecal isobutyrate concentrations were higher ($p<0.0001$) in dogs fed CBPM than those fed WGM or CGM. Fecal isobutyrate concentrations were also higher ($p<0.0001$) in dogs fed DC than those fed WGM. Fecal isovalerate concentrations were lower ($p=0.0004$) in dogs fed WGM than those fed the other 3 diets. Fecal concentrations were higher ($p<0.0001$) in dogs fed DC or CBPM than those fed WGM or CGM.

Fecal microbiota were shifted among dietary treatments. Alpha-diversity of fecal microbial communities (Figure 3.1), measured by observed OTU, was higher ($p<0.05$) in dogs fed CBPM than those fed DC or WGM. Alpha-diversity analysis assessed by Faith's PD was higher ($p<0.05$) in dogs fed CBPM than those fed WGM. Alpha-diversity analysis assessed by the Shannon diversity index was higher ($p<0.05$) in dogs fed CBPM than those fed DC. Although beta-diversity represented by PCoA plots of unweighted (Figure 3.2A) UniFrac distances were not different among diets, the PCoA plots of weighted (Figure 3.2B) UniFrac distances revealed that fecal microbial populations of dogs fed WG or CGM tended to shift away from those fed DC or CBPM. In terms of specific fecal microbiota taxa, the prominent phyla included Firmicutes, Fusobacteria, and Proteobacteria (Table 3.5). Dogs fed CGM had a higher ($p=0.003$) relative abundance of fecal Firmicutes and lower ($p<0.0001$) relative abundance of fecal Fusobacteria than dogs fed DC or CBPM. Dogs fed CBPM had higher ($p<0.001$) relative abundance of fecal Proteobacteria than those fed WGM and CGM. Relative abundances of fecal unclassified Lachnospiraceae and *Blautia* were higher ($p<0.0001$) in dogs fed WGM than those fed the other 3 diets. The relative abundance of fecal uncultured Lachnospiraceae was higher ($p=0.007$) in dogs fed DC or CGM than those fed CBPM. The relative abundances of fecal *Faecalibacterium* and *Peptoclostridium* were higher ($p<0.05$) in dogs fed CBPM than those fed WGM or CGM. The relative abundance of fecal *Romboutsia* was higher ($p=0.002$) in dogs fed CGM than those fed DC or CBPM. The relative abundance of fecal *Megamonas* was higher ($p=0.022$) in dogs fed WGM than those fed CBPM. The relative abundance of fecal *Fusobacterium* was higher ($p<0.0001$) in dogs fed DC or CBPM than those fed CGM. Lastly, the relative abundance of fecal *Parasutterella* was higher ($p<0.0001$) in dogs fed DC or CBPM than those fed WGM or CGM.

All serum chemistry markers were within the reference ranges except for creatinine concentrations, which were slightly lower in dogs fed WGM (Table 3.6). Serum creatinine

concentrations were lower ($p=0.0004$) in dogs fed WGM than those fed DC or CBPM. Serum creatinine concentrations were also lower ($p=0.0004$) in dogs fed CGM than those fed DC. Serum BUN:creatinine ratio was higher ($p<0.05$) in dogs fed WGM than those fed DC. Serum chloride concentrations were higher ($p<0.05$) in dogs fed WGM than those fed CBPM. Serum bilirubin concentrations were higher ($p<0.05$) in dogs fed CBPM than those fed DC. Serum creatine phosphokinase concentrations were higher ($p<0.05$) in dogs fed DC than those fed CGM. Hematology values were within the reference ranges for dogs in all treatments (Table 3.7). However, blood mean corpuscular hemoglobin concentrations were higher ($p<0.05$) in dogs fed DC than those fed WGM. Also, blood mean platelet volume was lower ($p<0.01$) in dogs fed CBPM than those fed the other 3 diets. Blood gene expression for all measured genes was not affected by diet (Table 3.8).

Discussion

The pet food industry is continually searching for and testing a variety of protein sources not only to meet the nutritional needs of pets, but also to align with pet owner preferences and beliefs. There is heightened consumer awareness of nutrition and health, and demand for sustainable and natural feeding approaches. In 2020, 41% of dog owners bought “premium” dog foods (Phillips-Donaldson, 2022). “Premium” has no regulatory definition, but in that survey, that term was used and compared to “basic/generic” food that typically contain by-product ingredients, or artificial colors and preservatives. Animal-based protein sources are typically the leading ingredients on premium pet food labels. Some consumers, however, are more selective than that, refusing to feed ingredients containing “by-product” in their title because they would not choose something that seems unfit for human consumption. Other pet owners are concerned about the sustainability of their pet food choices and may select plant-based options. While it is important

that the pet food industry provide options to meet diverse customer demands, it is essential that diets are complete and balanced. Manufacturers must conduct sufficient documented research studies to validate that diets are safe before reaching store shelves.

In the current study, two animal-based diets and two plant-based diets were evaluated for their nutritional value and how they impacted animal response. All four experimental diets were formulated to contain similar ingredient and nutrient composition, except for the primary source of protein. The final diets differed slightly in regard to nutrient composition, but were fairly similar. The dietary protein concentrations ranged from 39.8% - 40.7%, with all diets providing more than double the recommendation for adult maintenance by AAFCO (18%). The CBPM diet was also supplemented with taurine. Taurine is not an indispensable AA, but has been of concern to the pet food industry lately so it is commonly added (FDA. 2019). Taurine is a sulfur-containing beta-sulfonic acid that is present in highest concentrations in cardiac and skeletal muscle tissues of animals, and is lacking in plants (McCuster, 2014). This addition may have not been necessary due to the high taurine concentration measured in the final diet. In the DC diet, no additional AA were needed to create a complete and balanced formula. In addition to taurine, both plant-based protein diets were supplemented with L-lysine to meet AAFCO minimum concentrations for that indispensable AA. Previous research published by Reilly et al. (2021) reported that CGM had low DIAAS-like values for tryptophan (47.3%), with it being the first limiting AA of that ingredient. In the current study, the CGM diet contained a low tryptophan concentration (0.13%), which was slightly below the AAFCO minimum (0.16%). In the current study, all dietary treatments were considered well-digested by dogs, above the AAFCO and FEDIAF apparent protein digestibility minimum recommendations of 80%. Comparing just the animal-based diets in the current study, CP digestibility of the DC diet (89.85%) was 7 percentage units more digestible than the CBPM diet (82.59%). The plant-based diets, WGM and CGM, had high CP digestibilities (93.82% and

90.07%, respectively). This was likely due to the prior processing needed to separate these protein fractions from the other grain components and using the highly digestible crystalline AA to complete the diets. Both plant proteins used, WGM and CGM, are processed, removing the majority of starch and fiber, and concentrating protein. CGM is produced during the wet-milling of corn, which separates the corn kernel into starch, protein, and dietary fiber fractions (Moniruzzaman et al., 2020). Gross energy digestibility was lower in the CBPM diet, which was primarily due to the lower protein digestibility. Urrego et al. (2017) evaluated poultry meal- and wheat gluten-based diets in brachycephalic dogs. The wheat gluten and poultry meal diets in that study had an apparent CP digestibilities of 88% and 82.2%, respectively, which is similar to what was observed in the current study. Fecal scores of dogs fed the animal-based diets were higher (looser) than those fed the plant-based diets, but mean scores for all diets were considered ideal (between a score of 2-3).

