DETERMINATION OF MACRONUTRIENT COMPOSITION, AMINO ACID DIGESTIBILITY, AND OVERALL PROTEIN QUALITY OF SELECT NOVEL DIETARY PROTEINS FOR USE IN CANINE AND FELINE NUTRITION

BY

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THESIS

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ABSTRACT

Protein is an important component of companion animal diets as it is provides the foundation for normal bodily functions by supplying indispensable and dispensable amino acids (**AA**). The forms in which dietary protein is incorporated into diets is through common animal and plant-based ingredients such as beef, chicken, fish, and soybean based products. However, novel proteins have become a point of interest in the pet food market due to several factors including, the demand for more diverse products, owners prioritizing ingredient quality, and the ethos of purchasing pet foods that use sustainable ingredients. Despite the increased consumer attention towards novel protein sources, there is limited information in the literature regarding their chemical composition, AA profile, and AA digestibility. Therefore, the aim of this research was to analyze the macronutrient and AA composition of mammalian proteins (yak, wild boar, camel, and kangaroo as compared to beef), avian proteins (goose, quail, duck, and emu as compared to chicken), aquatic and reptile proteins (whitefish, carp slurry, eel, spirulina, and rattlesnake, as compared to salmon), and non-animal proteins (cricket meal, chocho, pumpkin seed powder, and hemp powder as compared to soybean meal) as well as to determine the standardized AA digestibility by using the precision-fed cecectomized rooster assay. This assay more accurately measures AA digestibility in the ileum by removing the ceca from roosters which minimizes the effect of microbial fermentation on AA. For this assay, the roosters are fasted and then tube fed 25g to 30g of substrate. The excreta is collected after 48 hours and analyzed for AA digestibility. Protein quality was further assessed by calculation of digestible indispensable amino acid (DIAAS-like) scores to compare against the nutrient profiles of the Association of American Feed Control Officials (**AAFCO**) and recommended allowances of the National Research Council (**NRC**) for adult dogs and cats at maintenance. For mammalian proteins, it was determined that each substrate was highly digestible by the roosters as no

standardized AA digestibility values were below 81%. Based on DIAAS-like scores, tryptophan and threonine were consistently the lowest scoring AA when compared with AAFCO and NRC references for both dogs and cats. Using NRC and AAFCO DIAAS-like values for adult cats, kangaroo, yak, and camel were scored as high-quality proteins as these ingredients contained no first-limiting AA as scores were all greater than 100%. For avian proteins, the standardized indispensable AA digestibility values from precision-fed cecectomized rooster assay ranged from 64.1% to 99.2% which determines the avian proteins to be moderate to highly digestible. Across all protein sources, tryptophan was the first-limiting AA, except for methionine being first-limiting in goose when compared with AAFCO nutrient profiles for dogs and cats. When compared to chicken, emu and goose were observed to be of higher quality for adult dogs using AAFCO and NRC comparisons. In contrast, chicken was the highest quality protein overall and was absent of any first-limiting AA according to DIAAS-like scores. In aquatic and reptile proteins, the standardized AA digestibility values for each ingredient were all greater than 80% and were considered to be highly digestible. Rattlesnake was consistently low quality for dogs and cats using AAFCO and NRC comparisons with tryptophan as first-limiting AA. Carp slurry and spirulina were determined to be high quality based on AAFCO and NRC comparisons; however, these proteins received moderate scores using NRC recommended allowance for adult dogs as the reference protein. In plant and insect proteins, standardized AA digestibility values were observed to be highly digestible, except for lysine in pumpkin seed powder (77.2%). Methionine was most often the first-limiting AA for dogs using NRC and AAFCO references. Cricket meal contained no limiting AA for cat AAFCO and NRC comparisons. In general, this research indicates that the novel proteins tested herein are of moderate to high quality. Data collected from these studies also provide justification for further in vivo research to determine

future use of these ingredients in more complex dietary matrices of treats and complete and balanced diets for companion animal.

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Mi orgullo es tambien de los suyos. Lo logramos.

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CHAPTER 1: INTRODUCTION

Dogs and cats have become integrated members of the family and the human-animal bond is a driving force for pet food innovation (Irvine and Cilia, 2017). A great focal point of companion animal nutrition is dietary protein and its sources. The recent demand for alternative proteins stems from the preference for diverse products, ingredient safety, and the perception that novel proteins are somehow better quality than traditional protein sources (Schleicher et al., 2019). This is observed through the industry trend of using fresh meat in the production of dry pet food in place of meat or fish meals (Montegiove et al., 2021). Owners have also initiated a shift from animal proteins towards plant-based options, and even insects such as the black soldier fly larvae, on the basis of sustainability (Dodd et al., 2019). With various pet foods and pet treats that are emerging on the market, it is necessary to provide nutritional adequate solutions for dogs and cats rather than maintaining an intense focus on ingredients alone.

Dietary proteins are required as a source of indispensable amino acids for companion animal diets; however, dogs and cats have different requirements. Not every amino acid (**AA**) is indispensable in both species as has been determined in cats where taurine is considered indispensable. The quality of proteins is highly dependent on their complete AA profile and AA digestibility. Several methods have been established to assess these parameters such as the precision-fed cecectomized rooster assay which has been validated to be an appropriate model for ileal cannulated dogs (Johnson et al., 1998). Minks have also been used as a model for dogs for determination of total tract digestibility of ingredients and diets (Tjernsbekk et al., 2014). To further evaluate the quality of a protein source, digestible indispensable amino acid (DIAAS-like) scores have been applied in companion animal nutrition (Oba et al., 2019; Do et al., 2020; Reilly et al., 2022). By the DIAAS-like method, comparisons can be made against the nutrient

profiles of the Association of American Feed Control Officials (**AAFCO**) and recommended allowances of the National Research Council (**NRC**) for dogs and cats at any specified life stage (e.g., growth, maintenance, gestation and lactation).

The objectives of this study were to determine the macronutrient compositions of select novel proteins of mammalian, avian, aquatic, reptile, insect species and plant-based ingredients, as well as to evaluate the quality of these proteins using the precision-fed cecectomized rooster assay to calculate standardized AA digestibility. Protein quality was further assessed by DIAAS-like calculations for adult dogs and cats based on AAFCO nutrient profiles and NRC recommended allowances.

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CHAPTER 2: LITERATURE REVIEW

Introduction

Pets have become deeply integrated in the lives of people. So much so that companion animals have become an extension of the owner's identify (Cheong and Yi, 2015). The humanization of pets has allowed market trends from the human world to trickle into the companion animal sector which is evident in recent innovations within the pet food industry such as the incorporation of novel proteins in canine and feline diets. Novel proteins are a topic of interest as people begin to prioritize their own health by pursuing optimal nutrition, rather than settling for adequate, while also making purchasing decisions from a sustainability standpoint (Karelakis et al., 2020). These positive consumer attitudes towards alternative proteins and the perceived high quality of these ingredients are encouraging the pet food industry to follow similar trends.

Traditional proteins in pet food include meat and poultry and their byproducts, as well as meals. Chicken, beef, soybean meal, and corn gluten meal are the most common protein sources seen in companion animal diets (Thomspon, 2008); however, due to shifting consumer attitudes away from traditional ingredients, these may not be perceived as nutritionally valuable or of high quality (Sanderson, 2021). Sustainability is also a factor that consumers have generally considered as valuable in recent years (Lopez et al., 2020) with plant, insect, and spirulina-derived proteins being regarded as a way to assuage concerns over the perceived environmental impact the animal production industry has compared to crop production (Alsaffar, 2016). Additionally, allergic reactions to common ingredients such as beef, fish, and dairy, lead to an increased demand for alternative proteins in canine and feline diets (Verlinden et al., 2007. Protein sources that are not as common may help in managing allergic reactions in companion

animals (Marchesi et al., 2017). Evidently, there are several reasons why pet owners would prefer to feed their companion animal a diet made with alternative proteins; however, there is limited scientific literature that describes chemical composition, amino acid digestibility, and overall quality of novel proteins; all of which are crucial to understanding different ingredients and properly formulating complete and balanced canine and feline diets.

Protein overview

Dietary proteins are composed of chains of 20 different amino acids (**AA**). These amino acid chains exist at various lengths and are linked by peptide bonds. Peptides are also molecules of linked amino acids that contain two or more AA residues and are shorter in length than a protein. The distinction of a protein from a peptide is the molecular weight, where the weight of 8,000 Daltons or higher designates a peptide as a protein (Hou et al., 2017). All proteins contain nitrogen, approximately 16%, as well as carbon, hydrogen, and oxygen (Case et al., 2011). Proteins contribute to several bodily functions aside from synthesizing muscle tissues. The AA in proteins are also responsible for modulating metabolic pathways, synthesizing hormones, transporting substrates, and supporting the immune system by synthesizing antibodies (Case et al., 2011).

Another function specific to pet food is the ability for AA to provide flavor to a product through Maillard reactions. These non-enzymatic browning reactions occur naturally when an amino group and a reducing sugar are heated during processing (Feiner, 2006). Lysine and cysteine are the amino acids that are most affected by this reaction and create more browning over shorter periods of time when compared to other compounds such as glycine and isoleucine (Hemmler et al., 2018). When flavor compounds are created, the flavor that is produced depends

on the specific amino acid that is reacting with the reducing sugar, for instance, the flavors and aromas that pertain to meat are derived by sulfur containing AA such as cysteine and methionine (Sasanam et al., 2022). Other factors that dictate flavor also include water, pH, and cooking temperature (van Boekel, 2006).

Dietary protein begins digestion in the gastric stomach by secretions of hydrochloric acid and proteases such as pepsin and is further degraded into AA and peptides in the small intestine where the AA, dipeptides, and tripeptides are primarily absorbed by enterocytes (Wu, 2021). Pepsin, an enzyme secreted by the stomach, functions ideally at pH of 3 and ceases activity in the lumen of small intestine where the environment is at pH 7. In the lumen of the small intestine, pancreatic enzymes trypsin, chymotrypsin, elastase, and procarboxypeptidase are activated and hydrolyze the peptide bonds between AA and primarily form peptides that are 6-8 AA long as well as some free AA residues. The resulting free AA are transported across the brush border membrane and into enterocytes by the corresponding AA transport system. The same occurs with peptides and peptide transport systems. The enterocyte cytoplasm is where peptides continue to be hydrolyzed by cytoplasmic peptidases to free amino acids and released into portal circulation (Bhutia and Ganapathy, 2018). Endogenous proteins appear from sloughed epithelial cells in the lumen of the intestine and the gut microbiota, as well as from gastric and pancreatic secretions which is of nutritional importance as endogenous proteins must be corrected in order to standardize AA digestibility coefficients (Adedokun et al., 2011).

Amino acids are classified as either indispensable (or essential) or dispensable (i.e., non-essential) where indispensable AA are required in the diet as they cannot be synthesized in sufficient amounts in animal cells. These AA for dogs and cats are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine; whereas the

dispensable AA are alanine, asparagine, aspartic acid, cysteine, glutamic acid, glycine, proline, serine, and tyrosine. Taurine is an AA that is synthesized from methionine and cysteine. It is considered to be conditionally indispensable for dogs, yet indispensable for cats. Cats have reduced activity of cysteine dioxygenase and cysteine sulfonic acid decarboxylase which are responsible for the synthesis of taurine. Due to this lowered enzyme activity, greater concentrations of cysteine are converted to pyruvate rather than taurine (Case et al., 2011). Taurine primarily regulates calcium and potassium channels in photoreceptor cells; therefore a taurine deficiency can cause feline central retinal degeneration and can lead to blindness (Hayes et al., 1975). Deficiencies can also lead to dilated cardiomyopathy (Pion et al., 1987) and unsuccessful reproduction in queens (Sturman and Messing, 1991). The NRC (2006) has established a recommended allowance for adult cats of 0.1% in extruded diets, and 0.17% in wet diets, if feeding a diet with high quality protein. For dry food, AAFCO (2020) nutrient profiles describe a minimum taurine inclusion of 0.1% in dry food and 0.2% in wet food. A taurine requirement has not been established in dogs.

Canines and felines have different requirements for dietary protein at different stages of life; therefore, the National Research Council (**NRC**) and the Association of American Feed Control Officials (**AAFCO**) have provided nutritional recommendations for diet formulations. When feeding adult dogs at maintenance, the NRC minimum requirement of crude protein (**CP**) is 8% on a DM basis for a diet with a caloric density equal or below 4,000 kcal. Adult cat maintenance diets have a minimum dietary CP requirement of 16% (NRC, 2006). According to AAFCO (2021) nutrient profile recommendations, diets for adult dogs at maintenance should have CP at a minimum of 45g/1000kcal ME (18% DMB) and cat maintenance diets are suggested to include CP at a minimum of 65 g/1000 kcal ME (26% DMB) (AAFCO, 2021). The

common sources of protein in commercial pet diets include meat by-products and meat by-product meals from animals such as fish, beef, swine, and chicken (Laflamme et al., 2014). And while diets that incorporate these ingredients have been shown to be highly digestible, 88-89% CP apparent total tract digestibility (**ATTD**) in dogs (Murray et al., 1997) and 81-93% CP ATTD in cats (Kerr et al., 2012), there persists a demand in the market for novel protein sources. The humanization of pets by their owners influences the decision of which proteins to feed as well as other factors such as nutritional quality and sensitivity to common pet food ingredients (Verlinden et al., 2007). The interest in incorporating alternative proteins in companion animal diets is also driven by the sustainability of traditional animal derived protein sources, which has encouraged consumers to turn toward insect and plant-based proteins (Tso et al., 2021).

Methods to evaluate protein quality

The quality of a protein is determined by AA content and AA digestibility. The AA profile of a protein can indicate the extent to which an ingredient can meet the AA requirements of an individual (Caire-Juvera et al., 2013) and the digestibility of AA is often quantified by measurement of ATTD of CP from animal feeding trials and feces collection (Duque-Saldarriaga et al., 2020). The greatest limitation with measuring ATTD of CP is that the calculation does not account for the fermentation that occurs in the hindgut of the animal that degrades AA (Fuller et al., 1994). To avoid microbial fermentation and inaccurate results, AA digestibility is best measured from the ileum as any AA that enter the large intestine are unlikely to be of additional benefit to the animal (Chung and Baker, 1992). This has been achieved through ileal cannulated pigs (Stein et al., 2005) and dogs (Bednar et al., 2000) and in the precision-fed cecectomized rooster assay (Parsons et al., 1982). Research by Johnson et al. (1998) has validated cecectomized roosters to replace ileal cannulated dogs as a model to study AA digestibility in

companion animals. This study compared the AA digestibility of canine diets by way of the precision-fed cecectomized rooster assay and by use of ileal cannulated dogs. Results indicated that the precision-fed cecectomized rooster assay could be used to evaluate AA digestibility due to high correlation with cannulated dog data. The same procedure has been attempted in felines; however, successful results were not obtained, and several complications occurred including skin inflammation and infection, cannula displacement, and cannula leakage (Mawby et al., 1999). Protein quality is also evaluated through two commonly used methods, the digestible indispensable amino acid score (**DIAAS**) and the protein digestibility corrected amino acid score (**PDCAAS**) which will be discussed further.

Protein digestibility corrected amino acid score

The protein digestibility corrected amino acid score (PDCAAS) is an established method that measures protein quality by assessing AA availability from fecal samples. The calculation is the ratio of the mg of the first limiting amino acid in 1g of a protein to the mg of the same amino acid in 1g of a reference protein. The true fecal nitrogen (**N**) digestibility is the difference between the intake of dietary N and the output divided by intake of dietary N (Darragh and Hodginson, 2000). There are limitations to the PDCAAS method, however. This method uses a true fecal N digestibility coefficient and although this coefficient includes a correction for fecal endogenous N, it does not account for the AA that were not absorbed in the ileum and were lost in the large intestine. Using fecal digestibility to evaluate AA availability yields inaccurate results because the unabsorbed dietary AA and endogenous AA are degraded in the hindgut by microbes. The lack of differentiation between dietary and endogenous AA leads to an overestimation of available AA because it is assumed that the AA was digested in the ileum

(Schaafsma, 2012). The scores obtained from PDCAAS are also truncated at 100% according to the idea that any excess of AA contributes no nutritional benefits (Schaafsma, 2005).

Digestible Indispensable Amino Acid Score

The digestible indispensable amino acid score (DIAAS) is a calculation that is used to evaluate the quality of a protein source. The score is the ratio of the mg of amino acid to the mg of the same amino acid in a reference protein and is typically calculated using the standardized amino acid digestibility values from ileal cannulated pigs and using the protein requirements of 2 to 5 year old children as a reference (Mathai, 2017). When analyzing proteins for pet foods the standardized AA digestibility values may be derived from ileal cannulated pigs or from excreta of cecectomized roosters. The reference protein often used is based on AAFCO nutrient profile or NRC recommended allowances. Due to the modification of the reference proteins, the calculated values are considered "DIAAS-like" as the values are not based on AA digestibility of cannulated pigs nor the protein requirements of children.

Long chain fatty acids

The use of fats in companion animal diets is an important component of a complete and balanced diet as these compounds play a vital role in overall pet health while also functioning as a palatant in pet foods which is crucial from a consumer standpoint. Long chain fatty acids are straight chain fatty acids (**FA**) that are composed of a carboxylic acid and 12 or more carbon atoms that create an aliphatic chain containing varying levels of saturation. These long chain FA are constituents of lipids and found in common food items such as soybean and olive oils, meats, eggs, and milk products (He et al., 2020). Lipids are high in energy and contribute heavily to the production of adenosine triphosphate by beta oxidation as well as provide structure to cell membranes and facilitate the absorption and storage of fat soluble vitamins (Nagy and Tiuca,

2017). To be digested, lipids undergo hydrolysis after exposure to lingual lipase which is secreted from the tongue. The stomach secretes gastric lipase to degrade triacylglycerols into fatty acids and diglycerides and to separate lipids from other stomach contents. Lipid digestion does not primarily take place in the stomach; therefore, fats pass to the small intestine and emulsification occurs. Bile, pancreatic lipase, and colipase are secreted from the pancreas into the small intestine to conduct lipolysis. This action results in free FA, monoglycerides, phospholipids, and free cholesterol which are absorbed by the enterocytes of the small intestine by passive diffusion (Carey et al., 1983). Within the enterocytes, triacylglycerols are formed and combine with cholesterol and phospholipids to create lipoproteins that enter the lymphatic system to be released into circulation (Nelson, Ackman, 1988). There are 4 classes of lipoproteins that include chylomicrons, VLDL, HDL, and LDL. In dogs, HDL serves in reverse cholesterol transport which is the removal of cholesterol from peripheral tissues and relocation to the liver to be metabolized (Bauer, 2004). Another way cholesterol can be cleared from peripheral tissues is by the exchange of cholesterol esters in HDL lipoproteins with triglycerides in VLDL or LDL lipoproteins by cholesteryl ester transfer protein (CETP) enzyme; however, activity of this enzyme is not found in dogs (Tsutsumi et al., 2001). To compensate for the lack of CETP enzyme, dogs form HDL_1, a subclass of HDL, composed of cholesterol esters and apoprotein E. The apoprotein E in HDL_1 binds to hepatic receptors to complete reverse cholesterol transport (Bauer, 2004).

Apart from being a source of energy, dietary fat is also a source of the essential FA, omega-3 and omega-6 FA. The common dietary omega-3 FA includes α-linolenic acid. Linoleic and arachidonic acids are dietary omega-6 FA (Bauer, 2016). Linoleic and α-linolenic acids are found in plant oils such as corn, soybean, and flaxseed and are converted into long chain

polyunsaturated FA in animals (Lenox, 2016). One of the most important long chain polyunsaturated FA animals can derive from linoleic acid is arachidonic acid which is a major component of cell membrane structure and precursor to eicosanoids such as prostaglandins and leukotrienes (Biagi et al., 2004). Eicosapentaenoic acid (**EPA**) is conversion of α-linolenic acid and is also a precursor to docosahexaenoic acid (**DHA**), another long chain polyunsaturated FA that is vital for retinal and neurological development as well as management of inflammation (Lenox, 2015) and may also play a role in preserving mental acuity in senior pets (Pan et al., 2013; Pan et al., 2018). Both EPA and DHA have been applied as nutraceutical agents to help manage pruritis and osteoarthritis and have renoprotective properties (Stice, 2019).

The FA profiles of dietary proteins, both animal and plant based, provide more details for the overall nutritional profile of a protein source, and may determine the levels at which to include a protein in a diet or assist in pairing complimentary proteins. As per the NRC, the recommended allowance for linoleic: α-linolenic ratio for adult dog maintenance diets is 25g/kg DM where linoleic acid is provided at 11 g DM basis and α-linolenic acid at 0.44 g DM basis. A maintenance requirement for α-linolenic acid for adult cats has yet to be established, therefore the NRC only has a recommended allowance for linoleic acid at 0.55% DM and arachidonic acid at 0.006% DM (NRC, 2006). The explanation for this difference is that cats lack the Δ6 desaturase concentrations to derive (EPA) from α-linolenic acid in significant amounts (Pawlosky et al., 1994). The maximum inclusion of omega-6:omega-3 polyunsaturated FA published by AAFCO is a ratio of 30:1 DM basis for adult dogs at maintenance. For adult cat maintenance diets, AAFCO recommends a minimum inclusion of linoleic acid at 0.6% DM basis and arachidonic acid at 0.02% DM basis and like NRC guidelines, there are no recommendations for α-linolenic acid (AAFCO, 2021).