Protein quality is dependent on the AA composition, digestibility, and bioavailability of the ingredient or diet in question. Apparent total tract CP digestibility is not a true representation of what the host digests because of the microbial metabolism that takes place in the hindgut, as well as endogenous protein losses that interfere with the calculations. Ileal-cannulated dogs or the cecectomized rooster assay provide accurate measures of CP and AA digestibility. Oba et al. (2018) evaluated the true nutrient digestibility and true metabolizable energy of chicken-based ingredients using a precision-based cecectomized rooster assay and reported that chicken meal had a lower DM digestibility (60.1%) compared with a low processed, steamed chicken (76.5%). This difference was thought to have been due to prior processing of the protein sources. CBPM goes through a high heat rendering process that may decrease protein quality. By-product protein quality can vary greatly by temperature at which the original material was processed, as well as the starting composition of the meat and tissues used in the rendering process. Some by-products may also

contain high amounts of connective tissues, which have constituents that analyze as fiber and are poorly digested by animals (Johnson et al., 1998).

All dogs remained healthy throughout the study and most serum chemistry and hematology values of dogs fed all diets were within references ranges for adult dogs, except for creatinine. Creatinine was slightly lower than reference range values for dogs fed the WGM diet, but no signs of clinical abnormality were observed during the study. High creatinine concentrations are a sign of kidney disease, but in combination with other biomarkers. The low creatinine concentrations in dogs fed the WGM diet lead to higher BUN:creatinine ratios compared with dogs fed the other diets. Blood Cl, bilirubin, and creatine phosphokinase differed among treatments, but still remained in healthy references ranges for dogs.

Final utilization of nutrients in the body is moderated by microbiota in the colon, where fermentation occurs. The gastrointestinal microbiome is a complex ecosystem that impacts host health. The production of fecal metabolites by microbiota can be influenced by substances entering the colon. Fecal SCFA are produced primarily from carbohydrate fermentation and are an important source of energy for colonocytes (Morrison and Preston, 2016). Higher SCFA concentrations are generally considered beneficial to the host. Fecal total SCFA concentrations were lowest in dogs fed the CGM diet, which could have been due to the lack of a dedicated fiber source (i.e., *Miscanthus* grass) that was present in the other 3 diets, and the lower overall TDF content of that diet. Fecal putrefactive compounds, namely ammonia and BCFA, are indicators of increased protein fermentation. Proteolytic fermentation takes place mainly in the distal large intestine, where ammonia is produced from the deamination of AA and hydrolysis of urea, whereas phenols are produced due to decarboxylation of AA (Jha et al., 2019). Ammonia can potentially have a negative impact on intestinal health and can contribute to fecal odor (Lee et al., 2022). Both animal-based diets in the current study resulted in higher fecal ammonia concentrations. Dogs fed

the CBPM diet had higher fecal indole concentrations compared with those fed WGM. Dogs fed the WGM diet had lower total BCFA concentrations. Due to the lower CP digestibility in the animal-based diets, more protein would have likely reached the colon, increasing proteolytic fermentation by gut microbiota. Previous research reported similar results, with high-protein poultry meal diets resulting in greater fecal concentrations of ammonia, BCFA, and indole compared with a high-protein wheat gluten diet fed to dogs (Nery et al., 2012).

Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, and Actinobacteria are the predominant microbial phyla in the gut of dogs (Wernimont et al., 2020; Deng and Swanson, 2015; Hoffmann et al., 2016). Changes to the fecal microbiome have been shown to occur quickly in response to dietary interventions. In the current study, three bacterial phyla, Firmicutes, Fusobacteriota, and Proteobacteria, shifted due to diet. Vegetable fiber content has been reported to increase the overall abundance of Firmicutes and decrease the abundance of Fusobacteria and Proteobacteria (Pilla and Suchodolski, 2020; Middelbos et al, 2010), however, we observed a similar shift in dogs fed the CGM diet that had the least amount of dietary fiber.

It is reported that *Megamonas* produces enzymes that result in ammonia production (Polanskey et al., 2016). However, *Megamonas* was found in highest abundance in dogs fed WGM, but had the lowest levels of fecal ammonia. *Megamonas* is also a key SCFA producing bacteria however, the current study SCFA levels did not change in the same direction as change in *Megamonas* relative abundance. Lee et al. (2022) also found that increases of relative abundance of *Megamonas* did not increase SCFA levels in healthy dogs, so it is possible unknown factors may be involved. Previous research evaluation of a raw diet, which was high in animal protein, reported high abundances of Proteobacteria and Fusobacteria (Sandri et al., 2018). Proteobacteria have been reported to be more abundant in dogs fed high-protein diets and be more variable among dogs than cats (Moon et al., 2018). Dogs fed the CBPM diet had a higher abundance of

Proteobacteria, which could be due to a greater amount of protein that entered the colon. *Parasutterella* has been shown to play a role in bile acid maintenance and cholesterol metabolism in mice (Ju et al., 2018). CGM fed dogs had the lowest abundance of Fusobacteria. *Fusobacterium* are associated with IBD and colorectal cancer in humans, but have not been associated with those conditions in dogs. In fact, it is usually the opposite, with healthy dogs and animals fed high-protein diets having high relative abundances of *Fusobacterium* (Felix et al., 2022). Unexpectedly, bacterial diversity was lower in dogs fed the DC diet than those fed the CBPM diet. Although reduced bacterial diversity is often associated with gastrointestinal disease (Xenoulis et al., 2008; Guard et al., 2015), stool quality was ideal, and no signs of disease were observed during this study. What is deemed "normal" for dogs gut microbiota is highly reliant on the diet being consumed at the time collection and demonstrates great flexibility (Do et al., 2021). Thus, shifts in microbial communities should be attributed to differences in protein sources, and not as an indication of gut dysbiosis because healthy dogs were used in this study, and these animals remained healthy and without any signs of gastrointestinal intolerance or discomfort in response to experimental diets.

In conclusion, all diets tested in this study were well tolerated and dogs remained healthy when fed both the animal- and plant-based diets. The deboned chicken diet was the only diet that did not have AA supplementation and was highly digestible, making it a high-quality protein diet. L-lysine supplementation was necessary to make sure that the plant-based diets were complete and balanced, but they had high nutrient digestibility and resulted in lower fecal concentrations of proteolytic fermentation metabolites. Three bacterial phyla and 9 bacterial genera in fecal samples were shifted among treatments, but fecal scores were maintained by all animals throughout the study so their impact on health are unknown. Because high-protein diets were tested in this study, impacts on health were likely difficult to measure. Therefore, research on diets containing

moderate concentrations of plant-based versus animal-based protein may be further investigated to more effectively evaluate how protein quality and AA concentrations impact canine health.

Literature Cited

Association of American Feed Control Officials (AAFCO). 2020. Official Publication. Oxford, IN: AAFCO.

Association of Official Analytical Chemists (AOAC). 2006. Official methods of analysis. 17th ed. Assoc. Off. Anal. Chem., Gaithersburg, MD.

American Association of Cereal Chemists (AACC). 1983. Approved methods. 8th ed. AACC, St. Paul, MN.

Budde, E. F. 1952. The determination of fat in baked biscuit type of dog foods. J. Assoc. Off. Agric. Chem. 35:799–805. doi:10.1093/jaoac/35.3.799

Bolyen, E., J. R. Rideout, M. R. Dillon, N. A. Bokulich, C. C. Abnet, G. A. Al-Ghalith, H. Alexander, E. J. Alm, M. Arumugam, F. Asnicar, Y. Bai, J. E. Bisanz, K. Bittinger, A. Brejnrod, C. J. Brislawn, C. T. Brown, B. J. Callahan, A. M. Caraballo-Rodríguez, J. Chase, E. K. Cope, R. Da Silva, C. Diener, P. C. Dorrestein, G. M. Douglas, D. M. Durall, C. Duvallet, C. F. Edwardson, M. Ernst, M. Estaki, J. Fouquier, J. M. Gauglitz, S. M. Gibbons, D. L. Gibson, A. Gonzalez, K. Gorlick, J. Guo, B. Hillmann, S. Holmes, H. Holste, C. Huttenhower, G. A. Huttley, S. Janssen, A. K. Jarmusch, L. Jiang, B. D. Kaehler, K. B. Kang, C. R. Keefe, P. Keim, S. T. Kelley, D. Knights, I. Koester, T. Kosciolek, J. Kreps, M. G. I. Langille, J. Lee, R. Ley, Y.-X. Liu, E. Loftfield, C. Lozupone, M. Maher, C. Marotz, B. D. Martin, D. McDonald, L. J. McIver, A. V. Melnik, J. L. Metcalf, S. C. Morgan, J. T. Morton, A. Turan Naimey, J. A. Navas-Molina, L. F. Nothias, S. B. Orchanian, T. Pearson, S. L. Peoples, D. Petras, M. L. Preuss, E.

Pruesse, L. B. Rasmussen, A. Rivers, M. S. Robeson II, P. Rosenthal, N. Segata, M. Shaffer, A. Shiffer, R. Sinha, S. J. Song, J. R. Spear, A. D. Swafford, L. R. Thompson, P. J. Torres, P. Trinh, A. Tripathi, P. J. Turnbaugh, S. Ul-Hasan, J. J. J. van der Hooft, F. Vargas, Y. Vázquez-Baeza, E. Vogtmann, M. von Hippel, W. Walters, Y. Wan, M. Wang, J. Warren, K. C. Weber, C. H. D. Williamson, A. D. Willis, Z. Z. Xu, J. R. Zaneveld, Y. Zhang, Q. Zhu, R. Knight, and J. G. Caporaso. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37:852–857. doi:10.1038/s41587-019-0209-9

Callahan, B. J., P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. Johnson, and S. P. Holmes. 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13:581–583. doi:10.1038/nmeth.3869

Chaney, A. L., and E. P. Marbach. 1962. Modified reagents for determination of urea and ammonia. Clin. Chem. 8:130–132. doi:10.1093/clinchem/8.2.130

Deng, P., and K. S. Swanson. 2015. Gut microbiota of humans, dogs and cats: current knowledge and future opportunities and challenges. Br. J. Nutr. 113:S6-S17. doi:10.1017/S0007114514002943

Erwin, E. S., G. J. Marco, and E. M. Emery. 1961. Volatile fatty acid analyses of blood and rumen fluid by gas chromatography. J. Dairy Sci. 44:1768–1771. doi:10.3168/jds.S0022-0302(61)89956-6

FEDIAF. 2021. Nutritional Guidelines for Complete and Complementary Pet Food for Cats and Dogs; The European Pet Food Industry Federation: Bruxelles, Belgium,

Félix, A. P., C. M. M. Souza, and S. G. de Oliveira. 2022. Biomarkers of gastrointestinal functionality in dogs: A systematic review and meta-analysis. Anim. Feed Sci. Tech. 283:115183. doi:10.1016/j.anifeedsci.2021.115183.

Flickinger, E. A., E. M. Schreijen, A. R. Patil, H. S. Hussein, C. M. Grieshop, N. R. Merchen, and G. C. Fahey, Jr. 2003. Nutrient digestibilities, microbial populations, and protein catabolites as affected by fructan supplementation of dog diets. J. Anim. Sci. 81:2008–2018. doi:10.2527/2003.8182008x

Guard, B. C., J. W. Barr, L. Reddivari, C. Klemashevich, A. Jayaraman, J. M. Steiner, J. Vanamala, and J. S. Suchodolski. 2015. Characterization of microbial dysbiosis and metabolomic changes in dogs with acute diarrhea. PLoS ONE. 10:0127259. doi:10.1371/journal.pone.0127259

Jha, R., J. M. Fouhse, U. P. Tiwari, L. Li, and B. P. Willing. 2019. Dietary fiber and intestinal health of monogastric animals. Front. Vet. Sci. 6:48. doi:10.3389/fvets.2019.00048

Johnson M. L., C. M. Parsons, G. C. Fahey, N. R. Merchen, and C. G. Aldrich. 1998. Effects of species raw material source, ash content, and processing temperature on amino acid digestibility of animal by-product meals by cecectomized roosters and ileally cannulated dogs. J. Anim. Sci. 76:1112–1122. doi:10.2527/1998.7641112x

Ju, T., J. Y. Kong, P. Stothard, and B. P. Willing. 2019. Defining the role of *Parasutterella*, a previously uncharacterized member of the core gut microbiota. ISME J. 13:1520–1534. doi:10.1038/s41396-019-0364-5.