Meat quality can also be influenced by FA concentrations by affecting the tenderness, taste, and preservation characteristics of several types of meat. The FA found in meat are influenced by what the animal was fed throughout production. For instance, grass feed beef contains higher amounts of omega-3 FA because the animal synthesizes omega-3 from the α-linolenic acid that is present in grass (Wood et al., 2002). The cut of meat also influences which FA would be in abundance because of the inherent differences between red and white muscle fibers. Williams (2007) observed how red meat from beef contains higher amounts of total polyunsaturated FA (44.8%) compared to skinless chicken breast and pork fillet (30% and 20%, respectively). The FA content of the beef in this study was an average of 9 cuts of beef (rump and round steak, topside roast, silverside roast, sirloin steak, fillet steak, T-bone steak, rib-eye, and blade steak). Lamb (forequarter chop, chump chop, loin chop, leg roast, shoulder roast, and lamb mini roast) and mutton (baking leg and casserole) FA profiles were also analyzed and were determined to be composed of 60% and 67% total polyunsaturated FA, respectively. A study that compared a variety of legumes (i.e., horse bean, common bean, broad bean, field pea, flat pea, kidney bean, lentil, pea, and soy bean) reported that linoleic acid was the major polyunsaturated FA with concentrations that ranged from 21% to 53% of the total FA content, with soybean being the highest. Compared with linoleic acid concentrations, legumes generally had lower concentrations of α-linolenic acid with broad bean containing the least, 3.6%, and kidney bean containing the most, 21.8% (Grela and Günter, 1995).

Mammalian proteins

Mammalian proteins are frequently used ingredients for commercial pet foods, a common choice includes beef which is typically in the form of meat and bone meal (Thompson, 2008). Meat and bone meals contain about 92% DM, 49-59% CP, 8.9-16% ether extract, 1-5% crude

fiber, and 18-24% ash (Hicks, Verbeek, 2016) with the crude fiber being determined by concentrations of hyaluronic acid and chondroitin sulfate present in connective tissue (Kannus, 2000). The predominant AA is proline at 11.3%, followed by glutamic acid, arginine, and leucine, 7.79%, 6-7%, and 6%, respectively (Hicks, Verbeek, 2016). Lean beef is another protein option for use in pet food and differs in content as it contains, 89.2% CP, 7.2% crude fat, and 4.8% ash on a DM basis (FAO, 2015). A study was conducted to evaluate the protein digestibility and amino acid bioavailability of beef loin using the cecectomized rooster assay and ileal-cannulated dogs. In dogs, beef loin had greater ileal total AA digestibility values (91.5%) as compared with chicken breast (90.4%).According to the results from the cecectomized rooster assay, the standardized digestibility of total AA for beef loin was 89.1% which was also higher than chicken breast (86.9%), therefore indicating that the beef protein was highly digestible and of good quality (Faber et al., 2010).

Yak (Bos grunniens). The yak is a herd animal that inhabits the mountainous regions of the Himalayas, mainly on the alpine grasslands of the Qinghai-Tibetan Plateau (Xiong et al., 2021). Nutrient composition varies slightly among yak meat samples depending on the cut of meat, age and sex of the animal, but generally DM, CP, crude fat, and ash are 24%, 85%, 10%, and 3.9%, respectively (Zi et al., 2004). The amino acid composition of yak meat is similar to beef; however, yak is higher in glutamic acid (15.65%) and remarkably lower in methionine (1.26%) (Zi et al., 2004). The main FA that have been found in the meat from grazed yak were palmitic acid (21%), stearic acid (21%), vaccenic acid (16%), linoleic acid (10%), and arachidonic acid (11%) (Liu et al., 2021). In a study on meat quality, yak meat contained higher concentrations of some omega-3 polyunsaturated FA such as α-linolenic acid (0.047% of meat) and eicosatetraenoic acid (0.043 of meat) compared with beef (Xiong et al., 2021). The high

levels of polyunsaturated FA found in this study portray yak meat as having the potential to be nutritionally valuable in that unsaturated fatty acids are believed to have protective effects over inflammation and cardiovascular disease (Lunn and Theobald, 2006). Comparable results were obtained by Smanalieva et al. (2019) where the unsaturated FA content, in relation to total FA, of yak meat and beef was 38% and 27%, respectively. There are several dog chews and treats that utilize yak meat as an ingredient. However, there is a lack of information regarding the nutrient digestibility and inclusion of yak meat in complete and balanced diets for dogs and cats.

Wild boar (Sus scrofa). The chemical analysis of wild boar meat consists of 23% DM, about 91% CP, 4-8% crude fat, and about 4% ash on a DM basis. The content for DM and CP are comparable to those seen in traditional pork (Szmanko et al., 2007; Batorska et al., 2018; Stanisz et al., 2018; Kaspryzyk et al., 2019; Soriano and Sanchez-Garcia, 2020; Machackova et al., 2021). Fresh boar meat has been observed to contain less fat than traditionally raised pork, where fat composition of the *Longissimus dorsi* muscle was 1.55% and 4.56%DM, respectively (Marisco et al., 2007), but a higher fat level in fresh wild boar meat of 2.62% DM was reported by Szmanko et al (2007) which could be attributed to the season in which the meat samples were taken (Sales and Kotrba, 2013). The transition from warmer to colder seasons is the point where animals increase their intake to gain weight thereby elevating the fat content of their meat. This is reflected in the meat sampled from wild boars hunted during the autumn and winter months which had a higher intramuscular fat content of 0.34 mm^2 when compared with the spring and summer meat samples that contained 0.28 mm^2 of intramuscular fat (Lachowicz et al., 2008). The most abundant FA found in those meat samples included palmitic (20.7-25%), stearic (10.5-11.28%), oleic (36.1-40.13%), and linoleic acids (11.37-15.9%) (Sales and Kotrba, 2013; Batorska et al., 2018). Predominant indispensable AAfound in wild boar meat include arginine

(12.40-14.02 g/kg), leucine (10.61-11.05 g/kg) threonine (8.25-8.99 g/kg), and lysine 11.86-12.38 g/kg), and for the dispensable AA, aspartic acid (15.50-17.43 g/kg), and glutamic acid (22.67-24.77 g/kg) were among the highest (Brudnicki et al., 2012).

Camel (Camelus). Camels inhabit the arid environments of the Middle East, North Africa, and Western Asia and have always existed as part of the local diets and cultures (Zappaterra et al., 2021). On average, the chemical composition of camel meat consists of about 25% DM, 85% CP, 6.8% crude fat, and 4% ash (Elsharawy et al., 2018; Mohammed et al., 2020, Baba et al., 2021). In a comparative study that included camel meat and beef, Mohammed et al. (2020) reported, camel meat as being significantly higher in CP at 21.83% and significantly lower in total fat at 1.51% than beef crude protein and total fat concentrations, 20.64% and 6.83%, respectively. Similar results were observed in the *Biceps femoris* muscle of camel meat and beef by Elsharawy et al. (2018), where the mean protein content of camel meat (21.3% DM) exceeded that of beef (18.1% DM). \ On average, FA concentration in camel meat is about 54% with the most predominant FA being palmitic, oleic, myristic, and stearic acids (about 26%, 32.2%, 6%, and 17.2%, respectively) (Shoman et al., 2019). An analysis of mineral content was performed, and the most abundant mineral was potassium with 762 mg/100 g of meat, but was significantly lower than in beef, 1326 mg/100 g. Other minerals inclduing phosphorus, sodium, magnesium, and calcium were present but not significantly different from levels found in beef (Kadim et al., 2008).

Kangaroo (Macropodidae). The use of kangaroo meat in pet food is already a widespread practice in Australia and domestic consumer attitudes continue to be in favor of it (Ampt and Owen, 2008; Foster, 2014; Spiegel and Wynn, 2014; Croft and Witte, 2021). Kangaroo meat is composed of approximately 27% DM, 84% CP, 10% crude fat, and 14% ash all on a DM basis

(Shul'gin et al., 2015; Luo et al., 2018). Fat content was analyzed by Shul'gin et al. (2015) and reported that industrial cuts of kangaroo meat (humeroscapular, dorsal, sternal, coxal, tail, and shank)contained significantly less fat than beef with concentrations of 2.6% and 15.8%, respectively. This same study also included an AA profile for kangaroo meat. The most abundant AA was glutamic acid (15.2%), followed by leucine (8.2%), aspartic acid (7.8%), and lysine (7.5%). In a study conducted by Food Science Australia (FSA, 2008), it was concluded that polyunsaturated FA comprised most of the total FA profile of kangaroo meat, with a mean of 37.5%. Out of total FA, the polyunsaturated FAobserved in abundance were linoleic acid (15-20%), arachidonic acid (6-10%) and α-linolenic acid (3-7%).

Avian proteins

Chicken (*Gallus gallus domesticus*) is the most used protein ingredient in pet foods as it is a high quality ingredient with about 86% standardized total indispensable AA digestibility in breast meat (Faber et al., 2010). The chemical composition of chicken breast meat consists of 25% DM, 88% CP, 4% fat, and 4% ash on a DM basis (Ali et al., 2007). Chicken meat is typically leaner and contains a higher proportion of unsaturated FA (Barroeta, 2007), which may be favorable to pet owners concerned with overall pet nutritional wellness. To further the idea of pet wellness, the use of raw protein ingredients has become a point of interest based on the customer perception that raw proteins are of higher quality than more traditional protein sources such as rendered animal by-product meals (Morgan et al., 2017; Thomas and Feng, 2020). Freeze dried (from raw) chicken by-product meals have demonstrated to be of moderate to high protein quality with 94.6% total indispensable AA digestibility and 92.6% total AA (both indispensable and dispensable) digestibility as determined by a rooster assay that utilized intact roosters (Cramer et al., 2007). In another study, raw mechanically separated chicken (MSC) and raw

salmon protein hydrolysate (SPH) were compared with poultry meal in kibble diets by Tijernsbek et al. (2017); however, no significant results were reported. This study partially replaced poultry meal with MSC so that MSC provided 25% of dietary CP in an extruded dog diet to examine its effects on amino acid digestibility and protein quality in minks. On a DM basis, MSC was composed of 35.8% DM, 42.1% CP, 14.1% crude fat, and 3.21% ash. The individual ingredients were also fed to minks and it was determined that MSC apparent total tract digestibility of total indispensable AA was significantly higher than that of poultry meal, 92% and 86%, respectively. However, apparent total tract digestibility of CP in MSC extruded diet was 81.3% which was not significantly different from the control poultry meal extruded diet, at 80.3% (Tijernsbek et al., 2017). To help meet demands for novel proteins, it would be beneficial to select ingredients that are comparable to chicken. Goose, quail, duck, and emu are avian species that are available for consumption by pets, yet the literature remains limited with nutritional information on these animals.

*Canada goose (Branta canadensis).*The chemical composition of raw goose meat is about 30% DM, 76% CP, 10% crude fat and 3% ash (Belinksy and Kuhnlein, 1998). Goose breast meat has a higher fat content compared with chicken breast, 9.2% and about 3%, respectively (Farrell, 2013; Oz and Celik, 2015). The breast meat of Canada geese contains a greater content of total monounsaturated FA (MUFA) at 54.12% when compared with total saturated FA, 32.24%, where the MUFA found in highest concentration is oleic acid and the lowest is myristic acid, about 44% and 0.02%, respectively. Total omega-3 PUFA are found in smaller amounts of 0.09% than total omega-6, 13.54%, with the most abundant omega-6 fatty acid being linoleic acid at 10%. (Belinksy and Kuhlein, 1998). The AA composition of goose breast meat resembles that of chicken, but, in g per 100g of protein, goose meat is higher in leucine (8.05 versus 7.60)

and lysine (7.44 versus 5.69). The most prevalent AA include leucine, aspartic acid (8.97), and glutamic acid (14.21), whereas tyrosine, methionine, and histidine were among the smallest concentrations, 3.82, 1.40, and 2.80, respectively (Spiegelaar et al., 2019).

Quail (Cortunix corturnix). Although not as commercially popular as chicken, Japanese quail is farmed for its meat throughout Europe and in the Americas (Danthi and Kalaikannan, 2017). Raw quail meat contains 29% DM, 58-79% CP, about 6-9% fat, and 3-6% ash (Hamm and Ang, 1982). The indispensableAA present in higher amounts in quail breast meatare leucine and valine, 1.77% and 1.34%, respectively, and the highest dispensable AA are glutamic and aspartic acids, 3.54% and 2.21%, respectively. Aspartic acid and glutamic acid have the highest values in relation to total amino acids (Cullere et al., 2017). In relation to total FA content, major FA in quail meat that have been reported are palmitic (17-21%), oleic (34%), and linoleic acids (24-27%) (Boni et al., 2010). In a cecectomized rooster study, the standardized total indispensable AA digestibility of whole prey quail was about 85% which is indicative of quail meat being a highly digestible protein source (Kerr et al., 2014).

Pekin duck (Anas platyrhynchos domesticus). The DM, CP, fat, and ash concentrations for duck breast and thigh meat is about 25%, 72-77%, 15-19%, and 5%, respectively (Galal et al., 2011). The FA with highest concentrations within duck breast and thigh meat are oleic (27% and 30%), palmitic (24% and 20%), and linoleic (13% and 17%) acids; as a percent of total fatty acid, pecking duck breast contains about 19% omega-3 and 22% omega-6 PUFA and in thigh meat, 14% and 24%, respectively (Aronal et al., 2012). The indispensable AA in breast meat that are found in largest concentrations are lysine (8.60%) and leucine (7.78%); there are also elevated levels of glutamic acid, aspartic acid and methionine, 15.21%, 9.57%, and 7.09%, respectively (Woloszyn et al., 2006; Aronal et al., 2012). Duck meal was evaluated by the

precision-fed cecectomized rooster assay to determine the AA digestibility for inclusion in dog and cat diets (Deng et al., 2016). The indispensable AA with the highest standardized digestibility was arginine at 90.3% and lowest was histidine, 73.4%; the limiting AA were determined to be methionine, cysteine, and tryptophan. The results for standardized digestibility of dispensable AA determined that the highest digestibility value was 82.4% for alanine and the lowest was 49.5% for cysteine.

Emu (Dromaius novaehollandiae). Emu meat has gained consumer interest as an alternative to red meat. On a DM basis, the chemical composition of emu meat consists of about 27% DM, 84.6% CP, 3% fat, and 6.7% ash (Naveena et al., 2013). The FA found at higher concentrations in various leg muscles are oleic (~21-26%), palmitic (~17-20%), arachidonic (~12-20%), linoleic (~16-18%), and stearic (~13-15%) acids (Buclaw et al., 2018) and 25% of total lipids is comprised of saturated fatty acids (Naveena and Kiran, 2013). Emu meat contains less cholesterol than beef, 0.058% and 0.067%, respectively, and total polyunsaturated FA is higher in emu than in beef, 0.024% in emu meat and 0.005% in beef (Horbańczuk and Wierzbicka, 2016). While there is lack of data over the AA composition of emu meat has been reported that concentrations of creatine in emu jerky, 0.022% DM, are significantly higher than beef jerky, 0.021% DM (Pegg et al., 2006). It has also been estimated that emu meat may have a PDCAAS value that is greater than beef (85%),, indicating that emu protein quality may be higher. This calculation was based on the greater CP content of emu meat compared to beef as reported by Adewumi et al. (2011). As was previously mentioned, PDCAAS values are truncated at 100%; therefore, it is important to note that emu protein quality might had been underestimated (Adewumi et al., 2011). Further research would be instrumental to fully understanding the bioavailability of AA in emu meat.

Aquatic proteins

In this category of protein ingredients, fish ingredients are commonly used in both dog and cat diets and are known to contain omega-3 FA (Aldrich, 2006). The chemical composition of common carp (*Cyprinus carpio*) contains about 24% DM, 62-75% CP, 8-12% fat, and 4% ash (Ashraf et al., 2011; Trbović et al., 2013). There are variations in composition due to the environmental conditions in which the fish were reared. This includes whether the fish were wild caught or farmed, as well as whether they were fed cereals or extruded pellets made of fish meal. In general, when compared to red meat, fish protein has greater lysine, methionine, and threonine concentration (Arino et al., 2003). The common FA found in carp include DHA, palmitic, oleic, , and linoleic acids (Ojagh et al., 2009). Additional aquatic dietary proteins already used in pet foods may include white fish, salmon, whereas spirulina and eel are more novel proteins. Spirulina has been used mostly in treats in the U.S., as this is not an approved ingredient to be used in pet foods. About a decade ago, New Zealand long-finned eels were being used in commercial cat food in California as a novel, earth-friendly and hypoallergenic dietary protein source. However, this became a controversial topic that gained headlines in the industry as researchers from Massey University in New Zealand reported concerns with this practice potentially endangering this species of eel that is considered a threatened species in their country (Petfood Industry, 2012; Radio New Zealand, 2012).

*Whitefish*This protein category can be comprised of various fish species that contain white meat (Seafood Source, 2014). A study by Ljubica et al. (2012) assess the average composition of several species of whitefish such as bream, barbel, crucian carp, catfish, tench, and silver carp. It was determined that whitefish contains about 25% DM, 56% CP, 26% crude fat, and 4% ash on a DM basis. A study sampled whitefish from the Great Lakes area of the U.S. and reported omega-3 FA present in 0.72% of wet fish sample and 0.27% of omega-6 FA

(Dellinger et al., 2019). In addition to the omega-3 FA, oleic and linoleic acids are seen in ample amounts, about 39% and 10%, respectively (Ljubica et al., 2002). A study evaluated the standardized AA digestibility of pollock (a species of whitefish) by-products (head, liver, milt, roe, and viscera), pollock hydrolysate, and whitefish meal by the precision-fed cecectomized rooster assay. All ingredients were compared to soybean meal. There was no difference in total indispensable AA digestibility among pollock by-products; however, differences were observed in indispensable AA digestibility when pollock by-products were compared to soybean meal. Standard digestibility of arginine in pollock head (86.1%) was significantly lower than soybean (92%) whereas pollock liver (98.7%) was significantly greater than soybean meal. Pollock liver produced lower standardized AA digestibility for threonine (82.8%), valine (91.8%) compared with soybean meal (91.4% for threonine and valine); however, higher values were observed in leucine (99.1%) compared with soybean meal at 89.2%. A profile of indispensable AA was also compiled and the AA that were present in the highest concentrations were lysine (4.72), leucine (4.43), and arginine (4.17%) along with the dispensable amino acids glutamic and aspartic acid (8.20% and 5.62%, respectively) (Folador et al., 2006).

Salmon (Salmo salar). Salmon meat contains approximately 28% DM, 75% CP, 5% fat, and 4% ash on a DM basis (Karrick and Thurston, 1964). Lipid content varies among wild caught and farmed salmon where the percent lipid found within farmed salmon has been shown to be significantly greater than in wild salmon at 16.6% and 6.4%, respectively (Hamilton et al., 2005). Both DHA and EPA have been identified in high concentrations in farmed and wild salmon. In wet weight, DHA concentrations were 1.6% and 0.63% in farmed and wild salmon, respectively; farmed salmon contained EPA at 1.1% and wild salmon at 0.41% (Hamilton et al., 2005) In a study by Colombo and Mazal (2020) 6 types of wild salmon were sampled (farmed

Atlantic, farmed organic Atlantic, farmed organic Chinook, wild Chinook, wild Pacific, and wild Sockeye). Major FA in farmed salmon include oleic acid (15.4%), linoleic acid (5.8%), and palmitic acid (5.0%). Concentrations for oleic and linoleic acids were significantly lower in that oleic acid ranged from 0.1% to 2.5% and 0.03% to 0.6% for linoleic acid. An in vivo study was conducted by Tjernsbekk et al. (2017) in which minks were fed extruded dog diets containing salmon protein hydrolysate (**SPH**) to determine the effects of partially replacing poultry meal with SPH on protein and AA digestibility. As individual ingredients, the ATTD of CP of SPH was 91.3%, which was significantly greater than the poultry meal ATTD of 80.9%. When these ingredients were added to extruded kibble diets, ATTD for CP in SPH was 79% which was not different from poultry meal at 80.3%. The total AA ATTD of SPH was 81.6%. The indispensable AA with highest ATTD in SPH diet were arginine (89.2%) and methionine (87.7%) and for dispensable AA, glutamic acid (87.6%) and alanine (85.7%). The results of this study suggest that salmon protein could be considered a high quality protein for extruded dog diets. In another study, Montegiove et al. (2021) performed an in vitro experiment with the aim of determining protein quality of fresh salmon fillet for pet food production. Crude protein concentration was assessed by the Kjeldahl method, and it was determined that salmon meal contained higher CP, 68% of DM, than fresh salmon, 38%; no significant difference was observed between fresh salmon and fresh chicken (40% CP). In vitro protein digestibility of fresh salmon was about 90% and chicken meal digestibility was about 70%. It was noted in this study that fresh salmon meat contained higher concentrations of taurine (0.17%) compared to salmon meal (0.11%). This is important as taurine is an essential amino acid for cats that needs to be supplemented in order to achieve a complete and balanced diet.

Spirulina (Arthrospira platensis). Spirulina is a filamentous cyanobacterium that is blue-green in color and grows in alkaline bodies of water and is now the most cultivated microalgae where 30% of the global production is used for animal feed (Ciferri, 1983; Becker, 2013; Lafarga 2020). This ingredient has a high CP content of 60-70% on a DM basis, exceeding that of soybean (40%) (Liu, 1997). It also contains 4-16% fat and 3-11% ash (Holman and Malau-Aduli, 2012). The total amount of fatty acids in spirulina is 8.12% of DM basis and the largest contributors are palmitic acid (46%), linoleic acid (31.5%), and γ-linolenic (12.9%) acids (Muhling et al., 2005; Liestianty et al., 2019). Among the indispensable AA, the highest concentrations are attributed to leucine and valine (5.5% and 4.5%, respectively) and the most abundant dispensable AA are glutamic acid, aspartic acid, and alanine (9.2%, 6%, 4.7%, respectively) (Liestianty et al., 2019). Many animal trials revolve around the dietary supplementation of spirulina to study its immunomodulatory effects (Satyaraj et al., 2021), antioxidant properties (Witkop et al., 2021), and hypocholesterolemic effects (Shamsudin et al., 2016); therefore, leaving a gap in knowledge about protein digestibility in companion animals. However, growth studies have been conducted in non-ruminant livestock such as pigs and chickens that indicate spirulina's capacity to maintain typical growth rates in chickens and increase weights of weaning pigs (Saxena et al., 1983; Grinstead et al., 2000). In the poultry study, spirulina replaced groundnut cake in chick feed at levels of 5.6%, 11.1%, and 16.6% (Saxena et al.. 1983). In pig diets, soybean meal was substituted by spirulina at levels of 0%, 0.2%, 0.5%, and 2% (Grinstead et al., 2000).