Laflamme, D. 1997. Development and validation of a body condition score system for cats: a clinical tool. Feline Pract. 25:13–18.

Lee, A. H., C. Y. Lin, S. Do, P. M. Oba, S. E. Belchik, A. J. Steelman, A. Schauwecker, and K. S. Swanson. 2022. Dietary supplementation with fiber, "biotics," and spray-dried plasma affects apparent total tract macronutrient digestibility and the fecal characteristics, fecal microbiota, and immune function of adult dogs. J. Anim. Sci. 100:skac048. doi:10.1093/jas/skac048

Lozupone, C., and R. Knight. 2005. UniFrac: A new phylogenetic method for comparing microbial communities. Appl. Environ. Microbiol. 71:8228–8235. doi:10.1128/AEM.71.12.8228-8235.2005

McCusker, S., P. R. Buff, Z. Yu, and A. J. Fascetti. 2014. Amino acid content of selected plant, algae and insect species: a search for alternative protein sources for use in pet foods. J. Nutr. Sci. 3:39. doi:10.1017/jns.2014.33

Middelbos, I. S., B. M. Vester 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 5:9768. doi:10.1371/journal.pone.0009768

Moniruzzaman, M., J. H. Damusaru, S. Won, S. J. Cho, K. H. Chang, and S. C. Bai. 2020. Effects of partial replacement of dietary fish meal by bioprocessed plant protein concentrates on growth performance, hematology, nutrient digestibility and digestive enzyme activities in juvenile Pacific white shrimp, *Litopenaeus vannamei*. J. Sci. Food Agric. 100:1285-1293. doi:10.1002/jsfa.10141

Moon, C. D., W. Young, P. H. Maclean, A. L. Cookson, and E. N. Bermingham. 2018. Metagenomic insights into the roles of Proteobacteria in the gastrointestinal microbiomes of healthy dogs and cats. Microbiol. Open 7:e00677. doi:10.1002/mbo3.677

Morrison, D. J., and T. Preston. 2016. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut Microbes 7:189–200. doi:10.1080/19490976.2015.1134082

Nery, J., R. Goudez, V. Biourge, C. Tournier, V. Leray, L. Martin, C. Thorin, P. Nguyen, and H. Dumon. 2012. Influence of dietary protein content and source on colonic fermentative

activity in dogs differing in body size and digestive tolerance. J. Anim. Sci. 90:2570–2580. doi:10.2527/jas.2011-4112

Oba, P. M., P. L. Utterback, C. M. Parsons, M. R. C. de Godoy, and K. S. Swanson. 2019. Chemical composition, true nutrient digestibility, and true metabolizable energy of chicken-based ingredients differing by processing method using the precision-fed cecectomized rooster assay. J. Anim. Sci. 97:998–1009. doi:10.1093/jas/sky461

Phillips-Donaldson, D. 2022. Pet food purchasing habits and trends of U.S. pet owners. Pet Food Ind. Retrieved from https://www.petfoodindustry.com/blogs/7-adventures-in-pet-food/post/11058-pet-food-purchasing-habits-and-trends-of-us-pet-owners.

Pilla, R., and J. S. Suchodolski. 2020. The role of the canine gut microbiome and metabolome in health and gastrointestinal disease. Front. Vet. Sci. 6:498. doi:10.3389/fvets.2019.00498

Polansky, O., Z. Sekelova, M. Faldynova, A. Sebkova, F. Sisak, and I. Rychlik. 2016. Important metabolic pathways and biological processes expressed by chicken cecal microbiota. Appl. Environ. Microbiol. 82:1569-1576. doi:10.1128/AEM.03473-15

Quast, C., E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, and F. O. Glöckner. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41:D590–D596. doi:10.1093/nar/gks1219

Reilly, L. M., P. C. von Schaumburg, J. M. Hoke, G. M. Davenport, P. L. Utterback, C. M. Parsons, and M. R. C. de Godoy. 2021. Use of the precision-fed cecectomized rooster assay to determine standardized amino acid digestibility, true metabolizable energy content, and digestible indispensable amino acid scores of plant-based protein by-products used in canine and feline diets. Transl. Anim. Sci. 5:2. doi:10.1093/tas/txab025

Rodrigues Hoffmann, A., L. M. Proctor, M. G. Surette, and J. S. Suchodolski. 2016. The microbiome: The trillions of microorganisms that maintain health and cause disease in humans and companion animals. Vet. Path. 53:10–21. doi:10.1177/0300985815595517

Sandri, M., S. Dal Monego, G. Conte, S. Sgorlon, and B. Stefanon. 2017. Raw meat based diet influences faecal microbiome and end products of fermentation in healthy dogs. BMC Vet. Res. 13:65. doi:10.1186/s12917-017-0981-z

U.S. Food and Drug Administration. 2019. FDA investigation into potential link between certain diets and canine dilated cardiomyopathy. Retrieved from https://www.fda.gov/animal-veterinary/outbreaks-and-advisories/fda-investigation-potential-link-between-certain-diets-and-canine-dilated-cardiomyopathy.

Urrego, M., L. Matheus, K. De Melo Santos, M. Ernandes, M. Monti, D. De Souza, and M. Brunetto. 2017. Effects of different protein sources on fermentation metabolites and nutrient digestibility of brachycephalic dogs. J. Nutr. 6:43. doi:10.1017/jns.2017.46

Xenoulis, P. G., B. Palculict, K. Allenspach, J. M. Steiner, A. M. Van house, and J. S. Suchodolski. 2008. Molecular-phylogenetic characterization of microbial communities imbalances in the small intestine of dogs with inflammatory bowel disease. FEMS Microbiol. Ecol. 66:579-589. doi:10.1111/j.1574-6941.2008.00556.x

Tables and Figures

Table 3.1 Ingredient and analyzed chemical composition of experimental diets differing in protein source[1]

	DC	CBPM	WGM	CGM
Ingredient		---------- % , as is --------		
Deboned chicken	41.00	0.00	0.00	0.00
Dried chicken	15.70	0.00	0.00	0.00
Spray dried chicken	6.50	0.00	0.00	0.00
Chicken byproduct meal	0.00	45.50	0.00	0.00
Wheat gluten protein (~70%)	0.00	0.00	46.29	0.00
Corn gluten meal (~63%)	0.00	0.00	0.00	51.80
Taurine	0.00	0.10	0.10	0.10
L-Lysine	0.00	0.00	0.05	0.01
Potato powder	25.63	37.63	24.17	21.37
Chicken fat	4.30	11.00	17.50	17.00
Liquid chicken palatant	2.50	2.50	2.50	2.50
Dry chicken palatant (dogs)	1.00	1.00	1.00	1.00
Monodicalcium phosphate 21%	1.20	0.00	3.20	3.00
CaCO3	0.00	0.00	2.00	1.50
Miscanthus grass	1.50	1.60	2.00	0.00
Salt	0.35	0.35	0.35	0.35
Potassium chloride	0.00	0.00	0.30	0.32
Choline chloride 60%	0.10	0.10	0.25	0.25
Trace mineral premix	0.07	0.07	0.13	0.12
Vitamin premix	0.10	0.10	0.11	0.11
Natural antioxidant, dry	0.05	0.05	0.05	0.05
Chemical composition				
Dry matter, %	88.4	90.3	92.8	89.6
		-------- % dry matter --------		
Organic matter, %	92.82	91.81	91.84	93.62
Ash, %	7.18	8.19	8.16	6.38
Acid-hydrolyzed fat, %	23.0	18.0	20.2	22.7
Crude protein, %	41.7	40.1	39.8	41.0
Total starch, %	27.37	27.54	24.24	23.64
Gelatinized starch, %	26.07	25.77	22.86	22.4
Cook, %	95.3	93.6	94.3	94.8
Total dietary fiber, %	7.16	7.62	8.10	6.98
Insoluble fiber, %	6.15	6.09	7.00	6.13
Soluble fiber, %	1.01	1.52	1.09	0.85
Nitrogen-free extract, %[2]	20.96	26.09	23.74	22.94
Gross energy, kcal/g	5.66	5.38	5.45	5.82
Metabolizable energy kcal/g[3]	4.15	3.85	3.94	4.17

[1]DC, deboned chicken diet; CBPM, chicken byproduct meal diet; WGM, wheat gluten meal diet; CGM, corn gluten meal diet.

Table 3.1 (cont.)
[2]Nitrogen-free extract, % = 100 – (acid-hydrolyzed fat {%} + crude protein {%} + ash {%} + TDF {%}).
[3]Metabolizable energy = 3.5 kcal/g x crude protein (%) + 8.5 kcal/g x acid-hydrolyzed fat (%) + 3.5 kcal/g nitrogen-free extract (%).

Table 3.2 Indispensable and dispensable amino acid (AA) concentrations (% DM) of experimental diets differing in protein source[1]

	DC	CBPM	WGM	CGM	AAFCO[2]
Indispensable					
Arginine	2.36	2.29	1.37	1.30	0.51
Histidine	1.02	0.81	0.80	0.80	0.19
Isoleucine	1.74	1.51	1.50	1.70	0.38
Leucine	2.82	2.56	2.71	5.52	0.68
Lysine	2.74	2.13	0.86	0.84	0.63
Methionine	0.84	0.68	0.60	0.82	0.33
Phenylalanine	1.58	1.52	1.96	2.47	0.45
Threonine	1.58	1.38	1.04	1.33	0.48
Tryptophan	0.35	0.24	0.36	**0.13**	0.16
Valine	1.98	1.86	1.66	1.93	0.49
Dispensable					
Alanine	2.26	2.23	1.17	3.07	---
Aspartic Acid	3.62	3.20	1.71	2.64	---
Cysteine	0.40	0.42	0.76	0.64	---
Glutamic Acid	5.75	5.85	12.0	8.20	---
Glycine	2.36	2.95	1.47	1.24	---
Hydroxylysine	0.21	0.24	0.11	0.14	---
Hydroxyproline	0.62	0.94	0.03	0.04	---
Lanthionine	0.01	0.01	0.00	0.00	---
Ornithine	0.06	0.07	0.02	0.04	---
Proline	1.96	2.47	4.20	3.46	---
Serine	1.31	1.33	1.56	1.73	---
Taurine	0.21	0.35	0.19	0.18	---
Tyrosine	1.07	0.95	1.07	1.51	---

[1]DC, deboned chicken diet; CBPM, chicken byproduct meal diet; WGM, wheat gluten meal diet; CGM, corn gluten meal diet.
[2]AAFCO nutrient profiles for adult dogs at maintenance.

Table 3.3 Food intake and fecal output of healthy adult dogs and apparent total tract macronutrient digestibility of experimental diets differing in protein source[1]

Item	DC	CBPM	WGM	CGM	SEM	p-value
Food Intake						
g food/d (as-is)	161.17[a]	157.58[a]	150.33[b]	145.67[b]	3.9891	**<0.0001**
g food/d (DM basis)	142.52[a]	142.25[a]	134.70[b]	135.24[b]	3.6043	**0.0009**
Fecal Output						
g feces/d (as-is)	59.81[b]	75.41[a]	49.54[c]	61.75[b]	3.6103	**<0.0001**
g feces/d (DM basis)	20.30[b]	26.24[a]	19.65[b]	20.83[b]	1.0268	**<0.0001**
Digestibility[2]						
Dry matter	85.71[a]	81.64[b]	85.44[a]	84.57[b]	0.5609	**<0.0001**
Organic matter	89.7[a]	85.75[b]	89.96[a]	88.33[a]	0.4538	**<0.0001**
Crude protein	89.85[b]	82.59[c]	93.82[a]	90.07[b]	0.5534	**<0.0001**
Acid -hydrolyzed fat	96.61	96.06	96.01	95.85	0.4029	0.3824
Energy	90.6[a]	86.76[b]	91.01[a]	89.49[a]	0.4071	**<0.0001**

[1]DC, deboned chicken diet; CBPM, chicken byproduct meal diet; WGM, wheat gluten meal diet; CGM, corn gluten meal diet.

[2]Digestibility data measured from total feces collected over five consecutive days.

[abc]Mean values within a row with unlike superscript letters differ ($p<0.05$). Bolded numbers are significant ($p<0.05$).