Eel (Anguilla japonica/Anguilla anguilla). Japanese eel is a popular protein that is readily consumed in East Asian countries with Japanese (*Anguilla japonica)* and European (*Anguilla anguilla)* eels being the two common species used for food (Hamidoghli et al., 2019; Gomez-

Limia et al., 2021). Eel is sought after for its rich FA profile and according to de Melo et al. (2013), the PUFA to saturated FA ratio that indicates higher quality fish must be greater than 0.45. The FA from European eel that were analyzed by Gomez-Limia et al. (2021) produced a PUFA to saturated FA ratio that ranged from 0.48 to 0.52. Another study compared the FA of wild caught and cultured Japanese eels. The FA that have been identified to be majorly present in cultured Japanese eel include oleic acid (37.6%) and palmitic acid (18.5%). The concentrations for the same FA in the wild eel were not significantly different, but wild eel contained significantly higher concentrations for linoleic acid (5.8%), α-linolenic acid (3.3%), and arachidonic acid (2.1%) (Oku et al., 2009). Eel meat is comprised of about 34% DM, 44-52% CP, 47% fat, and 1-3% ash on a DM basis (Wijayanti and Susilo, 2018; Gomez-Limia et al., 2021). The variability that has been documented exists between cultured eels could be attributed to different diets and farming practices as explained by Seo et al. (2013). In this study, 4 groups of cultured eels on the same farm were fed different formula feeds and there was no significant difference among the chemical composition of the eels sampled, having no effect on quality. However, there was a significant difference in crude fat content of eels across 5 different farms suggesting that physical environments (i.e., pool size, population size, activity levels) could affect fat content (Seo et al., 2013). Total indispensable AA in European eels is about 45% to 46% with the highest indispensable AA being leucine (1%), lysine (0.9%) and threonine (0.8%); glutamic acid (2%) is the highest dispensable AA as well as aspartic acid, arginine, and alanine, all three being about 1% (Gomez-Limia et al., 2021). Japanese eels contain leucine and lysine in high concentrations on a as is basis, 1.16% and 1.19%, respectively, and the highest dispensable AA include glutamic acid at 2.36% and aspartic acid at 1.46% (Damusaru et al., 2019).

Insect proteins

Cricket meal (Acheta domesticus). Proximate analyses that have been performed on cricket meal report that it is composed of about 89% DM, 60-70% CP, 17-22% fat, and 5% ash (Razak et al., 2012; Bosch et al., 2014; Kilburn et al., 2020). Crickets have a higher proportion of omega 3 and omega 6 FA (Kipkoech et al., 2017) and out of the total fatty acids present, palmitic acid, oleic acid, and linoleic acid contribute 90% (Osimani et al., 2018). The values of the highest AA within cricket meal are valine (3.2%), leucine (2.9%), tryptophan (2.8%), lysine (2.4%), and arginine (2.2%) on DM basis (Razak et al., 2012). In an in vivo study that was conducted in dogs investigated the ATTD of cricket meal at different inclusion levels (0%, 8%, 16%, or 24%) in dry dog diets (Kilburn et al., 2020). What the researchers found was that overall, the cricket meal was deemed acceptable to use as a protein source comparable to chicken based diets. The ATTD of CP ranged from 82.1% to 88.2%. Each treatment had an apparent fecal DM digestibility above 80% which is consistent with commercial diets.

A recent study evaluated digestibility of insect-based diets in companion animals. Reilly et al. (2022) used the precision-fed cecectomized rooster assay to determine standardized AA digestibility of speckled cockroach (SC), Madagascar hissing cockroach (MC), and superworm (SW). According to the precision-fed cecectomized rooster assay, indispensable AA digestibility of the 3 insect meals were >80%, indicating the AA were well digested. Lysine standardized digestibility was greater than 90% in all meals. Superworm meal had significantly greater digestibility values for histidine (93.3%), isoleucine (93.4%), and glutamate (95.2%) compared to SC and MC meals. These meals were also incorporated into retorted cat diets. There was no difference in ATTD of CP among diets with values that ranged from 86.31% to 88.62%. A study by Do et al. (2020) also evaluated the standardized AA digestibility of black soldier fly larvae of different ages by the precision-fed cecectomized rooster assay and DIAAS-like calculations. Day

0 larvae has significantly lower methionine and phenylalanine digestibility (89.4% and 85.8%, respectively) compared to larvae that were 23 days old (96% and 93.9%, respectively). Larvae that were 14, 18, and 23 days of age had indispensable AA digestibility that were >90% with the exception of histidine, threonine, and valine which ranged from 81.6% to 89.1%. The DIAAS-like values using AAFCO nutrient profiles for adult cats at maintenance were all greater than 100% as well when using NRC recommended allowances for adult cats.

Reptile proteins

Consuming reptile meat as a source of protein is already practiced in the Southern region of the U.S., specifically in the states of Georgia, Florida, Texas, and Louisiana (Domínguez et al., 2019). Alligator (*Alligator mississippiensis*) meat is one of the popular reptile proteins that is raised and mainly consumed in the South (Keul, 2018); alligator and crocodile meat have been farmed for centuries in other parts of the world as well, including Australia, Africa, Central America, and Asia (Klein et al., 2007). Alligator contains approximately 25% DM, 16-18% protein, 1% fat, and 1% ash (Ockerman and Basu, 2009). The FA profile of alligator meat is characterized by being rich in oleic acid, ranging between 33% and 55% of total FA composition, followed by palmitic and linoleic acids, at about 20% and 9%, respectively (Peplow et al., 1990). In a study by Deng et al. (2016), alligator meal was analyzed via the cecectomized rooster assay to evaluate the standardized protein digestibility and protein quality for use in companion animal diets. Total AA concentration of alligator meal was 54.59% on DM basis, with arginine being the indispensable AA at the highest concentration at 4.13%; followed lysine and leucine, 3.77% and 3.57%, respectively. Glutamic acid (7.84%,) and glycine (6.94%) were the most abundant dispensable AA. As determined by cecectomized roosters, the standardized AA digestibility of a majority of AA was above 80%, except for histidine (79.8%), which

suggests the high digestibility of alligator meal. Results from these studies support the need for more in vivo trials in both canines and felines to further assess the nutritional quality of reptile meat as a protein source in companion animal diets. Novel reptile proteins such as alligator and snake are of interest in the pet food industry as some owners express concern over their pets' allergic responses to more traditional protein sources (Viana et al., 2020).

Snakes. Reptiles from the suborder Serpents, rat snake, rattlesnakes, boa constrictors, cobra, sea snakes, and garden-type snakes are commonly eaten (Newman, 2001). There is limited literature on the proximate composition of specific snake species, information that will be reviewed will be data collected from the meat of different snake species such as the ball python (*Python regius*), spitting cobra (*Naja nigricollis*), and Cuban boa (*Chilabothrus angulifer*). On average, the snake meats being reviewed are composed of about 23% DM, 65% CP, 6% fat, and 5% ash (Abdule, 2007; Ockerman and Basu, 2009; Ogungbenle and Adaraniwon, 2013). The total AA content in cobra is 76.8% and is primarily comprised of glutamic acid (12.2%), aspartic acid (8.9%), and leucine (6%); the amount of total indispensable AA in cobra was also analyzed and determined to be 35.5% on a DM basis (Ogungbenle and Adaraniwon, 2013). When comparing with alligator meal, cobra has a greater total AA composition and is also higher in lysine and glutamic acid. These two proteins both contain glutamic acid in highest concentration. Erabu sea snakes (*Laticauda semifasciata*) were analyzed for omega-3 and omega-6 FA, and it was found that the snakes have higher omega-3 FA than omega-6 FA, where the most prominent omega-3 FA was DHA (~12%). Among the saturated fatty acids, palmitic acid had the greatest concentration at about 27% followed by stearic acid, about 9%. (Shirai et al., 2002). There remains a lack of digestibility data regarding snake meat ingredients for use in pet nutrition in the

literature. Thus, more investigation is required to determine their chemical composition, protein makeup and quality, and nutrient digestibility.

Plant-based proteins

Consumption of plant-based proteins has become popular globally; therefore, increasing the demand for new products (Joseph et al., 2020). As mentioned throughout this review, the pet food industry evolves to emulate trends seen in the human food market. In a survey of pet owners, 35% of total participants expressed interest in feeding their companion animals a diet containing plant-based proteins (Dodd et al., 2019). Common ways commercial diets utilize plant proteins are through the incorporation of bean, pea, lentil, and chickpea pulses (Reilly et al., 2020) as well as more extensively researched legumes such as soybeans (Clapper et al., 2001). A study by Clapper et al. (2001), evaluated soybean meals and soy protein concentrates in extruded dog diets to determine ATTD of macronutrients of soybean proteins in comparison with poultry meal. The ATTD of CP in soy protein concentrates ranged from 84.7% to 89.3% DM, and when compared to poultry meal (76.9% DM), the protein concentrate values were numerically greater, but not significant. . Thus, implying that soybean-derived ingredients are suitable plant-based protein options for extruded canine diets. Pulses were assessed as protein sources for dog and cat diets by Reilly et al. (2020) by calculation of DIAAS-like values. The DIAAS-like values for all indispensable AA, excluding methionine and tryptophan, ranged from 109.1% to 270.2% for adult dogs compared with AAFCO nutrient profile. For adult cats, DIAAS-like scores fell between 103.3% and 336.3%, indicating pulses are a high quality protein compared to AAFCO nutrient profile.

Chocho (*Lupinus mutabilis*). Chocho is a legume that is cultivated in the Andean region of South America (Atchison et al., 2016). There are several varieties of *Lupin,* with *L. mutabilis*

being the one that is most comparable to soybeans in oil and protein content. Chocho contains 91-93% DM, 40-47% CP, about 15% fat, and about 4% ash, on a DM basis (Schoeneberger et al., 1982, Gulisano et al., 2019, and Berru et al., 2021). The amino acids present in chocho closely resemble the amino acid profile of soybeans where the major AA are leucine (7%), lysine (5.8%), and isoleucine (4.2%); however, chocho is lower in methionine and tryptophan, both at 0.8% (Gulisano et al., 2019). Lupin seeds mainly contain unsaturated FA. The predominant unsaturated FA is oleic acid, at 53.8% of total FA whereas soybean contains 23% to 30%. The contrary is observed regarding linoleic acid, where the concentration in soybean, about 49%, is almost double that of chocho (Schoeneberger et al., 1982). The use of mixtures of different lupin varieties, rather than chocho alone, as a protein source has been studied in swine nutrition. A study by Zraly et al. (2008) showed that a blend of different white lupin varieties would be a suitable replacement for supplementary soybean in swine diets as long as the appropriate AA balance was maintained. Lupine seeds, both hulled and dehulled, replaced soybean meal by 50% or 100% in the diets used for that study. On average, there was no difference in body weight gain in pigs fed the lupine diets compared to soybean diets; values ranged from 0.82 kg/day to 0.86 kg/day. No difference was reported for average feed conversion ratio as all values ranged from 2.45 to 2.58 kg/kg. It would be beneficial to study the chocho variety alone to determine the efficacy of chocho as a sole protein source in monogastric nutrition.

Pumpkin (*Cucurbita pepo L*). Pumpkin seeds contain 95% DM and about 27% CP, 38% fat, and 5.5% ash on a DM basis (Elinge et al., 2012). About 98% of total FA content is composed of oleic (43.8%), linoleic (33.1%), palmitic (13.4%), and stearic (7.8%) acids (Elkholy et al., 2009). The AA in higher concentrations in pumpkin seeds are glutamic acid 19-23%, arginine14-16%, and aspartic acid about 9% (Idouraine et al., 1996). Both in vitro studies

(Fagbemi et al., 2005) and in vivo studies in rats (Pirman et al., 2007) have reported the apparent CP digestibility of pumpkin seed protein to be 72-88%. There is limited information on the nutrient digestibility of pumpkin seed protein powder, a plant-based protein that is becoming common in human nutrition in vegetarian, vegan and as an alternative protein in human nutrition. Similarly, there remains a gap in the literature regarding its use as a protein source for canine and feline nutrition.

Hemp (Cannabis sativa ssp. sativa). Currently, hemp is not approved by AAFCO; therefore, hemp may not be used in animal feed or pet food in the U.S. (AAFCO, 2022). Regardless, hemp seed has garnered interest for its nutritional content. Hemp seed powder contains about 25-40% CP, 8-11% fat, 30-42% dietary fiber, and 6-7.2% ash on a DM basis (Callaway, 2004; Leonard et al., 2019). The AA profile of a different species (*Cannabis sativa L.*) reflect lysine (~0.6-1%) as being the first limiting amino acid while glutamic acid appears to be the most abundant at 4.6% (Callaway, 2004; House et al., 2010). The fatty acid profile of hemp seed contains palmitic acid (~6%) as its predominant fatty acid, followed by stearic acid (~2%), and saturated fatty acids, myristiric, palmitic, stearic, arachidic, and lignoceric acids, make up no more than 10% of the total FA profile (Kiralan et al., 2010). In many animal studies, cannabidiol extract from hemp appears to be the ingredient of interest for pet products. Hemp is rich in omega-3 and omega-6 FA, specifically linoleic acid (about 56% of total FA) and α-linolenic acid (17.2% of total FA) (Rupasinghe et al., 2020). These FA can help promote skin health, and prevent cardiac disease in both dogs and cats (Freeman, 2010; Gedon, Mueller, 2018). Atopic dermatitis is a common skin disease with various forms of treatment including the more current option of supplementing essential FA in companion animal diets (Olivry et al., 2010). It is believed that inadequate synthesis of eicosanoids is partially responsible for the

inflammation that accompanies atopic dermatitis; therefore, the goal of supplementation as a treatment is to aid the animal in producing a greater concentration of eicosanoids through essential FA metabolism thus reducing signs of this skin disease (Sævik et al., 2004). The omega-3 FA also provide a cardioprotective effect through modulation of blood pressure, blood lipids, and heart arrhythmia thereby reducing the risk of developing cardiovascular disease (Innes, Calder, 2020). Hemp seed meal for nutritional supplementation is more often seen in livestock research. Khan et al. (2009) incorporated dried crushed hemp seeds to broiler chick diets to observe the effects of supplementation on carcass quality. This study reported that weight gain increased while feed intake decreased, which suggests hemp seed would be a quality protein supplement. Hemp seed was also fed to gestating sows and effects of hemp seed diets were assessed in sows and nursing piglets. Animals fed the hemp seed diets had improvement in oxidative status, as determined by an increase in antioxidant enzyme activity, compared to the animals fed a hemp-free control diet. Sows on the hemp diet maintained elevated levels of glutathione peroxidase enzyme over 21 days of gestation, peaking at day 21 at about 2000 nmol/min/ml whereas enzyme activity peaked at about 1500 nmol/min/ml at 21 days for sows on the hemp-free diet. Piglets produced a decreasing amount of reactive oxygen species over 21 day lactation period where day 1 levels were at about 8500 a.u. and were reduced to about 5500 a.u. on day 21. Piglets from sows that were fed a hemp free diet produced about 8800 a.u. on day 1 and about 6500 a.u. on day 21 (Palade et al., 2019).

Objectives and Hypothesis

The objectives of this study were to determine the chemical composition and assess the quality of select novel protein sources belonging to mammalian, avian, aquatic, reptile, insect

species and plant-based by determining their standardized AA digestibility through application of the precision-fed cecectomized rooster assay. Additionally, the novel protein sources were to be further assessed by the calculation of DIAAS-like values to compare against AAFCO nutrient profiles and NRC recommended allowances for adult dogs and cats at maintenance. It was hypothesized that the select novel proteins would be determined to be of high quality and have similar standardized AA digestibility to common ingredients used in companion animal diets such as chicken, beef, salmon, and soybean. It was also hypothesized that the DIAAS-like values would reflect a high quality protein that meets AAFCO and NRC indispensable AA recommendations.

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CHAPTER 3:

EVALUATION OF CHEMICAL COMPOSITION AND PROTEIN QUALITY OF EXOTIC MAMMAL PROTEIN SOURCES FOR USE IN CANINE AND FELINE NUTRITION.

Abstract

There has been a growing consumer demand for high quality ingredients in companion animal diets which has encouraged pet food manufacturers to begin including exotic meats in their products. Because of their novelty, there is little information available regarding proximate composition and assessment of protein quality and digestibility. The purpose of this study was to examine the macronutrient and amino acid (**AA**) composition, determine protein quality through standardized AA digestibility from the precision-fed cecectomized rooster assay, and calculation of digestible indispensable AA score (DIAAS)-like values of the following select novel proteins: yak, camel, kangaroo, and wild boar. Beef was also analyzed for the same parameters to serve as a traditional mammal protein comparison. Each protein was determined by the precision-fed cecectomized rooster assay to be highly digestible, with AA digestibility values falling no lower than 81% and differences ($P < 0.05$) observed in arginine and alanine digestibility. The protein references used for these DIAAS-like calculations were based on Association of American Feed Control officials (**AAFCO**) nutrient profile and National Research Council (**NRC**) recommended allowances. Generally, DIAAS-like values for cats were greater than for dogs based on both AAFCO and NRC recommendations. The majority of AA scores were well above 100%, the greatest being 503.31% for lysine in camel meat. Independent of the protein reference used, tryptophan and threonine were consistently the lowest scoring AA. The average scores for tryptophan and threonine for dogs according to AAFCO comparisons were 69.6% and 85.9%,

respectively; according to NRC comparisons the average scores were 58.9 for tryptophan and 71.8% for threonine. The average scores for tryptophan and threonine for adult cats using AAFCO comparisons were 87.7% for tryptophan and 89.6% for threonine; using NRC comparisons the averages for tryptophan and threonine were 83.1% and 88.1%, respectively. Based on NRC and AAFCO DIAAS-like data for adult cats at maintenance, kangaroo, yak, and camel contained no first-limiting AA as all scores were above 100%; therefore, designating these novel protein sources to be of high quality.

Introduction

Dietary proteins are commonly the spotlight of most pet food products and formulations. Pet owners often assess quality of pet foods by their protein content and sources. However, for the dog and cat, dietary proteins are important sources of indispensable AA and nitrogen for synthesis of non-essential (dispensable**)** AA and various proteins and other N-containing compounds (e.g., purines, hormones, catecholamines) in the body (NRC 2006).

Novel protein sources are becoming popular in both canine and feline diets as an alternative to more traditional ingredients such as beef and meat meals. Factors that have influenced the quest for searching for novel and alternative proteins may include pet owner's preference, need for hypoallergenic diets, concerns with sustainability, diversification of ingredients, among others. However, there is a lack of information regarding the nutritional composition and predictive digestibility of novel protein ingredients. The aim of this study was to analyze the chemical composition of four select novel protein sources (i.e., yak, camel, kangaroo, and wild boar) with beef as a traditional protein comparison and calculate DIAAS-like

values to determine protein quality based on AAFCO nutrient profile and NRC recommended allowances of dietary protein for adult dog and cat maintenance diets.

Materials and methods

Select novel protein sources

The novel proteins tested belonged to mammalian species, yak (*Bos grunnies*) stew meat (human-grade ingredient from Exotic Meat Market, USA), camel (genus *Camelus*) boneless stew meat (human-grade ingredient from Exotic Meat Market, USA), kangaroo meat (family Macropodidae; IMCD Group, Brampton, ON, Canada), and mechanically deboned wild boar (*Sus scrofa*; North Central Companies, USA), and were compared to mechanically deboned beef (genus *Bos*; Darling Ingredients, Inc., USA), a common protein in companion animal diets (Thompson, 2008). All samples were freeze dried and ground through a 2 mm sieve of a Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ, USA).

Chemical analyses

All proteins were sampled in duplicate with a 5% error as a threshold, otherwise analyses were repeated. Dry matter (**DM**), and ash were determined as per AOAC (2006; methods 934.01 and 942.05), organic matter (**OM**) was calculated by difference. Gross energy (**GE**) was analyzed by bomb calorimetry (Model 6200, Parr Instruments Co., Moline, IL) and crude protein (**CP**) was calculated based on total N concentration determined by Leco (TruMac N, Leco Corporation, St. Joseph, MI) according to AOAC (2006; method 992.15). Total fat content was measured by acid hydrolyzed fat (**AHF**) in accordance with AACC (1983) and Budde (1952). Amino acid content was determined according to AOAC (2007).

Long chain fatty acid analysis

For fatty acid (**FA**) profile analysis, each protein sample was weighed in duplicate at 0.1 g. The profiles were determined using modified methods of Lepage and Roy (1986) and Masood et al. (2005). Acetyl chloride, butylated hydroxytoluene (**BHT**), potassium carbonate, HPLC-grade methanol, and hexane were from Sigma-Aldrich (St. Louis, MO). To avoid fatty acid oxidation, BHT was added to methanol. The internal standard (nonadecanoic acid, 19:0) and external fatty acid methyl ester standards were purchased from Supelco Sigma-Aldrich (St. Louis, MO). Internal standard was dissolved in methane-BHT solution (50 µg BHT/ml methanol) at 0.1 mg/ml concentration and then added at 100 µl to test tubes that contained a 2mL methanol-hexane (4:1, v/v) mixture. Samples were then vortexed and placed on ice. Acetyl chloride (200 µl) was added drop wise while swirling the tubes. Samples were then capped under N. Protein samples were heated for 10 min at 100ºC, vortexed, and heated again for 50 min. After, the tubes were placed in ice to cool. Cooled samples were neutralized by adding 5 mL of 6% Na_2CO_3 solution and each tube was vortexed again for 1 min and centrifuged at 2300 *g* at 4 ºC for 3 min to separate the phases of the protein samples. The organic phase on the surface was extracted and collected in a test tube and the process was repeated by adding 0.5 mL of hexane, vortexing, and centrifuging at the same speed and temperature for 3 min. The surface organic phase was extracted and combined with the first extraction and evaporated under N to 300 µL, and transferred to a gas chromatography (**GC**) vial with a 300 µL glass insert, and crimped under N for fatty acid methyl ether (**FAME**) analysis by GC.

Gas chromatography for long chain fatty acid analysis

To analyze each FAME, a Thermo Scientific TRACE 1300 Gas Chromatograph coupled with FID was used. Exacts of 1 ul were injected into the GCC and separated on a fused silica capillary column (SP-2560, 100 m length, 0.25 mm I.D., 0.2 um film thickness). Helium was the carrier gas with a flow rate of 20 cm/sec, at a split-ratio of 100:1. The starting temperature was 140ºC for 5 min and then was increased by 4ºC every min to reach a final temperature of 240ºC which was held for 15 min. The injector temperature was 250ºC and detector temperature was 260ºC. Nonadecanoic acid (C19:0, Nuchek Prep, Elysian, MN) was the internal standard. The FAME standards (Supelco 37 Component FAME Mix, Sigma Aldrich) were used as the external standard for the identification of long chain fatty acid peaks by retention time comparisons.

Precision-fed cecectomized rooster assay

This assay was conducted as described by Parsons et al. (1982). Each protein was fed to four cecectomized Single Comb White Leghorn roosters housed in individual cages and fasted 26 hours prior to beginning of the trial. Approximately 20 g of each sample was mixed in a 1:1 ratio with corn to assist with the flow of fine and low-density ingredients. These ingredient mixtures were fed to the roosters by crop intubation. Excreta was collected during a 48 hour period, freeze dried, and ground to be analyzed further. The cecectomized rooster assay allows for the calculation of standardized AA digestibility by providing average endogenous AA values from the excreta of fasted roosters. The standardized AA digestibilities were calculated according to the methods described by Sibbald (1979).

Digestible indispensable amino acid score (DIAAS)-like values

Protein quality was measured based on DIAAS-like values that were calculated according to Mathai et al. (2017) using the standardized AA digestibility values obtained from the cecectomized rooster assay instead of ileal-cannulated pigs. The reference proteins in this study were based on the nutrient profile of the Association of American Feed Control Officials (**AAFCO**) and recommended allowances from the National Research Council (**NRC**) for adult dogs and cats at maintenance and were calculated by determining the amount, in mg, of each indispensable AA that is in 1g of protein from the values provided by the aforementioned publications. Likewise, this calculation was also done to determine mg of indispensable AA in 1 g of select proteins tested herein. The formula used to determine DIAAS-like values for each protein is as follows:

DIAAS-like (%) = [(mg of digestible indispensable AA in 1g dietary protein) / (mg of same indispensable AA in 1g reference protein)] x 100.

The overall quality of a protein source is reflected in the lowest DIAAS-like value calculated and the corresponding indispensable AA is considered the first limiting. The DIAAS-like values above 100% reflect a high quality protein source. Scores below 100%, but greater than 50%, are considered moderate, and scores below 50% are not considered to be a satisfactory source of that particular AA. Protein sources with DIAAS scores all equal or above 100% do not have a limiting AA in contrast with the reference protein.

Statistical analysis

All data were analyzed in SAS (SAS Institute Inc., version 9.4, Cary, NC) using the Mixed Models procedure. The procedure was conducted with protein ingredients as a fixed effect and roosters as a random effect. The Fisher-protected least significant difference test was used to determine the differences among treatments and was paired with a Tukey adjustment to control for type-1 experiment-wise error. Differences were considered statistically significant at $P < 0.05$.

Results and discussion

Chemical composition of select novel proteins

The chemical composition of these select novel proteins were reported as a percent on a DM basis (**Table 3.1**). Dry matter values ranged from 21.7% to 28.7%, with camel meat being the highest. Acid hydrolyzed fat values were the lowest relative to CP and OM, with the highest concentration belonging to beef at 57.27%. The lowest AHF concentration was 11.37% for yak meat, which is similar to results documented by Zi et al. (2004). Wild boar meat contained the highest OM content at 99.3% whereas kangaroo, beef, and camel ranged from 96.7% to 98%. Yak meat was highest in CP, about 84%, followed by camel (72.5%), kangaroo (67.3%), wild boar (50.3%), and beef (38.4%). The beef analyzed was mechanically deboned, a process by which residual meat from a bone is recovered using mechanical equipment that forces bones through a strainer (Akramzadeh et al., 2020). This process results in chemical compositions that are higher in fat and lower in protein as seen in a study by Serdaroğlu et al. (2005) where beef that was deboned by hand had 40% protein and 26% fat (DM basis) and mechanically deboned beef had 28% protein and 70% fat (DM basis), which is a pattern that is resembled in the current

study. Crude protein data of yak from the present study appear to be in agreement with previously reported CP concentration of 85% on a DM basis by Zi et al. (2004). In contrast, Hu et al. (2021) reported grazed yak and feedlot yak having 67.4% and 77.2% CP, respectively. When comparing the composition data of novel proteins with that of beef, the novel proteins generally reflected higher amounts of CP, lower fat, and lower GE. This pattern is supported by the findings of Elsharawy et al. (2018) and Mohammed et al. (2020), where camel meat surpassed beef in CP content, about 21% to 23.1% and 18.1% to 20% wet weight, respectively, and had lower fat concentrations, 1.5% to 1.7% in camel and about 6% to 12.2% in beef based on wet weight, as also reported for kangaroo steak meat (84.3% DM basis) in comparison with beef rump steak (82.5% DM basis) (Shul'gin et al., 2015; Luo et al., 2018). As is supported by the data of this study, the chemical composition of game meat typically reflects a leaner product, therefore having a lower fat content and higher, if not similar, CP concentration than domesticated production animals such as beef (Neethling et al., 2016). Variation in the chemical composition of the protein ingredients evaluated are likely due to differences in the types of meat cuts included, proportion of lean muscle to adipose tissue, but could also reflect the differences in carcass composition due to effects seasonally and reproductive cycle in the case of hunted (or not raised-farm) animals.

The complete AA profile was determined for each of the novel proteins and beef, and AA concentrations were reported as a percent by weight of protein source on DM basis (**Table 3.2**). Of the indispensable AA, the AA in highest concentration was lysine, 7.07%, in yak meat; camel and kangaroo were slightly lower, 6.31% and 5.21%, respectively. The highest concentration of arginine was also found in yak meat (5.32%), followed by camel (4.91%), kangaroo (4.43%), wild boar (2.66%), and beef (2.17%). The AA concentrations of beef were generally lower in the

current study than what has been previously reported by Faber et al. (2010) which could be attributed to the fact that only beef loin was tested whereas mechanically deboned beef was analyzed in our study. A study by Strazdina et al. (2014) contrasted the indispensable AA concentrations in wild boar against beef and pork to investigate the differences in AA composition between game meat and domesticated production animals. It was determined that wild boar and beef contained similar indispensable AA concentrations, about 24% and 27%, respectively whereas pork contained the lowest concentration at 12%. Compared to beef, camel has been reported to contain higher concentrations of arginine (8%), histidine (6.4%), isoleucine (6.4%), methionine (3.9%), valine (7.8%), and glutamic acid (19.3%) (Mohammed et al., 2020), which is supported by results from the current study in which the aforementioned AA were also greater (4.9%, 2.5%, 5.7%, 1.8%, 3.6%, and 10.7%, respectively) than beef. The AA composition of yak meat sourced from animals of different ages and sexes was analyzed and it was found that the levels of aspartic acid (about 8%), glutamic acid (about15%), lysine (about 5%) and leucine (about 7%) were prominent AA present in yak meat (Zi et al., 2004). In our study similar concentrations of those AA were also observed 7.4%, 12%, 7%, and 6.3%, respectively). Previous study has reported meats having the largest concentration of glutamic acid, about 17% (Williams, 2007); in the current study, yak meat has the highest concentration (12.02%) of this AA, followed by camel (10.78%), kangaroo (9.39%), wild boar (4.81%), and beef (3.79%).

Long chain FA in novel proteins were identified and are presented in **Table 3.3**. Beef contained the greatest concentrations of the most detected FA. This included myristic (1050.5 µg/g), palmitic (9457.7 µg/g), and oleic acids (15026.7 µg/g). Similar results have been obtained in the yak *Longissimus dorsi* muscle by Liu et al. (2021) and in the *Longissimus dorsi* muscle of

wild boar by Sales and Kotrba (2013) where palmitic acid was also found to be a prominent FA at 21%. and 25%, respectively, of total FA content. Palmitic and oleic acids were a major FA in the *Biceps femoris* muscle in camel at 26% and 32.2%, respectively, according to Shoman et al. (2019). Essential FA are members of the omega-3 and omega-6 FA and are required in the diets of companion animals as they have been evaluated to be beneficial for cardiovascular health, management of inflammation and skin diseases, and cognitive acuity (Pan et al., 2013; Lenox, 2015; Pan et al., 2018; Stice, 2019). The main FA found in meat include α-linolenic acid and linoleic acid (Wood et al., 2008). A study by Food Science Australia (2008) analyzed 4 cuts of meat from kangaroo (loin filet, knuckle, rump, and topside) and found that linoleic acid and α-linolenic acid were comprised about 20% and 7% of total polyunsaturated FA. The FA content was evaluated in the *Longissimus thoracis, Triceps brachii, semitendinosus, semimembranosus,* and *Biceps femoris* muscles in camel meat by Kadim et al. (2013) and it was determined that the palmitic acid was comprised the majority of the FA, ranging from 25% to 27%, which was also observed in our study. It was also found that camel meat had higher amounts of linoleic acid (about 7% to 8%) than α-linolenic acid (about 0.5% to 0.6%) (Kadim et al., 2013) which supports the findings in our study where linoleic acid was also greater than α-linolenic acid (301.6 µg/g and 100.8 µg/g, respectively). The highest concentration of stearic acid was observed in beef (8527.8 µg/g). It has been determined that rumen microbes convert linoleic acid to stearic acid (Kepler et al., 1966), which can explain the elevated concentration of stearic acid relative to camel (13.7 µg/g), wild boar (20.2 µg/g), and kangaroo (14.6 µg/g). Although yak is a ruminant, stearic acid concentration was lower than beef at 4.6 µg/g, which could be based on the cuts of meat. The cuts of yak meat received in our study were lean and for stewing in which various muscles are used (Pavan and Duckett, 2013) and may alter FA results.

Precision-fed cecectomized rooster assay

The data for standardized AA digestibility of indispensable AA and dispensable AA are presented in **Tables 3.4** and **3.5**, respectively. These data were calculated by application of the precision-fed cecectomized rooster assay, a method that has been validated by Johnson et al. (1998) to use as a direct comparison to AA digestibility in ileal-cannulated dogs. The microbial fermentation that occurs in the ceca of roosters can produce confounding results when determining AA digestibility as these are the main sites of microbial fermentation. Removal of the ceca allows for a more accurate estimation of ileal AA digestibility calculation by reducing AA degradation and synthesis (i.e, microbial cells) related to microbial fermentation and activity. This method also allows estimation of endogenous AA based on analysis of excreta from fasted birds (Elling-Staats et al., 2021). Ileal cannulated cat assays have been attempted (Mawby et al., 1999), but have been unsuccessful due to the temperamental nature of felines; however, it is assumed that the digestibility of protein in cats is comparable to dogs if protein digestibility is above 90% (Kendall et al., 1982). The standardized indispensable AA digestibility values were generally similar across the 5 protein sources; however, there was a significant difference only in the standardized digestibility of arginine. The arginine in yak, camel, and kangaroo meats were highly digestible, 94.72%, 94.58%, and 91.52%, respectively, but lower for beef (88.48%) and wild boar (81.48%). According to previous analysis, beef loin indispensable AA standardized digestibility values are greater than 80% (Faber et al., 2010) which is in alignment with results obtained from our study. The standardized dispensable AA digestibility values resemble those of indispensable AA, where alanine was the only AA differing among protein sources; yak meat had the greatest alanine digestibility (105.72%), followed by camel (103.51%), beef (96.04%),

kangaroo (95.86%), and wild boar (84.30%). Some standardized digestibility values for alanine were over 100%, but because these calculations are mainly an estimate, these greater percentages indicate that this particular AA was close to being 100% digestible by the roosters. A study by Deng et al. (2016) tested alternative proteins lamb meal, venison meal, and pork peptone using the precision fed cecectomized rooster study. Results indicated that the standardized AA digestibility for lamb meal was lower than other pseudo ruminants evaluated in the current study, camel and kangaroo. In Deng et al. (2016), tryptophan had the greatest standardized AA digestibility in lamb meal at 87.9% followed by methionine at 81.9%. All other indispensable AA were poorly digestible due to digestibility values being below 80%. Venison, however, had generally greater standardized AA digestibility where tryptophan digestibility was greater at 92.7% followed by arginine at 83.1%. The only indispensable AA that were poorly digestible were isoleucine (76.4%), histidine (77.3%), lysine (76.9%), and threonine (77.3%). When comparing those results with the AA digestibility of camel and kangaroo herein, results indicated that kangaroo and camel meats were highly digestible as all indispensable AA had digestibility values greater than 90%. The differences observed in the standardized AA digestibility between fresh meats and meat meals could be in part due to meat meal processing. Higher processing temperatures have been observed to decrease AA digestibility in meat and bone meals, especially in cysteine, methionine, lysine, and threonine (Wang and Parsons, 1998). Comparing results of pork peptone and wild boar standardized AA digestibility, arginine digestibility was higher in pork peptone (90.6%) than wild boar (81.4%). All other indispensable AA had lower digestibility values in pork peptone than in wild boar meat where all AA digestibility were greater than 90% except for arginine and valine (81.5%).

DIAAS-like values

The DIAAS is an adaptation of the protein digestibility corrected amino acid score (PDCAAS), a reference system used within the field of human nutrition to assign value to different dietary proteins. This method is recognized by the World Health Organization; however, it has been called into question because of its inherent limitations (Schaafsma, 2000). Calculations for PDCAAS use total tract digestibility, which does not account for AA losses to microbial fermentation as would ileal digestibility. In addition, the reference protein used does not reflect optimal AA intake as it is based on the minimum dietary protein requirements of various age groups. Furthermore, the calculated scores are truncated at 100% thereby underestimating high protein quality sources (Schaafsma, 2012).

Digestible indispensable amino acid scores are calculated using ileal digestibility values rather than total tract digestibility to achieve more accurate estimates of the bioavailability of individual AA. Ileal cannulated pigs have been used to determine protein quality by DIAAS calculations with reference proteins that were based on dietary protein requirements of infants, young children, adolescents, and adults (Mathai et al., 2017). In our study, in order to make a direct comparison to companion animal protein requirements, we applied the AAFCO nutrient profile and NRC recommended allowance as references to DIAAS-like calculations.

The DIAAS-like values of indispensable AA calculated using AAFCO nutrient profile and NRC recommended allowances as a reference protein for adult dogs at maintenance are reported in **Tables 3.6** and **3.7**. Most scores were above 100%, and in general camel, yak and kangaroo had DIAAS values greater than beef and wild boar. The lowest DIAAS-like score is considered first-limiting AA, if below 100%, and therefore determines the overall quality of a protein source. High quality proteins are characterized by the lowest AA being over 100%,

scores greater than 50% and less than 100% are moderate, and values lower than 50% designate the protein as insufficient as a source of the corresponding AA. When AAFCO nutrient profile of adult dogs was used as reference, tryptophan was the first-limiting AA in beef, wild boar, kangaroo (54.6%, 66.8%, and 87.6%, respectively), whereas yak and camel had no first limiting AA. The lowest score in yak meat was 116.74% for methionine, and in camel was tryptophan (134.2%). Beef, wild boar, and kangaroo are considered to be moderate quality while camel and yak are the high quality as all of their scores are above 100%. The DIAAS-like data pertaining to NRC recommended allowances for adult dogs at maintenance displayed more AA with scores less than 100%. Tryptophan was the first-limiting AA in beef (34.7%), wild boar (42.4%), and kangaroo (56.8%). The first-limiting AA in yak and camel meat was methionine (65.3% and 77.6%, respectively). According to our scoring system and NRC references, beef and wild boar would be insufficient sources of tryptophan for adult dogs at maintenance; yak and camel meat are of moderate quality. Additionally, the DIAAS-like score for tryptophan in camel meat was significantly higher than the same AA in the other four protein sources. The highest overall DIAAS-like value is in camel meat for lysine (244.46%).

The DIAAS-like calculations for adult cats at maintenance according to both AAFCO and NRC protein references were all generally higher than those of adult dogs at maintenance (**Tables 3.8** and **3.9**). Calculations based on AAFCO nutrient profile for adult cats resulted in tryptophan as the first-limiting AA for beef and wild boar, 78.9% and 96.5%, respectively. Kangaroo, yak, and camel contained no first-limiting AA although the lowest score for kangaroo meat was also tryptophan (126.6%) and threonine in yak and camel meat (130.2% and 155.2%, respectively). Similar to canine results, camel contained the highest ($p < 0.05$) DIAAS-like values for all indispensable AA with the highest overall being 332.1% in methionine. Based on

these results, wild boar and beef would be considered of moderate quality, whereas kangaroo, yak, and camel would be of high quality. The DIAAS-like values based on NRC recommended allowances for adult cats at maintenance were similar to results obtained using AAFCO as the protein reference. Tryptophan was the lowest DIAAS-like for beef (74.8%) and wild boar (91.4%). No first-limiting AA were present in kangaroo, yak, and camel thus labeling these meats as high quality. All DIAAS-like values for camel were significantly higher than the remaining novel protein sources and beef.

Calculating DIAAS scores for various protein sources is also important from a manufacturing standpoint due to the changes in protein structure induced by heat processing which naturally changes the quality of the meat. Bailey et al. (2020) calculated DIAAS scores for heat processed ribeye roast using swine standardized ileal digestibility values. Digestible indispensable AA scores for ribeye roast increased when cooked at 56ºC to 64ºC, however, DIAAS values decreased significantly when the meat was cooked at 72ºC. The first-limiting AA in the meat cooked to 72ºC were leucine and valine, both 99%, which designates this cut of meat as moderate quality whereas DIAAS of these same AA were 123% and 121%, respectively, in the meat cooked at 64ºC, making it a high quality protein.

Incongruities exist between dog and cat protein digestibility because of the differences in protein digestibility and absorption capacities. Golder et al. (2020), reported that cats have a significantly greater capacity to digest dietary protein compared to dogs. According to those authors, protein digestibility in cats fed either dry (95%) or wet (94%) diets were significantly greater than in dogs (86% and 89%, respectively).

Conclusion

The incorporation of novel protein sources in dog and cat diets has the potential to be advantageous because of the assumed higher quality of exotic meats as compared to more traditional dietary proteins already used in companion animal diets. The standardized AA digestibility from the cecectomized rooster assay indicate that all 5 proteins tested are highly digestible as none of the digestibility values fell below 81% in the indispensable AA category and 84% in the dispensable AA category. The DIAAS-like values based on AAFCO nutrient profile for both adult dogs and cats at maintenance revealed that all novel proteins tested are at least of moderate quality. Of the 4 novel proteins tested in the present study, yak and camel meats are believed to be high quality protein sources for adult dog maintenance diets and can be attractive novel proteins in pet food products; the same can be applied to cat diets for the corresponding life stage with the addition of kangaroo meat. The NRC DIAAS-like calculations yielded dissimilar results for adult dog maintenance because, according to NRC reference proteins, beef and wild boar are insufficient as a source of methionine. Counter to what has been reported for dogs, DIAAS-like values for cats based on NRC recommended allowance for adult cats remained relatively unchanged compared to AAFCO nutrient profile for cats. Specifically for cats, based on DIAAS-like scores, kangaroo, yak, and camel would be considered of high quality; whereas beef and wild boar moderate. Through this scoring method, the first-limiting AA have been identified as methionine and tryptophan for almost all proteins, meaning these AA may need to be supplemented with a compliment protein if used in future diet formulations. Future research is warranted to evaluate the nutrient digestibility of these novel protein sources once incorporated in pet food formulations, and their impact on palatability, nutrient digestibility, and fecal characteristics. In addition, very little is known about how current processing methods utilized in pet food manufacturing would affect the protein quality of these

ingredients. Since the vast majority of pet foods are processed using a wide range methods (e.g., extruded, retorted, lyophilized, baked, high pressure pasteurize, etc.) it would be important to understand potential trade-offs between nutritional value and safety of pet foods using these fresh meat ingredients.