Table 3.4 Fecal characteristics and metabolites of healthy adult dogs consuming experimental diets differing in protein source[1]

Items	**DC**	**CBPM**	**WGM**	**CGM**	**SEM**	**p-value**
Fecal scores[2]	2.75[a]	2.75[a]	2.25[b]	2.29[b]	0.1229	**0.0010**
pH	6.71	6.93	6.75	6.32	0.1615	0.0695
Dry matter (%)	31.1[b]	32.3[b]	35.5[a]	32.1[b]	0.7036	**<0.0001**
	---------- µmol/g (DMB) ----------					
Total phenol and indole	3.52	3.82	2.93	2.82	0.3603	0.0560
Phenol	0.46	0.28	0.39	0.02	0.1384	0.2182
Indole	3.06[ab]	3.54[a]	2.54[b]	2.80[ab]	0.3300	**0.0232**
Total SCFA[3]	422.0[a]	387.5[ab]	355.3[ab]	346.1[b]	24.957	**0.0309**
Acetate	238.4	228.1	194.0	195.5	14.850	0.1754
Propionate	119.5	101.5	108	94.7	7.8556	0.0566
Butyrate	64.1	57.8	53.3	55.9	4.9497	0.1966
Total BCFA[3]	24.0[a]	28.0[a]	17.7[b]	22.8[ab]	1.9649	**0.0002**
Isobutyrate	9.04[ab]	10.69[a]	7.08[c]	7.31[bc]	0.6817	**<0.0001**
Isovalerate	13.9[a]	16.2[a]	9.55[b]	14.3[a]	1.2461	**0.0004**
Valerate	1.13	1.18	1.08	1.25	0.1846	0.8608
Ammonia	207.0[a]	227.2[a]	123.6[b]	162.0[b]	12.562	**<0.0001**

[1]DC, deboned chicken diet; CBPM, chicken byproduct meal diet; WGM, wheat gluten meal diet; CGM, corn gluten meal diet.

[2]Fecal scores: 1 = hard, dry pellets; small hard mass; 2 = hard formed, dry stool; remains firm and soft; 3 = soft, formed and moist stool, retains shape; 4 = soft, unformed stool; assumes shape of container; 5 = watery, liquid that can be poured.

[3]Total short-chain fatty acids (SCFA) = acetate + propionate + butyrate; total branched-chain fatty acids (BCFA) = valerate + isovalerate + isobutyrate; total phenol and indole = phenol + indole.

[abc]Mean values within a row with unlike superscript letters differ ($p<0.05$). Bolded numbers are significant ($p<0.05$).

Table 3.5 Fecal bacterial phyla and genera (relative abundance, %) of healthy adult dogs consuming experimental diets differing in protein source[1]

Phyla	Genus	DC	CBPM	WGM	CGM	SEM	p-value
Firmicutes		49.4^{b}	50.4^{b}	56.4^{ab}	65.2^{a}	3.46	**0.003**
	Unclassified Lachnospiraceae	1.62^{b}	1.78^{b}	3.06^{a}	1.60^{b}	0.36	**<0.0001**
	Blautia	3.39^{b}	2.93^{b}	7.28^{a}	3.94^{b}	0.75	**<0.0001**
	Uncultured Lachnospiraceae	1.73^{a}	1.04^{b}	1.65^{ab}	1.87^{a}	0.34	**0.007**
	Faecalibacterium	6.21^{ab}	7.85^{a}	3.47^{b}	3.54^{b}	1.44	**0.017**
	Peptoclostridium	9.18^{ab}	10.3^{a}	6.25^{b}	6.65^{b}	1.15	**0.003**
	Romboutsia	1.14^{b}	1.03^{b}	1.83^{ab}	2.61^{a}	0.41	**0.002**
	Megamonas	3.37^{ab}	1.15^{b}	3.73^{a}	2.68^{ab}	1.07	**0.022**
	Allobaculum	3.99	12.8	5.57	4.04	2.9195	0.1467
	Uncultured Erysipelotrichaceae	1.01	9.09	3.14	7.06	2.3913	0.0658
	Lactobacillus	5.02	4.64	4.35	5.24	3.0135	0.1300
Fusobacteriota		27.5^{a}	23.1^{a}	21.2^{ab}	13.6^{b}	2.78	**<0.0001**
	Fusobacterium	27.5^{a}	23.2^{a}	21.2^{ab}	13.6^{b}	2.78	**<0.0001**
Proteobacteria		5.53^{ab}	6.83^{a}	3.33^{b}	4.12^{b}	0.63	**0.0010**
	Parasutterella	2.85^{a}	2.62^{a}	1.25^{b}	1.66^{b}	0.75	**<0.0001**
Actinobacteriota		1.94	2.15	3.02	1.78	0.491	0.1236
Bacteroidota		15.6	17.43	14.0	17.2	1.909	0.4245
	Bacteroides	9.43	10.19	7.50	13.0	1.5583	0.0995
	Alloprevotella	3.01	0.94	1.75	2.23	0.5038	0.2900

[1]DC, deboned chicken diet; CBPM, chicken byproduct meal diet; WGM, wheat gluten meal diet; CGM, corn gluten meal diet.
[abc]Mean values within a row with unlike superscript letters differ ($p<0.05$). Bolded numbers are significant ($p<0.05$).

Table 3.6 Serum chemistry of healthy adult dogs consuming experimental diets differing in protein source[1]