Tables

Table 3.1. Chemical composition of select novel protein sources

	Mammalian Protein Sources				
Item %	**Beef**	**Wild Boar**	**Kangaroo**	**Yak**	**Camel**
Dry matter, %	24.4	26.5	21.7	27.0	28.7
			%, DM basis[1]		
Organic matter, %	98.2	99.3	81.6	95.5	98.4
Crude protein, %	38.3	50.3	67.3	84.7	72.4
Acid hydrolyzed fat, %	57.2	37.0	17.1	11.3	19.4
Gross energy, kcal/g	6.6	5.8	5.0	5.8	6.1

[1]DM – dry matter

Table 3.2. Amino acid concentrations of select novel protein sources as a percentage of the total crude protein

% by weight, DM basis[1]	Mammalian Protein Sources				
	Beef	**Wild Boar**	**Kangaroo**	**Yak**	**Camel**
Indispensable AA					
Arginine	2.1	2.6	4.4	5.3	4.9
Histidine	1.4	0.9	1.6	2.8	2.5
Isoleucine	1.0	1.6	2.7	3.8	3.4
Leucine	1.9	2.9	4.7	6.3	5.7
Lysine	1.9	2.7	5.2	7.0	6.3
Methionine	0.5	0.8	1.5	2.1	1.8
Phenylalanine	1.1	1.7	2.6	3.3	3.0
Threonine	1.0	1.5	2.6	3.5	3.2
Tryptophan	0.2	0.3	0.5	1.0	0.8
Valine	1.2	2.0	3.0	4.0	3.6
Dispensable AA					
Alanine	2.2	2.7	4.3	4.7	4.4
Aspartic acid	2.3	3.4	5.8	7.4	6.6
Cysteine	0.2	0.4	0.6	0.9	0.8
Glutamic acid	3.7	4.8	9.3	12.0	10.7
Proline	2.5	2.8	4.1	3.6	3.6
Serine	1.0	1.5	2.3	2.7	2.6
Tyrosine	0.7	1.1	2.4	3.3	3.0

[1]DM – dry matter

Table 3.3. Long chain fatty acid (LCFA) concentrations of select mammalian proteins

	Mammalian Protein Sources				
LCFA Concentration (µg/g, DMB)	**Beef**	**Wild Boar**	**Kangaroo**	**Yak**	**Camel**
Caprylic (C8:0)	3.0	6.0	5.1	0.0	0.00
Capric (C10:0)	15.6	12.1	8.3	1.4	2.1
Undecanoic (C11:0)	0.0	10.0	9.2	0.0	0.0
Lauric (C12:0)	23.1	12.7	11.5	2.4	2.3
Myristic (C14:0)	1050.6	267.2	236.9	111.5	129.5
Myristoleic (C14:1)	135.6	7.5	6.7	8.9	9.8
Pentadecanoic (C15:0)	174.9	32.1	27.6	37.9	40.6
Palmitic (C16:0)	9457.8	5174.8	4572.3	1820.1	2234.3
Palmitoleic (C16:1)	85.5	120.7	378.5	191.0	265.9
Heptadecanoic (C17:0)	7.6	114.8	103.7	154.1	3.7
cis-10-Heptadecenoic (C17:1)	278.3	62.9	51.2	56.6	86.9
Stearic (C18:0)	8527.9	20.3	14.7	4.6	13.8
Oleic (C18:1n9c)	15026.7	9961.8	8624.9	2910.9	4657.0
Elaidic (C18:1n9t)	0.0	49.1	51.9	1977.1	2900.1
Linoleic (C18:2n6c)	22.0	17.1	15.8	337.0	301.6
Linolelaidic (C18:2n6t)	63.4	58.2	54.0	60.3	63.1
α-linolenic (C18:3n3)	67.7	67.1	51.4	93.0	100.8

Table 3.3. (continued) Long chain fatty acid (LCFA) concentrations of select mammalian proteins

	Mammalian Protein Sources				
LCFA Concentration (µg/g, DMB)	**Beef**	**Wild Boar**	**Kangaroo**	**Yak**	**Camel**
Eicosasdienoic (C20:2)	3.4	77.9	69.2	8.7	2.6
Eicosatrienoic (C20:3n6)	25.4	5.4	3.8	31.6	25.3
Eicosatrienoic (C20:3n3)	3.3	15.1	11.4	2.4	4.1
Arachidonic (C20:4n6)	18.0	13.6	5.2	119.7	94.4
Eicosapentaenoic (C20:5n3)	21.5	4.0	4.9	29.4	24.1
Henicosanoic (C21:0)	9.1	18.0	20.4	4.3	5.9
Behenic (C22:0)	18.6	18.2	14.1	6.9	8.2
Euric (C22:1n9)	3.8	5.2	5.1	0.0	0.0
Docosadienoic (C22:2)	2.3	4.4	18.1	4.3	4.2
Docosahexaenoic (C22:6n3)	10.6	18.9	16.2	4.3	3.5
Ligonoceric (C24:0)	11.6	33.0	17.0	8.5	7.3
Nervonic (C24:1n9)	5.6	7.8	10.5	3.6	3.1

Table 3.4. Standardized amino acid digestibility of select novel proteins calculated using cecectomized rooster assay[1]

	Mammalian Protein Sources						
Indispensable Amino Acids, %	**Beef**	**Wild Boar**	**Kangaroo**	**Yak**	**Camel**	**SEM[2]**	**P-value**
Arginine	88.4[ab]	81.4[b]	91.5[a]	94.7[a]	94.5[a]	1.7054	0.0003
Histidine	95.8	91.7	93.9	97.8	97.3	1.4806	0.0569
Isoleucine	94.6	94.1	96.2	98.3	97.1	1.2902	0.1832
Leucine	94.1	93.4	95.3	98.5	96.6	1.6258	0.2396
Lysine	94.9	93.7	95.1	98.5	96.3	1.7105	0.3806
Methionine	96.6	94.8	95.9	99.5	97.4	2.0094	0.5606
Phenylalanine	93.3	90.8	94.9	98.8	97.0	2.7177	0.3124
Threonine	91.5	93.1	96.2	99.5	97.7	3.6112	0.5349
Tryptophan	94.1	95.1	95.9	98.7	97.4	1.4957	0.2400
Valine	89.9	81.5	90.5	95.2	90.4	7.1390	0.7470

[1]n = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-b] means within a row with different superscripts are significantly different at $P < 0.05$

Table 3.5. Standardized amino acid digestibility of select novel proteins calculated using cecectomized rooster assay[1]

	Mammalian Protein Sources						
Dispensable Amino Acids, %	**Beef**	**Wild Boar**	**Kangaroo**	**Yak**	**Camel**	**SEM[2]**	**P-value**
Alanine	96.0[ab]	84.3[b]	95.8[ab]	105.7[a]	103.5[a]	3.0454	0.0015
Aspartic acid	93.2	91.6	95.5	98.6	96.9	1.7482	0.0824
Cysteine	93.8	93.3	95.4	98.7	91.2	1.4710	0.0932
Glutamic acid	95.1	93.3	96.2	99.0	97.6	1.4205	0.0909
Proline	96.0	95.1	96.5	97.6	99.1	2.0700	0.6991
Serine	90.5	92.1	94.0	99.7	97.1	3.3223	0.3226
Tyrosine	95.9	94.5	95.9	99.3	97.6	1.6869	0.3594

[1]n = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-b] means within a row with different superscripts are significantly different at $P < 0.05$

Table 3.6. Digestible indispensable amino acid score (DIAAS)-like[1] values for select novel proteins compared with AAFCO nutrient profile for adult dogs at maintenance

	Mammalian Protein Sources						
Indispensable Amino Acids	**Beef**	**Wild Boar**	**Kangaroo**	**Yak**	**Camel**	**SEM[2]**	**P-value**
Arginine	177.6[d]	170.1[d]	214.0[b]	191.9[c]	234.4[a]	2.8834	0.0001
Histidine	131.8[d]	135.1[d]	201.4[c]	260.8[b]	318.0[a]	3.0635	0.0001
Isoleucine	116.2[d]	137.2[c]	180.4[b]	186.7[b]	216.6[a]	2.2208	0.0001
Leucine	119.1[d]	143.7[c]	172.1[b]	174.3[b]	205.3[a]	2.2424	0.0001
Lysine	133.1[c]	137.0[c]	199.6[b]	204.5[b]	243.6[a]	2.5492	0.0001
Methionine	66.8[d]	78.0[c]	114.0[b]	116.7[b]	138.7[a]	1.0586	0.0001
Phenylalanine	107.4[d]	126.8[c]	142.9[b]	137.1[b]	161.5[a]	2.0481	0.0001
Threonine	85.9[d]	103.4[c]	136.7[b]	137.5[b]	163.8[a]	3.5083	0.0001
Tryptophan	54.6[e]	66.8[d]	87.6[c]	119.1[b]	134.2[a]	1.4568	0.0001
Valine	109.5[d]	135.8[c]	152.2[b]	151.7[b]	179.0[a]	2.4317	0.0001
Met + Cys	84.1[d]	102.3[c]	139.0[b]	143.5[b]	168.8[a]	4.1194	0.0001
Phe + Tyr	119.6[d]	141.5[c]	185.4[b]	187.0[b]	221.1[a]	3.0536	0.0001

[1]n = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-e] means within a row with different superscripts are significantly different at $P < 0.05$

Table 3.7. Digestible indispensable amino acid score (DIAAS)-like[1] values for select novel proteins compared with NRC recommended allowances for adult dogs at maintenance

	Mammalian Protein Sources						
Indispensable Amino Acids	**Beef**	**Wild Boar**	**Kangaroo**	**Yak**	**Camel**	**SEM[2]**	**P-value**
Arginine	144.3^{d}	138.7^{d}	173.9^{b}	155.9^{c}	190.5^{a}	2.3438	0.0001
Histidine	74.0^{d}	75.8^{d}	113.0^{c}	146.4^{b}	178.5^{a}	1.7195	0.0001
Isoleucine	64.5^{d}	76.2^{c}	100.2^{b}	103.7^{b}	120.3^{a}	1.2341	0.0001
Leucine	66.1^{d}	79.8^{c}	95.6^{b}	96.8^{b}	114.1^{a}	1.2461	0.0001
Lysine	133.5^{c}	137.5^{c}	200.2^{b}	205.1^{b}	244.4^{a}	2.5575	0.0001
Methionine	37.3^{d}	43.6^{c}	63.7^{b}	65.3^{b}	77.6^{a}	0.5916	0.0001
Phenylalanine	59.9^{d}	70.7^{c}	79.7^{b}	76.5^{b}	90.1^{a}	1.1431	0.0001
Threonine	53.2^{d}	64.1^{c}	84.8^{b}	85.3^{b}	101.6^{a}	2.1747	0.0001
Tryptophan	34.7^{e}	42.4^{d}	56.8^{c}	75.6^{b}	85.2^{a}	0.8684	0.0001
Valine	61.1^{d}	75.7^{c}	84.9^{b}	84.6^{b}	99.8^{a}	1.3560	0.0001
Met + Cys	54.7^{d}	66.6^{c}	90.5^{b}	93.4^{b}	109.8^{a}	2.6807	0.0001
Phe + Tyr	119.3^{d}	141.2^{c}	185.0^{b}	186.6^{b}	220.7^{a}	3.0484	0.0001

[1]n = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-e] means within a row with different superscripts are significantly different at $P < 0.05$

Table 3.8. Digestible indispensable amino acid score (DIAAS)-like[1] values for select novel proteins compared with AAFCO nutrient profile for adult cats at maintenance

	Mammalian Protein Sources						
Indispensable Amino Acids	**Beef**	**Wild Boar**	**Kangaroo**	**Yak**	**Camel**	**SEM[2]**	**P-value**
Arginine	126.3[d]	121.4[d]	152.1[b]	136.4[c]	166.7[a]	2.0496	0.0001
Histidine	117.2[d]	120.0[d]	179.0[c]	231.8[b]	282.7[a]	2.7230	0.0001
Isoleucine	122.7[d]	144.8[c]	190.5[b]	197.0[b]	228.7[a]	2.3447	0.0001
Leucine	94.3[d]	113.8[c]	136.3[b]	138.1[b]	162.6[a]	1.7764	0.0001
Lysine	146.0[c]	150.4[c]	219.0[b]	224.4[b]	267.3[a]	2.7972	0.0001
Methionine	160.3[d]	187.2[c]	273.3[b]	279.9b	332.7[a]	2.5396	0.0001
Phenylalanine	166.9[d]	197.1[c]	222.1[b]	213.1[b]	251.0[a]	3.1841	0.0001
Threonine	81.3[d]	98.0[c]	129.5[b]	130.2[b]	155.2[a]	3.3225	0.0001
Tryptophan	78.9[e]	96.5[d]	126.6[c]	172.1[b]	193.9[a]	2.1055	0.0001
Valine	125.6[a]	155.7[c]	174.5[b]	173.9[b]	205.2[a]	2.7861	0.0001
Met + Cys	84.1[d]	102.3[c]	139.0[b]	143.5[b]	168.8[a]	4.1194	0.0001
Phe + Tyr	119.6[d]	141.5[c]	185.4[b]	187.0[b]	221.1[a]	3.0536	0.0001

[1]n = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-e] means within a row with different superscripts are significantly different at $P < 0.05$

Table 3.9. Digestible indispensable amino acid score (DIAAS)-like[1] values for select novel proteins compared with NRC recommended allowances for adult cats at maintenance

	Mammalian Protein Sources						
Indispensable Amino Acids	**Beef**	**Wild Boar**	**Kangaroo**	**Yak**	**Camel**	**SEM[2]**	**P-value**
Arginine	131.2[d]	126.1[d]	158.1[b]	141.8[c]	173.2[a]	2.1307	0.0001
Histidine	108.2[d]	110.8[d]	165.2[c]	214.0[b]	260.9[a]	2.5131	0.0001
Isoleucine	114.1[d]	134.7[c]	177.2[b]	183.3[b]	212.7[a]	2.1803	0.0001
Leucine	88.2[d]	106.4[c]	127.5[b]	129.1[b]	152.1[a]	1.6617	0.0001
Lysine	274.9[c]	283.1[c]	412.2[b]	422.4[b]	503.3[a]	5.2662	0.0001
Methionine	145.1[d]	169.4[c]	247.3[b]	253.3[b]	301.1[a]	2.2976	0.0001
Phenylalanine	134.8[d]	159.2[c]	179.4[b]	172.1[b]	202.7[a]	2.5713	0.0001
Threonine	88.1[d]	106.1[c]	140.2[b]	141.0[b]	168.0[a]	3.5973	0.0001
Tryptophan	74.7[e]	91.44[d]	122.3[c]	162.9[b]	183.5[a]	1.8700	0.0001
Valine	117.4[d]	145.6[c]	163.2[b]	162.6[b]	191.9[a]	2.6052	0.0001
Met + Cys	209.4[d]	254.6[c]	346.1[b]	357.3[b]	420.1[a]	10.2503	0.0001
Phe + Tyr	115.4[d]	136.6[c]	179.0[b]	180.5[b]	213.5[a]	2.9482	0.0001

[1]n = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-e] means within a row with different superscripts are significantly different at $P < 0.05$

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CHAPTER 4:

ASSESSING PROTEIN QUALITY OF SELECT NOVEL AVIAN PROTEIN SOURCES THROUGH STANDARDIZED AMINO ACID DIGESTIBILITY AND DIGESTIBLE INDISPENSABLE AMINO ACID SCORES FOR INCLUSION IN COMPANION ANIMAL DIETS.

Abstract

To meet the needs of consumers wanting to incorporate alternative protein sources in the diets of their companion animals, more products are being marketed to appeal to the idea of feeding pets the primitive diets of their ancestors. There is a lack of information available to characterize the macronutrient composition, amino acid (**AA**) content and protein digestibility and quality of exotic and novel proteins. The purpose of this study was to analyze the chemical composition and AA quality of chicken, goose, quail, duck, and emu meats by using the cecectomized rooster as a model. These parameters were also analyzed through calculation of DIAAS-like scores from standardized amino acid digestibility from the precision-fed cecectomized rooster assay. Chicken served as a reference for traditional avian proteins. Dry matter (**DM**) concentration across all protein sources ranging from 28.0% to 37.5%. On a DM basis, crude protein (**CP**) was lowest in goose (42%) and highest in emu meat (95.8%), whereas acid hydrolyzed fat (**AHF**) was lowest in emu (4.7%) and highest in quail (42.4%). Gross energy (**GE**) values ranged from 4.9 to 7.3 kcal/g. Standardized AA digestibility of select protein sources did not fall below 81% for dispensable AA and remained above 60% for indispensable AA. Differences ($P < 0.05$) were observed in the digestibility of arginine, histidine, isoleucine which were lower in duck than the remaining novel proteins. The protein references used for

DIAAS-like calculations were based on Association of American Feed Control officials (**AAFCO**) recommended values and National Research Council (**NRC**) recommended allowances for dogs and cats at maintenance. The DIAAS-like values for cats were generally higher compared to dogs where many of the AA coefficients for both AAFCO and NRC were well above 100%. When using reference protein values for adult cats, higher DIAAS-like scores were observed in contrast with DIAAS-like scores for adult dogs, with values well above 100%, According to the DIAAS-like system, the first-limiting AA was tryptophan across all protein sources, except for methionine being the first-limiting AA for goose meat when compared with NRC recommended allowance for adult dogs. For adult cats at maintenance, the chicken reference protein was the highest quality protein source overall along with goose and quail; these being absent of any first-limiting AA.

Introduction

The pet food market has been known to mirror the trends that arise in human nutrition. Consumers are favoring dietary proteins perceived as higher quality and thereby projecting these preferences onto their pets by choosing to feed diets that incorporate novel proteins. There is the perception that poultry by-products in pet food create a low quality diet which encourages the use of whole cuts of poultry as a protein alternative and makes human grade ingredients attractive to consumers (Deng and Swanson, 2015). Processing has been demonstrated to alter protein quality (Bellagamba et al., 2015) and has been attached to negative connotations which has inspired the implementation of different methods such as freeze drying. Freeze drying can offer a variety of benefits including preventing microbial proliferation to create a more stable shelf life and maintaining protein integrity and quality (Babić et al., 2009) therefore preserving the composition of raw ingredients that are intended for pet foods. Chicken that has been

processed by freeze drying has not been observed to be nutritionally different from fresh cuts (Harper and Tappel, 1957) which may provide insight into how other avian species may compare under identical processing conditions.

The avian proteins being tested herein are commonly consumed in parts of Europe, Asia, and Australia, but do not currently have a high demand in the U.S. market. There is information available on the chemical compositions of these protein sources; however, they are only in the context of human nutrition. Studies have been performed to investigate the protein digestibility of quail and duck (Kerr et al., 2014), but is limited to feline nutrition. Due to this more recent niche in the pet food market, there is a need for information on alternative protein sources such as chemical composition, amino acid (**AA**) profile, and assessment of protein quality and protein digestibility. At this moment, there have been no published scientific studies that use the precision-fed cecectomized rooster assay to estimate standardized AA digestibility as well as calculate digestible indispensable amino acid scores (**DIAAS**-like) to assign protein quality to raw or freeze dried goose, quail, duck, emu, and chicken. The purpose of this study was to determine the chemical composition of four select novel avian protein sources and compare with chicken which served as a control for commonly known dietary avian protein. This study utilized the calculation of standardized AA digestibility from the precision-fed cecectomized rooster assay to derive DIAAS-like values to estimate protein quality relative to AA nutrient profile values for adult dogs and cats at maintenance based on Association of American Feed Control officials (**AAFCO**) and the AA recommended allowances of the National Research Council (**NRC**).

Materials and methods

Select novel protein sources and sample preparation

The 4 select novel proteins analyzed in the present study are avian species and are compared with chicken which is a common dietary protein in pet diets. The novel proteins tested were goose (*Branta canadensis*; Exotic Meat Market. USA), quail (*Coturnix coturnix*; Exotic Meat Market. USA), duck (*Anas platyrhynchos domesticus*; Exotic Meat Market. USA), and emu (*Dromaius novaehollandiae*; Exotic Meat Market, USA). Each sample was freeze dried (FreeZone Bulk Tray Dryer, Labconco Corporation, Kansas City, MO) and ground to a uniform consistency in a Wiley mill (Arthur H Thomas Co. Wiley Mini Mill, 5206 MP) through a 2 mm sieve.

Chemical analyses

All 5 protein sources were weighed and analyzed in duplicate. Dry matter (**DM**) and ash concentrations were determined following AOAC (2006); methods 934.01 and 942.05. In addition, crude protein (**CP**) was evaluated by measuring total nitrogen with LECO (TruMac N, Leco Corporation, St. Joseph, MI), gross energy (**GE**) was measured by bomb calorimetry (Model 6200, Parr Instruments Co., Moline, IL), and total fat content was extracted by acid hydrolyzed fat (**AHF**) according to Budde (1952) and AACC (1983). A complete amino acid (**AA**) profile was compiled according to AOAC (2007) as well as a fatty acid profile.

Long chain fatty acid analysis

Each ingredient was weighed in duplicate (0.1 g) for fatty acid profile analysis. The fatty acid profiles were determined using modified methods of Lepage and Roy (1986) and Masood et al. (2005). Acetyl chloride, butylated hydroxytoluene (**BHT**), potassium carbonate, HPLC-grade methanol, and hexane were from Sigma-Aldrich (St. Louis, MO). To protect fatty acids from oxidation, BHT was added to methanol. Internal standard (nonadecanoic acid, 19:0) and external fatty acid methyl ester standards were from Supelco Sigma-Aldrich (St. Louis, MO). Internal standard was dissolved in methane-BHT solution (50 µg BHT/ml methanol) at 0.1 mg/ml concentration. The internal standard was added at 100 µl to test tubes that contained a 2mL methanol-hexane (4:1, v/v) mixture and were then vortexed and placed on ice. Tubes were swirled while slowly adding 200 µl of acetyl chloride, dropwise, and capped under N. Ingredient samples were heated for 10 min at 100ºC, vortexed, and heated again for 50 min; after, the tubes were placed in ice to cool. Cooled samples were neutralized by adding 5 mL of 6% Na_2CO_3 solution and vortexed again for 1 min each and centrifuged at 2300 *g* at 4 ºC for 3 min to separate the phases of the ingredient samples. The top organic phase was extracted and collected in a test tube and the process was repeated by adding 0.5 mL of hexane, vortexing, and centrifuging at the same speed and temperature for 3 min. The organic phase was extracted and combined with the first extraction and evaporated under N to 300 µL, and transferred to a gas chromatography (GC) vial with a 300 µL glass insert, and crimped under N for fatty acid methyl ether (**FAME**) analysis by GC.

Gas chromatography for long chain fatty acid analysis

To analyze every FAME, a Thermo Scientific TRACE 1300 Gas Chromatograph coupled with FID was used. Exacts of 1 ul were injected into the GCC and separated on a fused silica capillary column (SP-2560, 100 m length, 0.25 mm I.D., 0.2 um film thickness). The carrier gas, helium, had a flow rate of 20 cm/sec, at a split-ratio of 100:1. The starting temperature was 140ºC for 5 min and then was increased by 4ºC /min to reach a final temperature of 240ºC that was held for 15 min. The injector temperature was 250ºC and detector temperature was 260ºC. Nonadecanoic acid (C19:0, Nuchek Prep, Elysian, MN) was the internal standard. The FAME standards (Supelco 37 Component FAME Mix, Sigma Aldrich) were used as the external standard for the identification of long chain fatty acid peaks by retention time comparisons.

Precision-fed cecectomized rooster assay

The precision-fed cecectomized rooster assay was performed according to Parsons et al. (1982). All ground proteins were fed to four cecectomized Single Comb White Leghorn roosters that were kept in individual cages. The birds were fasted 26 hours before the trial. The roosters were crop intubated and fed a 1:1 mixture of protein sample and corn. Over 48 h, excreta were collected and at the end of the collection period the samples were freeze dried and ground into a powder for future analysis. The precision-fed cecectomized rooster assay is used to obtain a standardized amino acid digestibility measurement to estimate protein digestibility as described by Sibbald (1979).

Digestible indispensable amino acid score (DIAAS)-like values

Protein quality was determined through calculation of DIAAS-like values as described by Reilly et al. (2020). This method requires the use of a reference protein; therefore, in this study we used National Research Council (**NRC**, 2006) recommended allowances and Association of American Feed Control Officials (**AAFCO**, 2021) recommended nutrient profile for adult canine and feline. The DIAAS equation is formulated as amount in mg of digestible indispensable AA in 1g of dietary protein divided by amount in mg of the same indispensable AA in 1 g of reference dietary protein, multiplied by 100 and expressed as a percent. The lowest result from this calculation serves to estimate the overall quality of a protein source, referred to as the first-limiting amino acid. Scores of individual AA > 100% are characterized as high quality. Proteins with results that are greater than 50%, but below 100% are of moderate quality. Lastly, DIAAS-like values < 50% indicate that the protein in question does not qualify as a sufficient source of the corresponding amino acid. It is important to note that there is no first-limiting AA if all DIAAS-like scores are over 100%.

Statistical analysis

Data were analyzed in SAS (SAS Institute Inc., version 9.4, Cary, NC) using the Mixed Models procedure. Statistical significance was set at $P < 0.05$. This procedure was conducted with proteins as a fixed effect of treatment and roosters as a random effect. The Fisher-protected least significant difference test served to determine differences among treatments and worked in tandem with Tukey adjustment to control for experiment wise error.

Results and discussion

Chemical composition of select novel proteins

The chemical composition of the 4 select novel proteins and chicken were reported as a percent on a DM basis (**Table 4.1**). Dry matter varied from 27.9% to 37.5% for emu and quail, respectively. Concentration of AHF varied widely; being lowest for emu (4.7%) and greatest for quail at 42.5%. Crude protein values were generally similar among chicken (45.6%) and goose (42.0%), but lower than quail (56.2%), duck (67.8%), and emu that had the greatest concentration at 95.9%. The macronutrient composition of emu meat has been previously reported by Naveena et al. (2013) and supports the findings of fat and ash from the current study, 3% and 6.7%, respectively. However, Naveena et al. reported 84.6% protein which is lower than the value from our study. A similar determination was made where fresh emu meat contained about 86% CP (Pegg et al., 2006). Depending on the cuts of emu meat that were received and due to grinding and mixing different cuts together, protein concentration could be affected as there are higher proportions of lean meat in the leg muscles of emus as was noted by Sales and Horbanczuk(1998). Similar concentrations of CP in duck have been noted in the literature. Galal et al. (2011) reported about 74% CP in duck thigh meat and 76% CP in duck breast meat (DM basis). In contrast, Hamm and Ang (1982) found that CP concentration for quail was greater, about 74% (DM basis).

A complete AA profile was generated for all proteins tested herein where AA are expressed as a percent by weight of protein source on DM basis (**Table 4.2**). The most abundant AA in the indispensable AA category was arginine in emu meat comprising 5.9%, and lysine in chicken (3.3%), quail (3.6%), goose (2.7%), and duck (4.1%). Birds reared for meat consumption are highly dependent on dietary lysine for optimum muscle development. These

animals are typically fed diets that surpass the lysine requirement as lysine has been identified as the second limiting AA in common production bird diets (Siqueira et al., 2021), which could explain the elevated levels of lysine in the avian proteins tested. Among the dispensable AA category, all bird meats contained glutamic acid in the greatest concentration. Emu had the highest, 12.9%, followed by duck (7.3%), quail (6.1%), chicken (5.6%), and goose (4.3%).

A long chain fatty acid profile was created for each protein source and is presented in **Table 3.3**. Overall, emu meat had a lower fatty acid composition compared to the other proteins tested, it also lacked caprylic, capric, undecanoic, lauric, eicosatrienoic, euric, or docosadienoic acids. Furthermore, it was also the protein source with the lowest amounts of myristic and palmitic acids, 7.96 µg/g and 392.38 µg/g, respectively. Goose had the greatest amount of palmitic acid (8760.3 μg/g) as well as oleic acid (19553.59 µg/g). Goose and duck had the highest amounts of stearic acid, 2226.4 µg/g and 1344.9 µg/g, respectively. Concentration of eicosapentaenoic acid (**EPA**) were lower in chicken (4.4 µg/g), goose (2.6 µg/g), quail (5.7 µg/g), and duck (1.8 µg/g) compared to emu (23.5 µg/g). Docosahexaenoic acid (**DHA**) composition was similar in chicken (34.0 µg/g), goose (24.6 µg/g), quail (30.6 µg/g), and emu (39.0 µg/g); but lower in duck (8.8 µg/g). Alpha-linolenic acid (**ALA**) was highest in goose at 113.7 µg/g and the lowest in emu, 3.6 µg/g. Chicken was found to contain the least amount of linoleic acid (6.97 µg/g) aside from goose which had a concentration of 0 µg/g. Duck had the highest level of linoleic acid at 643.8 µg/g followed by quail (266.02 µg/g) and emu (53.6 µg/g). Goose and chicken had similar concentrations of arachidonic acid (6.2 µg/g and 7.7 µg/g, respectively) as did quail and duck (37.7 µg/g and 42.1 µg/g, respectively); however, emu contained the greatest at 174.6 µg/g. EPA and DHA are essential in canine and feline nutrition depending on species and life stage. According to AAFCO and NRC, EPA and DHA are required in the diets of puppies and kittens.

The NRC recommended allowances for EPA and DHA are lower for dog maintenance diets; however, an amount has not been established for dog maintenance by AAFCO. In cats, the NRC recommended allowance is the same as kittens. The AAFCO nutrient profiles do not have determined amount for cat maintenance diets (Bauer, 2008). Additionally, EPA and DHA are believed to possess anti-inflammatory qualities that aid in the management of osteo-arthritis (Johnson et al., 2020) and support cognitive ability (Tynes and Landsberg, 2021).

Precision-fed cecectomized rooster assay

Results for standardized AA digestibility of indispensable AA and dispensable AA of select novel proteins are displayed in **Table 4.4** and **Table** 4.**5** on an as fed basis, respectively. Testing avian meats for AA digestibility through the precision-fed cecectomized rooster assay has been performed prior to this study by Kerr et al. (2014) and Oba et al. (2019). Ground chicken, ground duck, and whole prey quail were analyzed for protein quality by Kerr et al. (2014) and the results indicated that there was no difference among chicken, duck, and quail for lysine standardized digestibility, which is similar to what was found in our study. There were no differences between duck and chicken for arginine and histidine standardized digestibilities (Kerr et al., 2014). In our study, chicken and duck had similar digestibilities for arginine (77.7% and 83.5%, respectively) and histidine, (86.5% and 87.1%, respectively); however, the digestibilities of these AA were greater ($P < 0.05$) in quail meat, 84.4% and 93%, respectively.

Standardized AA digestibility of raw chicken was evaluated by Oba et al. (20…) using the precision-fed cecectomized rooster assay. What authors found was that all indispensable AA were highly digestible with values greater than 80%, except for histidine (79.8%). In our study,

arginine was the only indispensable AA that had a digestibility value less than 80%. All others were highly digestible. The digestibility of arginine from emu and goose meat (95% and 84.5%, respectively) are higher ($P < 0.05$) than the remaining proteins. The histidine digestibility was higher ($P < 0.05$) in emu meat at 97.8%, whereas the digestibility of isoleucine in chicken was higher ($P < 0.05$), 99.2%, than the novel proteins. Significant differences in AA digestibility were also observed in the dispensable AA category, specifically in alanine and cysteine (**Table 4.5**). Emu and chicken had greater ($P < 0.05$) cysteine digestibility (97.2% and 98.1%, respectively) in contrast with goose (94.2%), quail (92.7%), and duck (87.1%). Alanine digestibility values were greater than 100% for chicken (101.9%) and goose (100.5%), but due to standardized AA digestibility being regarded as an estimate, these larger digestibility coefficients imply that alanine was highly digestible by the cecectomized rooster model.

DIAAS-like values

The DIAAS-like results for adult dogs at maintenance are presented in **Tables 4.6 and 4.7** and results for adult cats at maintenance are presented in **Tables 4.8 and 4.9**. According to DIAAS scoring system, the overall quality of a protein source is determined by the first-limiting AA, characterized by its smallest DIAAS-like value. The first-limiting AA was tryptophan for all protein sources tested, with the exception of methionine in goose meat when using NRC recommended allowances for adult dogs.

The AAFCO comparison for adult dogs reflects tryptophan as the first-limiting AA for all select protein sources. Duck had the lowest ($P < 0.05$) DIAAS-like coefficient, 63.5%. Emu and goose meat were the highest ($P < 0.05$) quality proteins with tryptophan DIAAS-like values of

99.7% and 97.7%, respectively. According to NRC recommended allowances for adult dogs, tryptophan was the first-limiting AA for chicken (57.8%), quail (49%), duck (40.1%), and emu (55.9%), while methionine was first-limiting in goose (58.5%). Based on these DIAAS-like scores, it can be determined that chicken, emu, and goose are comparable in protein quality, and can be determined to be of moderate quality.

The results for AAFCO and NRC comparisons for adult cats at maintenance indicate that tryptophan was the first-limiting AA for only duck and emu. All DIAAS-like values for chicken, goose, and quail were > 100%. The greatest DIAAS-like values ($P < 0.05$) using AAFCO recommendations were for methionine and were found in chicken and goose, 265.2% and 251.2%, respectively. Using NRC recommendations for adult cats, the greatest value ($P < 0.05$) reported was for lysine, 574.4%, in emu meat.

As a response to the limitations that are present in PDCAAS, the DIAAS scoring method was developed. By using DIAAS calculations, underestimation of protein quality is minimized by not truncating scores at 100% and endogenous AA losses are corrected for by using standardized ileal AA digestibility values from ileal-cannulated pigs (Schaafsma, 2012). In the present study, DIAAS calculations were modified using the standardized indispensable AA digestibility values from the cecectomized rooster assay and using AAFCO (2021) recommended values and NRC (2006) recommended allowances for CP of dog and cat maintenance diets as the reference proteins. These modifications allow for a better comparison with the species of interest resulting in DIAAS-like values. The use of DIAAS-like calculations within companion animal nutrition has increased in prevalence in recent years with research conducted by Oba et al. (2019). Referring to a study by Oba et al. (2019) where different chicken ingredients were evaluated by DIAAS-like scores, it was found that, when using AAFCO comparisons for adult

dogs, raw chicken had lower DIAAS-like values in all indispensable AA, except for histidine (184.7%) and tryptophan (111.8%), compared to the DIAAS-like values for chicken in the current study. Using NRC comparisons, taw chicken contained methionine as first-limiting (69%). For adult cats using both AAFCO and NRC comparisons, raw chicken contained no first-limiting AA. In our study, however, tryptophan was most often the first-limiting AA.

Conclusion

With a market that is becoming increasingly interested in alternative protein sources more data are emerging that characterizes the macronutrient composition and AA profiles of novel proteins for inclusion in canine and feline diets. The select novel proteins evaluated in the present study show promising results for their use in pet nutrition. Based on chemical composition alone, the results from novel proteins were comparable to chicken. Duck and emu contained the least amount of fat, emu had the greatest concentration of CP. When compared to chicken, a common protein source, emu and goose meat were found to be comparable to chicken based on DIAAS-like values using AAFCO and NRC comparisons for adult dogs and considered to be moderate quality. Based on this same criteria, quail and duck were determined to be low quality. For adult cats at maintenance goose and quail were determined to be the high quality protein sources that were most comparable to chicken. Goose and quail had no first-limiting AA according to NRC recommended allowances and AAFCO nutrient profile. Duck and emu were considered to be moderate quality based on DIAAS-like values that were less than 100% but greater than 50%. More research is warranted to determine the effects of these novel protein once incorporated on complete and balanced food on fecal characteristics, macronutrient digestibility, and overall diet acceptability of dogs and cats.

Tables

Table 4.1. Chemical composition of select avian protein sources

	Avian Protein Sources				
Item %, DMB[1]	**Chicken**	**Goose**	**Quail**	**Duck**	**Emu**
Dry matter, %	36.4	36.1	37.5	29.5	27.9
			%, DMB[1]		
Organic matter, %	97.6	97.5	85.7	95.9	95.3
Crude protein, %	45.5	42.0	56.1	67.8	95.8
Acid hydrolyzed fat, %	37.8	41.0	42.4	19.5	4.7
Gross energy, kcal/g	6.2	7.3	6.0	4.9	5.5

[1]DMB = dry matter basis

Table 4.2. Amino acid concentrations of select avian protein sources as a percentage of the total amino acids

% by weight, DM basis	Avian Protein Sources				
	Chicken	**Goose**	**Quail**	**Duck**	**Emu**
Indispensable AA					
Arginine	2.9	2.4	3.0	3.9	5.9
Histidine	1.1	0.8	1.1	1.2	2.8
Isoleucine	1.9	1.5	2.1	2.2	4.0
Leucine	3.0	2.5	3.4	3.8	6.8
Lysine	3.2	2.7	3.6	4.1	7.4
Methionine	0.9	0.8	1.0	1.2	2.0
Phenylalanine	1.7	1.5	2.0	2.2	3.6
Threonine	1.6	1.4	1.9	2.1	3.6
Tryptophan	0.3	0.3	0.4	0.4	1.0
Valine	2.0	1.7	2.2	2.5	4.4
Dispensable AA					
Alanine	2.9	2.2	2.8	4.0	5.7
Aspartic acid	3.5	3.0	4.1	4.6	8.0
Cysteine	0.3	0.4	0.4	0.5	0.9
Glutamic acid	5.6	4.3	6.1	7.3	12.9
Proline	2.4	2.0	2.3	3.8	5.0
Serine	1.3	1.2	1.6	1.9	3.0
Tyrosine	0.9	1.1	1.6	1.7	3.4

Table 4.3. Long chain fatty acid (LCFA) concentrations of select avian proteins

	Avian Protein Sources				
LCFA Concentration (µg/g, DM basis)	**Chicken**	**Goose**	**Quail**	**Duck**	**Emu**
Caprylic (C8:0)	13.3	8.0	25.2	2.5	0.0
Capric (C10:0)	4.3	2.8	7.0	1.6	0.0
Undecanoic (C11:0)	2.3	3.4	7.2	3.6	0.0
Lauric (C12:0)	7.7	19.8	9.9	3.2	0.0
Myristic (C14:0)	148.3	155.5	137.9	75.2	17.9
Myristoleic (C14:1)	21.6	11.1	15.4	8.8	18.9
Pentadecanoic (C15:0)	31.1	26.0	40.6	20.3	14.1
Palmitic (C16:0)	5109.8	8760.3	3173.9	3420.6	392.3
Palmitoleic (C16:1)	66.9	128.5	54.7	405.4	52.9
Heptadecanoic (C17:0)	47.0	30.0	41.6	9.7	61.9
cis-10-Heptadecenoic (C17:1)	27.7	26.7	19.1	13.1	27.4
Stearic (C18:0)	34.2	2226.4	37.2	1344.9	932.7
Oleic (C18:1n9c)	32.0	19553.5	5187.6	5938.3	40.8
Elaidic (C18:1n9t)	310.8	0.0	375.7	19.8	190.6
Linoleic (C18:2n6c)	6.9	0.0	266.0	643.8	53.6
Linolelaidic (C18:2n6t)	48.0	42.5	49.5	49.2	24.3
α-linolenic (C18:3n3)	16.0	113.7	8.1	20.0	3.6

Table 4.3. (continued) Long chain fatty acid (LCFA) concentrations of select avian proteins

LCFA Concentration (µg/g, DM basis)	Avian Protein Sources				
	Chicken	**Goose**	**Quail**	**Duck**	**Emu**
Eicosasdienoic (C20:2)	4.4	65.5	23.7	22.0	3.6
Eicosatrienoic (C20:3n6)	2.8	16.1	4.3	7.1	27.8
Eicosatrienoic (C20:3n3)	1.9	21.3	15.2	0.9	0.0
Arachidonic (C20:4n6)	7.7	6.2	37.7	42.1	174.6
Eicosapentaenoic (C20:5n3)	4.4	2.6	5.7	1.8	23.5
Henicosanoic (C21:0)	30.1	12.2	26.3	10.0	4.1
Behenic (C22:0)	14.2	15.8	25.0	21.4	13.1
Euric (C22:1n9)	5.1	4.8	2.6	4.4	0.0
Docosadienoic (C22:2)	4.0	2.3	0.0	5.8	0.0
Docosahexaenoic (C22:6n3)	34.0	24.6	30.6	8.8	39.0
Ligonoceric (C24:0)	7.0	7.1	18.4	17.5	10.9
Nervonic (C24:1n9)	13.5	8.5	22.2	29.5	4.9

Table 4.4. Standardized amino acid digestibility of select avian proteins calculated using cecectomized rooster assay[1]

Indispensable Amino Acids, %	Avian Protein Sources					SEM[2]	P-value
	Chicken	Goose	Quail	Duck	Emu		
Arginine	77.7[b]	84.5[a]	84.4[ab]	83.5[b]	95.0[a]	2.4814	0.0034
Histidine	86.5[b]	93.1[ab]	93.0[ab]	87.1[b]	97.8[a]	2.2939	0.0175
Isoleucine	99.2[a]	94.9[ab]	93.9[ab]	91.7[b]	98.1[ab]	1.6027	0.0296
Leucine	95.5	93.7	92.6	90.0	97.5	2.2371	0.2167
Lysine	97.0	93.3	92.5	88.4	96.8	2.5000	0.1461
Methionine	97.4	94.3	94.3	90.2	97.7	2.8618	0.3453
Phenylalanine	90.7	90.5	93.1	87.6	97.2	3.3334	0.3696
Threonine	92.5	90.8	89.8	87.8	97.1	3.1851	0.3463
Tryptophan	94.9	95.7	94.9	88.0	98.1	2.2343	0.0610
Valine	82.9	72.9	64.1	69.8	90.0	8.7794	0.2785

[1]*n* = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-b]Means within a row with different superscripts are significantly different at $P < 0.05$

Table 4.5. Standardized amino acid digestibility of select novel proteins calculated using cecectomized rooster assay[1]

	Avian Protein Sources						
Dispensable Amino Acids, %	**Chicken**	**Goose**	**Quail**	**Duck**	**Emu**	**SEM[2]**	**P-value**
Alanine	101.9[a]	100.5[ab]	81.4[c]	81.7[bc]	98.9[ab]	4.3309	0.0052
Aspartic acid	92.0	92.7	92.7	88.4	97.5	2.2738	0.1413
Cysteine	98.1[a]	94.2[ab]	92.7[ab]	87.1[b]	97.2[a]	2.3073	0.0306
Glutamic acid	96.1	93.1	93.6	88.9	98.2	2.2338	0.0909
Proline	96.3	95.5	94.1	86.7	94.6	3.2594	0.2851
Serine	95.1	90.0	89.4	83.2	96.8	4.2667	0.2332
Tyrosine	98.1	94.5	93.9	90.3	98.1	2.3963	0.1721

[1]*n* = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-c]Means within a row with different superscripts are significantly different at $P < 0.05$

Table 4.6. Digestible indispensable amino acid score (DIAAS)-like[1] values for select novel proteins compared with AAFCO nutrient profile for adult dogs at maintenance

	Avian Protein Sources						
Indispensable Amino Acids, %	**Chicken**	**Goose**	**Quail**	**Duck**	**Emu**	**SEM[2]**	**P-value**
Arginine	219.8^{a}	197.8^{b}	178.0^{c}	174.3^{c}	208.3^{ab}	4.1260	0.0001
Histidine	178.7^{b}	166.9^{b}	167.6^{b}	139.3^{c}	257.4^{a}	4.5087	0.0001
Isoleucine	191.3^{b}	168.1^{c}	163.2^{c}	137.5^{d}	374.5^{a}	3.7102	0.0001
Leucine	173.6^{b}	155.2^{bc}	148.8^{cd}	133.4^{d}	643.2^{a}	4.8377	0.0001
Lysine	179.2^{b}	173.7^{b}	167.9^{b}	147.3^{c}	700.1^{a}	4.3752	0.0001
Methionine	110.6^{b}	104.7^{b}	97.4^{c}	87.6^{d}	196.9^{a}	1.5180	0.0001
Phenylalanine	145.6^{b}	140.8^{b}	130.5^{bc}	115.8^{c}	337.5^{a}	3.5691	0.0001
Threonine	124.5^{b}	120.1^{bc}	114.3^{bc}	102.1^{c}	345.2^{a}	4.4198	0.0001
Tryptophan	91.1^{b}	97.7^{a}	77.2^{c}	63.2^{d}	99.7^{a}	1.4064	0.0001
Valine	158.6^{b}	141.5^{cb}	134.6^{cd}	121.6^{d}	420.7^{a}	4.3236	0.0001
Met+Cys	143.6^{ab}	137.8^{ab}	119.7^{b}	115.1b	162.6^{a}	4.9686	0.0001
Phe+Tyr	151.3^{bc}	161.7^{b}	160.7^{b}	138.0^{c}	193.7^{a}	4.8935	0.0001

[1]*n* = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-d]Means within a row with different superscripts are significantly different at $P < 0.05$

Table 4.7. Digestible indispensable amino acid score (DIAAS)-like[1] values for select novel proteins compared with NRC recommended allowances for adult dogs at maintenance