Item	Reference Range	DC	CBPM	WGM	CGM	SEM	p-value
Creatinine, mg/dL	0.5–1.5	0.63[a]	0.57[ab]	0.46[c]	0.51[bc]	0.055	**0.0004**
Blood urea nitrogen (BUN), mg/dL	6–30	15.3	16.0	15.6	15.8	1.295	0.9014
BUN:creatinine ratio		25.6[b]	30.0[ab]	35.1[a]	33.2[ab]	2.848	**0.0145**
Total protein, g/dL	5.1–7.0	5.99	6.05	5.96	5.96	0.098	0.5856
Albumin, g/dL	2.5–3.8	3.29	3.28	3.28	3.27	0.067	0.9412
Globulin, g/dL	2.7–4.4	2.70	2.78	2.68	2.69	0.075	0.3485
Albumin:globulin ratio	0.6–1.1	1.23	1.19	1.24	1.23	0.047	0.4051
Ca, mg/dL	7.6–11.4	9.88	10.07	9.90	9.83	0.105	0.1066
P, mg/dL	2.7–5.2	3.13	3.11	2.81	3.08	0.193	0.4023
Na, mmol/L	141–152	144.8	144.9	145.4	145.1	0.656	0.9847
K, mmol/L	3.9–5.5	3.95	3.92	4.00	3.99	0.068	0.6909
Na:K ratio	28–36	36.7	37.2	36.5	36.5	0.634	0.7889
Cl, mmol/L	107–118	110.5[ab]	109.4[b]	111.4[a]	110.8[ab]	0.729	**0.0282**
Glucose, mg/dL	68–126	91.1	87.2	88.0	90.1	2.093	0.3926
Alkaline phosphatase, U/L	7–92	36.8	33.8	49.8	38.2	11.42	0.6267
Corticosteroid-induced ALP, U/L	0–40	8.75	7.50	19.8	11.6	9.677	0.4102
Alanine transaminase, U/L	8–65	24.7	22.6	20.7	22.5	1.795	0.1100
Gamma glutamyltransferase, U/L	0–7	2.83	3.08	3.25	3.17	0.256	0.4539
Total bilirubin, mg/dL	0.1–0.3	0.15[b]	0.21[a]	0.17[ab]	0.16[ab]	0.020	**0.0251**
Creatine phosphokinase, U/L	26–310	113.0[a]	102.7[ab]	91.0[ab]	86.1[b]	9.427	**0.0454**
Cholesterol, mg/dL	129–297	243.8	236.4	250.3	233.1	19.44	0.8940
Triglycerides, mg/dL	32–154	81.5	62.3	108.8	58.8	26.56	0.2346
Bicarbonate, mmol/L	16–24	37.4	22.3	37.1	21.8	11.89	0.2945
Anion gap	8–25	17.4	17.1	17.6	16.5	0.460	0.2718

[1]DC, deboned chicken diet; CBPM, chicken byproduct meal diet; WGM, wheat gluten meal diet; CGM, corn gluten meal diet.

Table 3.6 (cont.)
[abc]Mean values within a row with unlike superscript letters differ ($p<0.05$). Bolded numbers are significant ($p<0.05$).

Table 3.7 Hematology of healthy adult dogs consuming experimental diets differing in protein source[1]

Item	Reference Range	**DC**	**CBPM**	**WGM**	**CGM**	**SEM**	**p-value**
Red blood cells (10^6/μl)	5.50-8.50	7.16	7.08	7.00	7.14	0.164	0.7316
Reticulocytes(%)		0.35	0.34	0.43	0.36	0.046	0.0625
Reticulocytes(μl)		25071	24864	30944	26467	3866	0.1424
Hemoglobin (g/dL)	12.0-18.0	16.0	15.7	15.5	15.8	0.303	0.5272
Hematocrit (%)	35.0-52.0	47.7	47.0	46.9	47.4	0.846	0.7801
Mean cell volume (fl)	58.76.0	66.6	66.5	67.0	66.5	0.486	0.1633
MCH[2] (pg)	20.0-25.0	22.3	22.2	22.2	22.2	0.221	0.3468
MCHC[2] (g/dL)	33.0-38.6	33.5^{a}	33.4^{ab}	33.1^{b}	33.3^{ab}	0.175	**0.0302**
Mean platelet volume (fl)		10.6^{a}	10.4^{b}	10.7^{a}	10.6^{a}	0.261	**0.0013**
Platelets (10^3/μl)	200-700	282.3	299.8	309.8	277.3	17.56	0.3602
White blood cell count (10^3/μl)	6.00-17.00	5.51	5.06	5.86	5.41	0.359	0.6118
Lymphocytes (10^3/μl)		1.08	1.11	1.12	1.11	0.148	0.9996
Monocytes (10^3/μl)		0.27	0.31	0.28	0.27	0.041	0.7366
Eosinophils (10^3/μl)		0.13	0.15	0.15	0.18	0.027	0.6037
Lymphocytes (%)		19.0	23.1	18.7	20.5	2.039	0.0524
Monocytes (%)		4.69	5.20	4.73	4.91	0.428	0.7112
Eosinophils (%)		2.03	3.31	2.68	3.33	0.534	0.1496
Basophils (%)		0.13	0.25	0.10	0.26	0.054	0.2203

[1]DC, deboned chicken diet; CBPM, chicken byproduct meal diet; WGM, wheat gluten meal diet; CGM, corn gluten meal diet.

[2]MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration.

[abc]Mean values within a row with unlike superscript letters differ ($p<0.05$). Bolded numbers are significant ($p<0.05$).

Table 3.8 Blood mRNA expression of healthy adult dogs consuming experimental diets differing in protein source[1]

Gene Symbol	**DC**	**CBPM**	**WGM**	**CGM**	**SEM**	**p-value**
mTOR	1.01	1.06	1.01	1.04	0.101	0.9802
IGF-1	2.04	3.33	1.78	2.83	0.953	0.8250
MMP3	0.85	1.46	0.62	11.59	4.923	0.1265
HSPA1	1.12	1.04	1.23	1.54	0.174	0.1733
HSP90AA1	1.02	1.05	1.08	0.98	0.104	0.8022
PGC-1α	0.92	1.21	1.22	2.33	0.522	0.3371
RPS6a5	1.20	1.14	1.14	0.98	0.148	0.5347
SREBP1	1.22	1.07	1.15	2.94	0.747	0.7577

[1]DC, deboned chicken diet; CBPM, chicken byproduct meal diet; WGM, wheat gluten meal diet; CGM, corn gluten meal diet.

[2]Statistics were conducted using ΔΔCt values to generate p-values; data are reported as fold change in relation to a housekeeping gene and CBPM ($2^{-\Delta\Delta Ct}$).

[3]*mTOR*, mammalian target of rapamycin; *IGF-1*, insulin-like growth factor-1; *MMP3*, matrix metallopeptidase-3; *HSPA1*, heat shock protein (HSP)-A1; *HSP90AA1*, heat shock protein-90AA1; *PGC-1α*, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; *RPS6a5*, ribosomal protein S6 kinase; *SREBP1*, sterol regulatory element-binding transcription factor-1.

Figure 3.1 Fecal alpha diversity indices of healthy adult dogs consuming experimental diets differing in protein source (DC, deboned chicken diet; CBPM, chicken byproduct meal diet; WGM, wheat gluten meal diet; CGM, corn gluten meal diet).