	Avian Protein Sources						
Indispensable Amino Acids, %	**Chicken**	**Goose**	**Quail**	**Duck**	**Emu**	**SEM[2]**	**P-value**
Arginine	178.6^{a}	160.8^{b}	144.7^{c}	141.6^{c}	169.3^{ab}	3.3526	0.0001
Histidine	100.3^{b}	93.7^{b}	94.1^{b}	78.2^{c}	144.5^{a}	2.5313	0.0001
Isoleucine	106.2^{b}	93.4^{c}	90.6^{c}	76.4^{d}	210.3^{a}	2.0645	0.0001
Leucine	96.4^{b}	86.2^{bc}	82.7^{cd}	74.1^{d}	361.1^{a}	2.6984	0.0001
Lysine	179.7^{b}	174.3^{b}	168.4^{b}	147.8^{c}	393.0^{a}	4.0151	0.0001
Methionine	61.8^{b}	58.5^{b}	54.4^{c}	48.9^{d}	110.5^{a}	0.8486	0.0001
Phenylalanine	81.2^{b}	78.6^{b}	72.8^{bc}	64.6^{c}	189.4^{a}	1.9945	0.0001
Threonine	77.2^{b}	74.5^{bc}	70.8^{bc}	63.3^{c}	193.8^{a}	2.6392	0.0001
Tryptophan	57.8^{b}	62.0^{a}	49.0^{c}	40.1^{d}	55.9^{b}	0.8792	0.0001
Valine	88.5^{b}	78.9^{bc}	75.1^{cd}	67.8^{d}	236.2^{a}	2.4163	0.0001
Met+Cys	80.0^{ab}	76.8^{ab}	66.7^{b}	64.1^{b}	90.6^{a}	4.4398	0.0001
Phe+Tyr	147.7^{bc}	157.8^{b}	156.8^{b}	134.7^{c}	189.0^{a}	4.7761	0.0001

[1]*n* = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-d]Means within a row with different superscripts are significantly different at $P < 0.05$

Table 4.8. Digestible indispensable amino acid score (DIAAS)-like[1] values for select novel proteins compared with AAFCO nutrient profile for adult cats at maintenance

	Avian Protein Sources						
Indispensable Amino Acids, %	**Chicken**	**Goose**	**Quail**	**Duck**	**Emu**	**SEM[2]**	**P-value**
Arginine	156.3^{a}	140.6^{b}	126.6^{c}	123.9^{c}	148.1^{ab}	2.9343	0.0001
Histidine	158.8^{b}	148.3^{b}	149.0^{b}	123.8^{c}	228.8^{a}	4.0072	0.0001
Isoleucine	201.9^{b}	177.4^{c}	172.2^{c}	145.1^{d}	332.9^{a}	3.8202	0.0001
Leucine	137.5^{b}	123.0^{bc}	117.9^{c}	105.6^{c}	571.7^{a}	1.0036	0.0001
Lysine	196.6^{b}	190.6^{b}	184.2^{b}	161.7^{c}	622.3^{a}	4.6001	0.0001
Methionine	265.2^{a}	251.2^{a}	233.7^{b}	210.0^{c}	175.0^{d}	3.5177	0.0001
Phenylalanine	226.4^{b}	218.9^{bc}	202.8^{c}	180.0^{d}	300.0^{a}	5.0718	0.0001
Threonine	117.9^{b}	113.7^{bc}	108.2^{bc}	96.7^{c}	306.8^{a}	4.0839	0.0001
Tryptophan	131.7^{b}	141.1^{a}	111.5^{c}	91.3^{d}	88.6^{d}	1.9368	0.0001
Valine	181.9^{b}	162.3^{bc}	154.3^{cd}	139.4^{d}	373.9^{a}	4.6478	0.0001
Met+Cys	122.9^{ab}	118.0^{ab}	102.5^{b}	98.5^{b}	139.2^{a}	6.8216	0.0001
Phe+Tyr	148.0^{bc}	158.1^{b}	157.2^{b}	134.9^{c}	189.4^{a}	4.1857	0.0001

[1]*n* = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-d]Means within a row with different superscripts are significantly different at $P < 0.05$

Table 4.9. Digestible indispensable amino acid score (DIAAS)-like[1] values for select novel proteins compared with NRC recommended allowances for adult cats at maintenance

	Avian Protein Sources						
Indispensable Amino Acids, %	**Chicken**	**Goose**	**Quail**	**Duck**	**Emu**	**SEM[2]**	**P-value**
Arginine	162.4^{a}	146.1^{b}	131.5^{c}	128.8^{c}	153.9^{ab}	3.0480	0.0001
Histidine	146.6^{b}	139.9^{b}	137.5^{b}	114.3^{c}	211.2^{a}	3.6995	0.0001
Isoleucine	187.8^{b}	165.0^{c}	160.2^{c}	135.0^{d}	307.3^{a}	3.5504	0.0001
Leucine	128.6^{b}	115.0^{bc}	110.2^{c}	98.8^{c}	527.7^{a}	3.7242	0.0001
Lysine	370.1^{b}	358.9^{b}	346.8^{b}	304.4^{c}	574.4^{a}	8.0807	0.0001
Methionine	240.0^{a}	227.3^{a}	211.5^{b}	190.1^{c}	161.6^{d}	3.1830	0.0001
Phenylalanine	182.8^{b}	176.8^{bc}	163.8^{c}	145.4^{d}	276.9^{a}	4.1561	0.0001
Threonine	127.7^{b}	123.2^{b}	117.2^{bc}	104.8^{c}	283.2^{a}	4.1908	0.0001
Tryptophan	124.6^{b}	133.6^{a}	105.5^{c}	86.5^{d}	81.8^{d}	1.8313	0.0001
Valine	170.1^{b}	151.7^{bc}	144.3^{cd}	130.3^{d}	345.2^{a}	4.3351	0.0001
Met+Cys	306.0^{ab}	293.7^{ab}	255.1^{b}	245.3^{b}	346.5^{a}	16.9775	0.0001
Phe+Tyr	142.8^{bc}	152.6^{b}	151.7^{b}	130.3^{c}	182.8^{a}	4.6205	0.0001

[1]*n* = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-d]Means within a row with different superscripts are significantly different at $P < 0.05$

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CHAPTER 5:

DETERMINING PROTEIN QUALITY OF SELECT NOVEL AQUATIC AND REPTILE PROTEIN SOURCES FOR APPLICATION IN CANINE AND FELINE NUTRITION USING THE PRECISION-FED CECECTOMIZED ROOSTER ASSAY AND DIGESTIBLE INDISPENSABLE AMINO ACID SCORES

Abstract

The objective of this study was to determine the chemical composition and amino acid (**AA**) quality of carp slurry, white fish, spirulina, eel, salmon, and rattlesnake by application of the cecectomized rooster model. Through this model, standardized AA digestibility was determined and digestible indispensable AA (DIAAS)-like scores were calculated. Dry matter (**DM**) values ranged from 22.1% to 34.2% for fish and rattlesnake ingredients, while DM of spirulina was higher at 95.3%. Crude protein (**CP**) was highest in carp slurry and spirulina (72.5% and 72.8% DM basis, respectively) and lowest in salmon (43.5%). Acid hydrolyzed fat (**AHF**) concentrations were variable as rattlesnake had the lowest amount of total fat at 1.8% and almon contained the most at 57%. The standardized AA digestibility values for all protein sources remained above 80% for both indispensable and dispensable AA and were considered to be highly digestible. To obtain DIAAS-like calculations, reference proteins were used for adult dogs and cats at maintenance according to the nutrient profiles of the Association of American Feed Control officials (**AAFCO**) and the National Research Council (**NRC**) recommended allowances. Rattlesnake was consistently low quality for both dogs and cats using AAFCO and NRC comparisons and tryptophan was determined to be the first-limiting AA. DIAAS-like values for tryptophan using AAFCO comparisons for dogs and cats were 39% and 27.7%,

respectively and values using NRC comparisons for dogs and cats were 31.7% and 28.8%, respectively. Carp slurry and spirulina were determined to be high quality according to each comparison, except when using NRC recommended allowance as the reference protein for dogs at maintenance where these proteins received moderate scores, 79.5% and 63.3%, respectively.

Introduction

There has been no decline in amount of pet owners in the U.S. for several years, with the most recent surge in animal adoption brought on by the COVID-19 pandemic (Ho et al., 2020). With more time spent at home and a greater emotional connection with pets (Applebaum et al., 2021), pet owners have been inclined to devote more attention to what they are feeding their companions. Although sustainability has tag appeal to consumers, pet food purchasing decisions have also become ingredient focused (Schleicher et al., 2019; Kwak and Cha, 2021). While new avenues are being explored, it is still important to ensure that the dietary requirements of companion animals are being met. Protein is the most expensive ingredient; therefore, emphasizing the need for further investigation into alternative sources that are both environmentally and economically feasible while still meeting nutrient requirements.

Whitefish and salmon are some aquatic proteins that are already being used in pet products whereas more novel sources include eel and spirulina. Whitefish protein may contain one or more of fish species with white meat, these include pollock, haddock, hoki, hake, cod, redfish, roughies, whiting and Chilean seabass (World Wild Life, 2012). Many novel ingredients have not been approved for use in pet foods thereby limiting use to only treats such as spirulina. An interesting aspect of spirulina is that it can grow in a variety of environments and is able to efficiently utilize less water which facilitates its cultivation on a commercial scale (Afroz and

Singh, 2021). There is some concern regarding the possibility of the over-exploitation of some novel proteins as they may be considered an endangered species in other communities. One such animal protein is eel meat (Radio New Zealand, 2012) which further emphasizes the need for research regarding the nutritional composition of farm raised eels, perhaps of various species, and how it compares to that of wild caught in order to avoid the over consumption of a limited resource. Common carp is an invasive species that inhabits the shallow waters of North America (Pearson et al., 2019) and is known for being reproductively successful and disrupting local aquatic ecosystems (Dauphinais et al., 2018). Removal and management of common carp has been an ongoing project since the early 20th century and products for human use such as fertilizers, fish bait, and fish meal have been derived from these fish to combat population growth (Weber et al., 2011). Incorporation of common carp in pet food may be a potential solution to mitigating the impact of this invasive species. Reptiles are rare pet food ingredients; however, alligator is becoming incorporated into specialty diets. Snake species are not common in pet food, but there are edible species that include rattlesnakes, boa constrictors, cobra, and garden-type snakes (Newman, 2001).

There is limited information available on the nutrient composition of these ingredients. However, there has yet to be data available concerning standardized AA digestibility and digestible indispensable amino acid (**DIAAS**)-like scores for determination of protein quality for possible application in companion animal nutrition. Therefore, the purpose of this study was to further analyze the chemical composition of novel sources of aquatic and reptile proteins as compared with more traditional aquatic protein sources like salmon and whitefish. Standardized AA digestibility was calculated using the precision-fed cecectomized rooster assay to obtain DIAAS-like coefficients to determine protein quality in relation to the nutritional requirements of

adult dogs and cats at maintenance following recommended allowance and nutrient profile as provided by the Association of American Feed Control officials (**AAFCO**) and the National Research Council (**NRC**), respectively.

Materials and methods

Select novel protein sources and sample preparation

The select novel proteins analyzed in this study were raw aquatic and reptile species, powdered Spirulina (*Athrospira*; sourced from Chippin Inc., Silver Spring, MD, USA), carp slurry (*Cyprinus carpio*; sourced from Chippin Inc., Silver Spring, MD, USA), ground eel (Anguilloformes; human-grade ingredient from Exotic meat Market, USA), rattlesnake (Crotalinea; human-grade ingredient from Exotic Meat Market, USA). These protein ingredients were compared with ground whitefish (Northern Pelagic Group LLC, New Bedford, MA, USA), and ground salmon (*Salmo salar*; Jaemar Inc., Carlsbad, CA, USA) which are common protein ingredients in companion animal diets. All proteins were freeze dried (FreeZone Bulk Tray Dryer, Labconco Corporation, Kansas City, MO) and finely ground through a 2 mm sieve of a Wiley mill (Arthur H Thomas Co. Wiley Mini Mill, 5206 MP).

Chemical analyses

All select novel proteins were weighed and analyzed in duplicate. Dry matter (**DM**) and ash content were determined according to AOAC (2006); methods 934.01 and 942.05. Bomb calorimetry (Model 6200, Parr Instruments Co., Moline, IL) was used to measure gross energy

(**GE**). Crude protein (**CP**) was determined by measuring total N with LECO (TruMac N, Leco Corporation, St. Joseph, MI). Total fat content was extracted by acid hydrolyzed fat (**AHF**) as described by Budde (1952) and AACC (1983). Total dietary fiber (**TDF**) was measured for spirulina according to Prosky et al. (1992). Spirulina was the only aquatic protein chosen to be analyzed for TDF due to the intrinsic characteristic of the ingredient. Complete amino acid (AA) profiles were generated according to AOAC (2007) as well as a long chain fatty acid profile as described below.

Long chain fatty acid analysis

A 0.1 g sample of each ingredient was weighed in duplicate for fatty acid profile analysis. Ingredient fatty acid profiles were determined using modified methods of Lepage and Roy (1986) and Masood et al. (2005). Acetyl chloride, butylated hydroxytoluene (**BHT**), potassium carbonate, HPLC-grade methanol, and hexane were purchased from Sigma-Aldrich (St. Louis, MO). To prevent fatty acid oxidation, BHT was added to methanol. Internal standard (nonadecanoic acid, 19:0) and external fatty acid methyl ester standards were obtained from Supelco Sigma-Aldrich (St. Louis, MO). Internal standard was dissolved in methane-BHT solution (50 µg BHT/ml methanol) at 0.1 mg/ml concentration. The internal standard was added at 100 µl to test tubes that contained a 2mL methanol-hexane (4:1, v/v) mixture and were then vortexed and placed on ice. Tubes were swirled while slowly adding 200 µl of acetyl chloride, dropwise, and capped under N. Ingredient samples were heated for 10 min at 100ºC, vortexed, and heated again for 50 min; after, the tubes were placed in ice to cool. Cooled samples were neutralized by adding 5 mL of 6% Na_2CO_3 solution and vortexed again for 1 min each and

centrifuged at 2300 *g* at 4 ºC for 3 min to separate the phases of the ingredient samples. The organic phase at the top was extracted and collected in a test tube and the process was repeated by adding 0.5 mL of hexane, vortexing, and centrifuging at the same speed and temperature for 3 min. The organic phase was extracted and combined with the first extraction and evaporated under N to 300 µL, and transferred to a gas chromatography (GC) vial with a 300 µL glass insert, and crimped under N for fatty acid methyl ether (**FAME**) analysis by GC.

Gas chromatography for long chain fatty acid analysis

To analyze individual FAME, a Thermo Scientific TRACE 1300 Gas Chromatograph coupled with FID was used. Exacts of 1 ul were injected into the GCC and separated on a fused silica capillary column (SP-2560, 100 m length, 0.25 mm I.D., 0.2 um film thickness). The carrier gas, helium, had a flow rate of 20 cm/sec, at a split-ratio of 100:1. The starting temperature was at 140ºC for 5 min and then was increased in increments of 4ºC per minute to reach a final temperature of 240ºC that was held for 15 min. The injector temperature was 250ºC and detector temperature was 260ºC. Nonadecanoic acid (C19:0, Nuchek Prep, Elysian, MN) was the internal standard. The FAME standards (Supelco 37 Component FAME Mix, Sigma Aldrich) were used as the external standard to facilitate the identification of long chain fatty acid peaks by retention time comparisons.

Precision-fed cecectomized rooster assay

The precision-fed cecectomized rooster assay was conducted as described by Parsons et al. (1982). Four cecectomized Single Comb White Leghorn roosters were fed each of the finely ground proteins after being fasted for 26 hours before the trial. The birds were crop intubated and a 1:1 mixture of protein sample and ground corn were fed. Excreta were collected over 48 hours, freeze dried, and ground to be analyzed. This assay was used to calculate standardized AA digestibility according to Sibbald (1979).

Digestible indispensable amino acid score (DIAAS)-like values

Through calculation of DIAAS-like values, the quality of the novel protein sources was determined as described by Reilly et al. (2021). Reference proteins were required for this calculation; therefore, we applied the recommended nutrient profile of the Association of American Feed Control Officials (**AAFCO,** 2021) and recommended allowances from the National Research Council (**NRC**, 2006) for adult dogs and cats. DIAAS-like values are expressed as a percent and were determined by multiplying the ratio of mg of digestible indispensable AA in 1g dietary protein to mg of the same indispensable AA in 1g of reference protein by 100. The first-limiting AA is the lowest DIAAS-like value and describes the overall quality of a protein. Amino acids that are scored greater than 100% are considered high quality, scores less than 100% but greater than 50% are moderate quality, and scores less than 50% are characterized as being an insufficient source of the respective AA. If all DIAAS-like values are over 100%, then there is no first-limiting AA.

Statistical analysis

Data were analyzed in SAS (SAS Institute Inc., version 9.4, Cary, NC). Mixed Models procedure was applied, and statistical significance was set at $P < 0.05$. This procedure was conducted with roosters as a random effect and proteins as a mixed effect of treatment. The Fisher-protected least significant difference test allowed determination of differences among treatments and operated with Tukey adjustment control for experiment wise error.

Results and discussion

Chemical composition of select novel proteins

The chemical composition of the tested protein sources is displayed in **Table 5.1** and reported on a DM basis as a percent. Spirulina contained the greatest DM overall at 95.3% due to being received as a powder. Spirulina was followed by salmon at 34.2%, eel at 30.5%, rattlesnake at 30.1%, whitefish at 22.8%, and carp slurry at 22.1%. Total dietary fiber was analyzed for spirulina with an insoluble portion of 18.2% and soluble portion of 1.8% which is higher than what has been presented in the literature where the insoluble and soluble fractions were determined to be 6.8% and 2%, respectively; however, CP values in the literature are in agreement with what has been determined in the current study (71.3% and 72.8%, respectively) (Raczyk et al., 2022). The amount of CP was similar in carp slurry (72.5%), whitefish (70.3%), and spirulina (72.8), but was lowest in salmon at 43.5%, and intermediate for eel (58.7%) and rattlesnake (60.9%). A wider range was observed for AHF, where salmon contained the largest amount, 57%, and rattlesnake the lowest, 1.8%. Rattlesnake and eel samples still contained bones at the time of grinding, which may explain the elevated ash concentrations (38.3% and 13%,

respectively) compared to the lower values that have been reported by Ljubica et al. (2002), Ockerman and Basu (2009), and Wijauanti and Susilo (2018). In prior research, CP in spirulina has been reported to be as high as 70% (Liu, 1997) and fat concentrations range from 4% to 16% (Holman and Malau-Aduli, 2012). These data support the findings in this study with a CP value that is slightly greater and a comparable fat concentration at 7.1%. The CP value for salmon was the lowest among the protein sources; however, similar results were presented by Aas et al. (2019) where CP for salmon fillet and whole salmon were 51% and 41% (DM basis), respectively. The literature on rattlesnake proximate composition is limited; therefore comparisons must be made with existing data gathered from meats of different snake species. On average, DM content is about 23%, CP is about 65%, fat is about 6%, and ash is about 5% (Abulude, 2007; Ockerman and Basu, 2009; Ogungbenle and Adaraniwon, 2013). The rattlesnake meat tested herein contained a greater DM content at 30.1%, similar CP, and was lower in fat at 1.8%. Complete AA profiles were compiled for every protein and data were reported on DM basis as a percent by weight of protein ingredient (**Table 5.2**). Lysine and glutamic acid were present in greater concentrations in rattlesnake, 7.1% and 11.9%, respectively. The AA profile of cobra also reflects lysine (5.8%) and glutamic acid (12.3%) as having greater concentrations as well as aspartic acid (8.9%) and leucine (6%) which are similar to aspartic acid and leucine concentrations in rattlesnake, 7.4% and 5.9%, respectively (Ogungbenle and Adaraniwon, 2013). Arginine and lysine were found in higher concentrations in eel meat, 3.9% and 4.4%, respectively, and are greater compared to results from prior research where levels of arginine and lysine were 1% and 0.9%, respectively (Gomez-Limia et al., 2021). Lysine was highest indispensable AA in carp slurry (6.6%), whitefish (4.2%), and eel (4.4%). Fresh carp meat was evaluated by Manik et al. (2019), lysine concentration corresponded to

4.5% of total indispensable AA and glutamic acid at 7.9% of total dispensable AA which was lower than what was observed in the current study. Methionine concentration was the lowest (1.4%) in fresh carp meat compared to carp slurry (2%). Leucine was the indispensable AA with the highest concentration in spirulina (6.1%), which is similar to the concentration that has been reported by Liestianty et al. (2019) at 5.5%. In general, glutamic acid was the overall most abundant dispensable AA. Alanine and aspartic acid were also present in high amounts compared to other dispensable AA, with spirulina containing the most, 5.1% and 6.8%, respectively. Variations observed in AA composition may be due to the species, dietary and environmental practices, which is also dependent on whether the fish were wild caught or farm raised. Generally, when compared to red meat, the AA composition of fish has greater lysine content (Arino et al., 2003) which is reflected in the results of the current study.