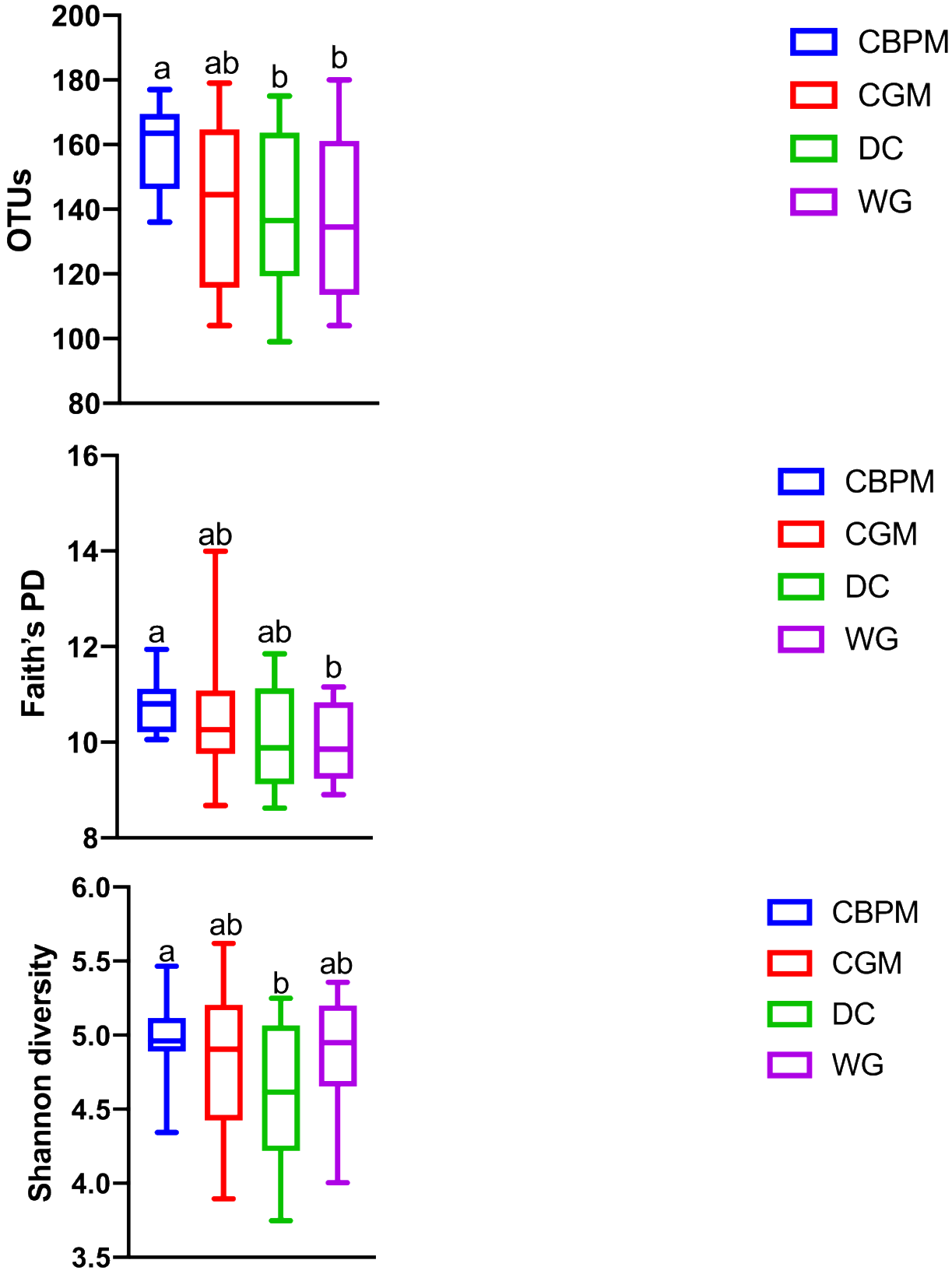

Figure 3.2 Unweighted principal coordinate analysis (PCoA) plot of healthy adult dogs consuming experimental diets differing in protein source (DC, deboned chicken diet; CBPM, chicken byproduct meal diet; WGM, wheat gluten meal diet; CGM, corn gluten meal diet).

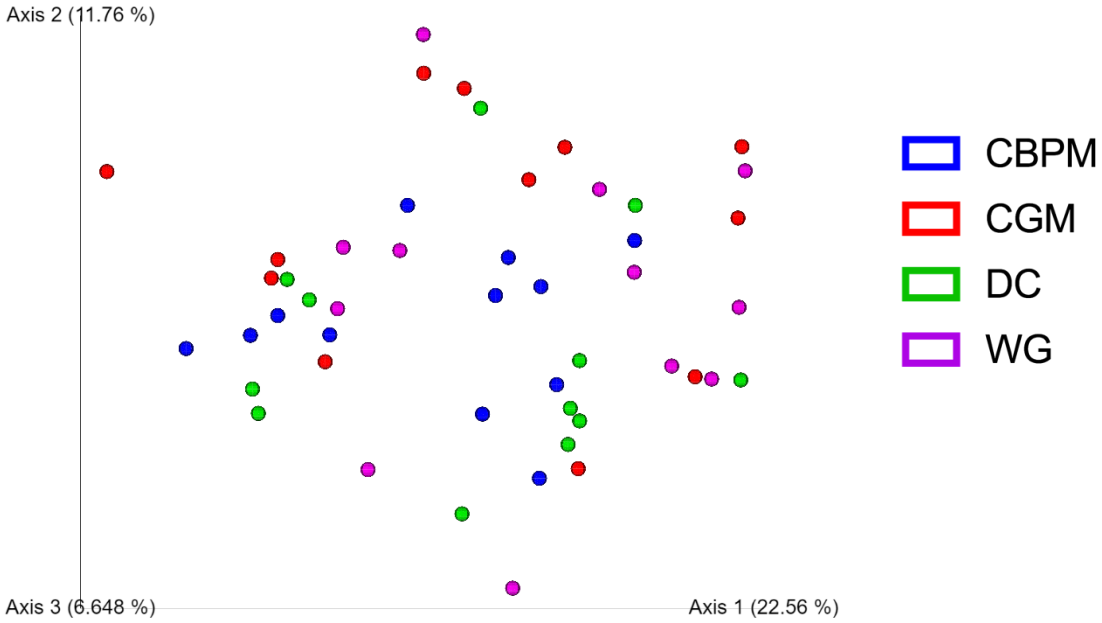

Figure 3.3 Weighted principal coordinate analysis (PCoA) plot of healthy adult dogs consuming experimental diets differing in protein source (DC, deboned chicken diet; CBPM, chicken byproduct meal diet; WGM, wheat gluten meal diet; CGM, corn gluten meal diet).