Each protein was analyzed for a long chain fatty acid profile (**Table 5.3**). Rattlesnake meat had the lowest overall fatty acid concentration, linoleic (266 ug/g DM basis), palmitic (249 ug/g), and oleic (242 ug/g) acids were most abundant. Both EPA and DHA were highest in salmon from the current study, 1026.8 µg/g and 873 µg/g, respectively which is in alignment with comparisons made with other marine fish species. Farmed Atlantic salmon had the greatest concentration of total DHA and EPA at 2.1% which was greater than mackerel (1.2%) and herring (2%). The farmed Atlantic salmon concentrations were also greater than wild salmon, Chinook salmon, and sockeye salmon, 1.8%, 1.7%, and 1.2%, respectively (Lee et al., 2009). Rattlesnake contained greater DHA levels than carp slurry, 49.3 µg/g and 27.4 µg/g respectively; however was lower in EPA (9.41 µg/g). Concentrations of DHA and EPA have been observed to be higher in farmed Japanese eel muscle (7.2% and 4%, respectively) than in wild Japanese eel (4.2% and 2.8%, respectively) (Oku et al., 2009) which, from a sustainability standpoint, may be

justification to use farmed eel for product diversification. Fatty acid compositions in muscle tissues of farmed aquatic species has been determined to be affected by higher lipid content in finishing diets compared to wild-caught counterparts (Mourente and Bell, 2006) which can explain the variability that is observed in the ways the fish are sourced. Linoleic acid is also an essential fatty acid in canine and feline nutrition (Bauer, 2008) and was found in the greatest concentration of 4354.6 µg/g in salmon. Linoleic acid concentration in carp slurry was similar to whitefish (59.3 µg/g and 58.8 µg/g, respectively) and the concentration of linoleic acid in spirulina was similar to that of rattlesnake (266 µg/g and 265.9 µg/g, respectively); however, eel had no detectable concentration of linoleic acid. Alpha-linolenic acid is a precursor for EPA and was highest in salmon at 2643.8 µg/g, followed by eel (237 µg/g), carp slurry (57.7 µg/g), whitefish (44.1 µg/g), rattlesnake (24.5 µg/g), and spirulina (8.1 µg/g). Palmitic and oleic acids have been determined to be among the most abundant fatty acids present in spirulina, eel, and salmon (Muhling et al., 2005; Oku et al., 2009; Foroutani et al., 2018) which is supported by our data where concentration of palmitic acid was highest in eel at 4187.4 µg/g followed by similar concentrations observed in spirulina and salmon (3173.9 µg/g and 3072.7 µg/g, respectively). Oleic acid was highest in salmon at 12497 µg/g followed by eel at 7485.5 µg/g/ and spirulina at 5187.6 µg/g.

Precision-fed cecectomized rooster assay

Data for standardized AA digestibility of indispensable AA and dispensable AA of our select novel proteins are presented on an as fed basis in **Table 5.4** and **Table 5.5**, respectively. All indispensable AA were highly digestible across all protein sources with no individual AA digestibility value falling below 81.2%. Significant differences ($P < 0.05$) were observed in all

indispensable AA except for isoleucine, phenylalanine, and threonine where all standardized AA digestibility values were greater than 90%. Arginine standardized digestibility was greatest ($P < 0.05$) in eel at 98.4% and lowest ($P < 0.05$) in spirulina at 91.1%; there were no differences in arginine digestibility among carp slurry, whitefish, salmon, and rattlesnake. Spirulina also had the lowest ($P < 0.05$) standardized digestibility for leucine, tryptophan, and valine, 91.1%, 91%, 94.4%, and 88.6%, respectively. Eel and salmon had the greatest histidine digestibility at 92.4% and 92.1%, respectively. Results for eel and salmon tryptophan standardized digestibility were above 100% (100.4% and 100.9%, respectively). Standardized AA digestibility values are estimations which allows these results to be accepted as being highly digestible as determined by the cecectomized rooster model. Elevated results may be due to underestimation of endogenous AA. The dispensable AA standardized digestibility did not differ ($P > 0.05$) among protein sources, except for alanine and glutamic acid digestibility. Digestibility of alanine from carp slurry (98.3%), whitefish (97.9%), eel (97.8%), and salmon (96.4%) were higher ($P < 0.05$) than spirulina (89.5%); however, rattlesnake did not differ from any (93%). For glutamic acid digestibility eel (97.6%) and carp slurry (97.8%) were higher ($P < 0.05$) than spirulina (90.3%), but not different than whitefish (96.6%), salmon (95.8%), rattlesnake (94.9%). In the dispensable AA category, no standardized digestibility coefficient fell below 80.8% (the digestibility of cysteine in eel meat). The precision-fed cecectomized rooster assay has been performed to determine the standardized AA digestibility of salmon meal with crushed bones and whitefish meal (Folador et al., 2006). The standardized indispensable AA digestibility ranged from 80.3% to 93.6% in whitefish meal and 82.9% to 93.3% in salmon meal with crushed bones, and, therefore, were determined to be highly digestible which is in agreement with the results of the present study where whitefish indispensable AA digestibility ranged from 81.2% to 98.8% and

92.1% to 100.9% in salmon. Alligator meal was analyzed for standardized AA digestibility by the precision-fed cecectomized rooster assay (Deng et al., 2016). All indispensable AA were highly digestible except for histidine (79.8%). Histidine was also the AA with the lowest indispensable AA digestibility from rattlesnake meat in the current study (91.2%). Additionally, no standardized AA digestibility values exceeded 90.4% in alligator meal whereas in rattlesnake, all AA digestibility values were greater than 90% with the only exception being proline at 89.8%.

DIAAS-like values

The DIAAS-like data calculated according AAFCO nutrient profile and NRC recommended allowances for adult dogs at maintenance are summarized in **Table 5.6** and **Table 5.7** and data using the same reference proteins for adult cats at maintenance are summarized in **Table 5.8** and **Table 5.9**, respectively. According to this scoring method, the quality of a protein source is based on the lowest DIAAS-like score, referred to as the first-limiting AA. Rattlesnake was the lowest quality protein source among all tested novel protein sources for both dogs and cats using AAFCO and NRC comparisons and tryptophan (< 40%) was the most often first-limiting AA. Using AAFCO recommendations for adult dogs, carp slurry and spirulina were the only protein sources to receive a high quality score as all DIAAS-like values were greater than 100%. Eel, salmon, and whitefish were determined to be moderate quality with DIAAS-like scores of 86.5%, 70.4% and 66.8%, respectively, for tryptophan. Although rattlesnake was characterized as a low quality protein source due to having a DIAAS-like coefficient of 39% for tryptophan, it contained greater ($P < 0.05$) scores for arginine (268.5%), leucine (310%), lysine (370.9%), phenylalanine (162.9%), and threonine (171%) compared to the remaining protein

sources. According to NRC recommended allowances for adult dogs, tryptophan was the first-limiting AA for all protein sources tested except for spirulina. Methionine was the lowest DIAAS-like value for spirulina at 63.3% which designates spirulina as a moderate quality protein. Carp slurry and eel were also moderate quality with tryptophan DIAAS-like values of 78.2% and 54.9%, respectively. Whitefish (42.4%), salmon (44.7%), and rattlesnake (31.7%) were low quality proteins.

Using AAFCO recommended values for adult cats, it was determined that carp slurry, spirulina, and eel are high quality proteins with DIAAS-like coefficients >100% compared towhitefish and salmon which were considered to be moderate quality with the first-limiting AA for whitefish being tryptophan (96.6%) and leucine for salmon (85.2%). The greatest DIAAS-like values were observed in methionine across all proteins, excluding rattlesnake which produced the lowest scores overall therefore being characterized as a low quality protein source. Carp slurry produced the highest ($P < 0.05$) DIAAS-like value for methionine at 341.1%. Similar results were obtained when NRC comparisons were used. Carp slurry, spirulina, and eel had DIAAS-like values that were all over 100%. Tryptophan was first-limiting for whitefish at 91.4% and leucine was first-limiting for salmon at 79.6%. Lysine DIAAS-like value for carp slurry was the greatest ($P < 0.05$) coefficient at 486%.

The method of DIAAS-like calculation and its application within canine and feline nutrition has been previously studied by Oba et al. (2019) where chicken meal, retorted chicken, steamed chicken, and raw chicken. Chicken meal is a common protein ingredient in companion animal diets and what the authors found was that when using AAFCO and NRC comparisons for adult dogs, methionine was the first-limiting AA with DIAAS-like values of 78.5% and 43.6%, respectively. For cats, threonine was the first-limiting AA with DIAAS-like coefficients of

91.5% and 98.8% using AAFCO and NRC comparisons, respectively. When raw chicken was evaluated, the first-limiting AA was methionine, 58.9%, using NRC comparisons for dogs; there were no first-limiting AA using AAFCO comparisons. The DIAAS-like values for raw chicken for cats using AAFCO and NRC comparisons reflected no first-limiting AA. When comparing these results with other common pet food ingredients from our study, such as salmon, it was determined that salmon was of similar quality as chicken meal based on AAFCO and NRC comparisons for adult dogs; however, for adult cats, salmon was of lower quality than chicken meal and had isoleucine as the first-limiting AA (Oba et al., 2019). The DIAAS-like results for raw chicken offer a closer comparison to the raw ingredients tested in our study. Using AAFCO comparisons for adult dogs, values for methionine were greater in carp slurry (142.2%), whitefish (114.3%), spirulina (113.3%), and eel (125.5%) than raw chicken (106.1%)(Oba et al., 2019); however, salmon and rattlesnake results were lower, 103.4% and 105.7%, respectively. Based on NRC comparisons for adult dogs, the tryptophan DIAAS-like values for whitefish (42.4%), eel (54.9%), salmon (44.7%) and rattlesnake (31.7%) were lower than tryptophan DIAAS-like values in raw chicken (71%; Oba et al., 2019). Again, when using NRC recommended allowances for adult dogs to compare with DIAAS-like values for methionine in raw chicken (58.9%) as reported by Oba et al. (2019), all proteins tested in our study had greater DIAAS-like coefficients for methionine, except for salmon which was slightly lower at 57.7%. Based on the comparisons between these studies, raw aquatic and raw reptile proteins may be paired with raw chicken to supplement lower methionine availability in raw chicken and tryptophan in fish and reptiles for use in companion animal diets.

Other methods have been applied to estimate protein quality, such as the immobilized digestive enzyme assay (**IDEA**) as performed by Faber et al. (2010). This assay measured the

amount of free AA present after enzyme hydrolysis from in vitro digestion of pollock fillet (a type of whitefish) and salmon fillet by calculation of an IDEA score. The IDEA scores reported by the authors predicted that pollock fillet would have greater AA digestibility than salmon fillet, 0.71 and 0.64, respectively, with indispensable AA digestibility values predicted to range from 93.2 to 101.8 in pollock and 91.3 to 96.9 in salmon. When the substrates were tested in vivo by cecectomized roosters, authors observed that pollock fillet generally had greater indispensable AA digestibility values than salmon fillet, with values for pollock in a range of 83.2% to 93.9% and for salmon, a range of 84.5% to 90.8%. Compared to results from our study, whitefish and salmon had similar standardized indispensable AA digestibility and the DIAAS-like values were generally greater in whitefish than in salmon.

Conclusion

Sustainability, product diversification, and demand for novel proteins are driving factors that impact the purchasing decisions of pet owners which inspires the innovation of new pet food products. There is limited information surrounding the chemical composition and protein quality of these novel proteins in the literature, justifying the analyses performed herein. The results obtained from this study indicate that these tested novel proteins may be beneficial for canine and feline diets as they contain elevated concentrations of omega-3 and omega-6 fatty acids and, according to the precision-fed cecectomized rooster assay, have an AA profile that is generally highly digestible. Carp slurry and spirulina contained the greatest CP concentrations and salmon possessed the largest amount of omega-3 and omega-6 fatty among all novel proteins. When compared to carp slurry, rattlesnake produced the lowest DIAAS-like scores for both AAFCO nutrient profile and NRC recommended allowances for adult dogs and cats which would

characterize the protein as low quality. Carp slurry and spirulina were consistently high or moderate quality and tryptophan was the common first-limiting AA. Focal points of future research may involve 1. studying potential health benefits derived from intrinsic omega-3 and omega-6 concentrations in some of these novel ingredients, and 2. investigating the overall acceptability of diets and optimal levels of inclusion to of these novel protein sources, to optimize nutrient digestibility and fecal quality, and impact on fecal metabolites and microbiota.

Tables

Table 5.1. Chemical composition of select novel protein sources

	Aquatic and Reptile Protein Sources					
Item %, DMB	**Salmon**	**Whitefish**	**Carp Slurry**	**Eel**	**Spirulina**	**Rattlesnake**
Dry matter, %	34.2	22.8	22.1	30.5	95.3	30.1
			%, DM basis			
Organic matter, %	92.3	95.2	95.8	87.0	92.9	61.7
Crude protein, %	43.5	70.3	72.5	58.7	72.8	60.9
Total dietary fiber, %	NA[1]	NA	NA	NA	20.0	NA
Insoluble	NA	NA	NA	NA	18.2	NA
Soluble	NA	NA	NA	NA	1.8	NA
Acid hydrolyzed fat, %	57.0	10.4	18.3	32.2	7.1	1.8
Gross energy, kcal/g	7.0	4.4	6.0	6.3	5.4	3.6

[1]NA – not analyzed

Table 5.2. Amino acid concentrations of select novel protein sources as a percentage of the total crude protein

	Aquatic and Reptile Protein Sources					
% by weight, DM basis	**Salmon**	**Whitefish**	**Carp Slurry**	**Eel**	**Spirulina**	**Rattlesnake**
Indispensable AA						
Arginine	2.2	4.0	4.3	3.9	5.0	5.2
Histidine	0.7	1.2	2.0	1.7	1.2	2.0
Isoleucine	1.1	2.3	3.5	2.3	4.2	3.7
Leucine	1.8	3.9	5.7	3.7	6.1	5.9
Lysine	2.1	4.2	6.6	4.4	3.4	7.1
Methionine	0.8	1.6	2.0	1.4	1.6	2.0
Phenylalanine	1.1	2.3	3.0	2.3	3.3	3.1
Threonine	1.2	2.4	3.0	2.2	3.3	3.3
Tryptophan	0.2	0.4	0.8	0.4	0.7	0.7
Valine	0.2	2.7	3.7	2.4	4.4	3.5
Dispensable AA						
Alanine	2.2	4.1	4.1	4.0	5.1	4.9
Aspartic acid	2.7	5.4	7.1	5.0	6.8	7.4
Cysteine	0.2	0.5	0.7	0.5	0.7	0.9
Glutamic acid	3.6	7.9	10.3	7.1	10.0	11.9
Proline	2.2	3.5	2.5	3.6	2.6	4.4
Serine	1.3	2.6	2.5	2.0	2.8	3.3
Tyrosine	1.0	1.7	2.3	1.9	3.0	2.8

Table 5.3. Long chain fatty acid (LCFA) concentrations of select aquatic and reptile protein sources

	Aquatic and Reptile Protein Sources					
LCFA Concentration (ug/g, DM basis)	**Salmon**	**Whitefish**	**Carp Slurry**	**Eel**	**Spirulina**	**Rattlesnake**
Caprylic (C8:0)	0.3	1.9	5.9	0.6	25.2	0.0
Capric (C10:0)	1.7	0.0	0.8	1.8	7.0	0.0
Undecanoic (C11:0)	0.0	0.0	2.7	1.5	7.2	0.0
Lauric (C12:0)	39.2	5.0	22.0	59.7	9.9	0.0
Myristic (C14:0)	670.5	502.2	705.5	824.0	137.9	6.5
Myristoleic (C14:1)	4.4	6.8	15.3	48.2	15.4	0.0
Pentadecanoic (C15:0)	52.5	43.0	135.7	83.9	40.6	3.0
Palmitic (C16:0)	3072.7	1228.0	2449.4	4187.4	3173.9	249.4
Palmitoleic (C16:1)	49.9	402.0	1418.2	333.1	54.7	19.2
Heptadecanoic (C17:0)	25.5	36.4	74.7	110.9	41.6	7.9
cis-10-Heptadecenoic (C17:1)	31.5	43.6	46.0	146.6	19.1	38.0
Stearic (C18:0)	81.2	424.5	518.1	1058.8	37.2	139.0
Oleic (C18:1n9c)	12497.0	1424.1	1366.9	7485.5	5187.6	242.3
Elaidic (C18:1n9t)	940.9	15.3	78.6	0.0	375.7	5.5
Linoleic (C18:2n6c)	4354.6	58.8	59.3	0.0	266.0	265.9
Linolelaidic (C18:2n6t)	314.8	1.9	11.6	53.5	49.5	35.7
α-linolenic (C18:3n3)	2643.8	44.1	57.7	237.7	8.1	24.5

Table 5.3. (continued) Long chain fatty acid concentrations of select aquatic and reptile protein sources

	Aquatic and Reptile Protein Sources					
LCFA Concentration (ug/g, DM basis)	**Salmon**	**Whitefish**	**Carp Slurry**	**Eel**	**Spirulina**	**Rattlesnake**
Eicosasdienoic (C20:2)	205.5	16.3	12.7	16.1	23.7	2.9
Eicosatrienoic (C20:3n6)	69.9	0.0	7.7	3.6	4.3	6.7
Eicosatrienoic (C20:3n3)	309.2	14.7	15.0	15.1	15.2	0.0
Arachidonic (C20:4n6)	9.4	17.1	21.2	45.6	37.7	129.4
Eicosapentaenoic (C20:5n3)	873.0	60.5	32.9	196.5	5.7	9.41
Henicosanoic (C21:0)	3.6	5.0	12.5	7.0	26.3	0.0
Behenic (C22:0)	2.6	11.4	12.3	22.0	25.0	3.3
Euric (C22:1n9)	582.5	847.6	12.7	23.9	2.6	4.4
Docosadienoic (C22:2)	247.3	5.4	7.7	49.3	0.0	0.0
Docosahexaenoic (C22:6n3)	1026.8	91.2	27.4	331.8	30.6	49.3
Ligonoceric (C24:0)	3.0	5.9	4.4	0.0	18.4	4.4
Nervonic (C24:1n9)	7.9	107.5	12.7	24.9	22.2	28.6

Table 5.4. Standardized amino acid digestibility of select novel proteins calculated using cecectomized rooster assay[1]

	Aquatic and Reptile Protein Sources							
Indispensable Amino Acids, %	**Salmon**	**Whitefish**	**Carp Slurry**	**Eel**	**Spirulina**	**Rattlesnake**	**SEM[2]**	**P-value**
Arginine	97.3^{ab}	96.8^{ab}	97.1^{ab}	98.4^{a}	91.1^{b}	94.7^{ab}	1.3909	0.0190
Histidine	92.1^{a}	81.2^{b}	90.1^{ab}	92.4^{a}	89.9^{ab}	91.2^{ab}	2.2993	0.0270
Isoleucine	95.2	95.8	97.2	96.3	90.9	95.4	1.3876	0.0699
Leucine	96.0^{ab}	98.0^{a}	98.4^{a}	97.7^{a}	91.0^{b}	95.9^{ab}	1.4465	0.0207
Lysine	97.2^{ab}	91.3^{b}	96.6^{ab}	98.8^{a}	92.6^{ab}	95.7^{ab}	1.5692	0.0271
Methionine	96.4^{b}	98.8^{ab}	99.6^{a}	97.0^{ab}	96.9^{b}	96.4^{b}	0.7815	0.0417
Phenylalanine	94.9	97.2	97.5	96.6	93.9	94.7	1.4056	0.3922
Threonine	93.9	95.0	97.2	97.5	91.8	94.3	1.79	0.25
Tryptophan	100.9^{a}	94.9^{b}	98.0^{ab}	100.4^{a}	94.4^{b}	96.6^{ab}	1.0829	0.0016
Valine	96.9^{a}	97.7^{a}	97.9^{a}	97.5^{a}	88.6^{b}	95.0^{ab}	1.7635	0.0111

[1]*n* = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-b]Means within a row with different superscripts are significantly different at $P < 0.05$

Table 5.5. Standardized amino acid digestibility of select novel proteins calculated using cecectomized rooster assay[1]

	Aquatic and Reptile Protein Sources							
Dispensable Amino Acids, %	**Salmon**	**Whitefish**	**Carp Slurry**	**Eel**	**Spirulina**	**Rattlesnake**	**SEM[2]**	**P-value**
Alanine	96.4[a]	97.9[a]	98.3[a]	97.8[a]	89.5[b]	93.0[ab]	1.3539	0.0011
Aspartic acid	95.0	94.5	95.8	95.5	90.3	93.7	1.37	0.10
Cysteine	85.3	88.5	91.7	80.8	93.8	90.9	5.2338	0.5421
Glutamic acid	95.8[ab]	96.6[ab]	97.8[a]	97.6[a]	90.3[b]	94.9[ab]	1.4199	0.0157
Proline	98.9	97.0	94.8	97.4	95.2	89.8	2.2757	0.1374
Serine	92.9	96.5	97.2	96.7	90.0	92.9	2.3157	0.2183
Tyrosine	95.0	94.4	98.1	95.7	94.2	94.6	1.9175	0.7172

[1]*n* = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-b]Means within a row with different superscripts are significantly different at $P < 0.05$

Table 5.6. Digestible indispensable amino acid score (DIAAS)-like[1] values for select novel proteins compared with AAFCO nutrient profile for adult dogs at maintenance

	Aquatic and Reptile Protein Sources							
Indispensable Amino Acids, %	**Salmon**	**Whitefish**	**Carp Slurry**	**Eel**	**Spirulina**	**Rattlesnake**	**SEM[2]**	**P-value**
Arginine	179.3^{c}	179.7^{c}	193.1^{c}	218.2^{b}	213.4^{b}	268.5^{a}	3.0786	0.0001
Histidine	139.0^{c}	120.2^{d}	218.0^{b}	242.1^{a}	136.6^{cd}	101.6^{e}	3.8903	0.0001
Isoleucine	118.2^{f}	140.1^{e}	213.5^{b}	169.4^{d}	237.3^{a}	191.7^{c}	2.9886	0.0001
Leucine	107.5^{e}	131.4^{d}	192.6^{b}	153.8^{c}	192.7^{b}	310.0^{a}	2.7606	0.0001
Lysine	134.4^{d}	143.5^{d}	235.3^{b}	198.0^{c}	118.9^{e}	370.9^{a}	2.7204	0.0001
Methionine	103.4^{d}	114.3^{c}	142.2^{a}	125.5^{b}	113.3^{c}	105.7^{d}	0.9603	0.0001
Phenylalanine	97.3^{d}	116.7^{c}	152.1^{b}	142.9^{b}	163.3^{a}	162.9^{a}	2.1391	0.0001
Threonine	100.4^{e}	114.1^{d}	145.4^{b}	131.4^{c}	151.3^{b}	171.0^{a}	2.5669	0.0001
Tryptophan	70.4^{d}	66.8^{d}	123.1^{a}	86.5^{c}	109.5^{b}	39.0^{e}	0.8780	0.0001
Valine	106.8^{e}	126.6^{d}	174.0^{b}	141.6^{c}	190.6^{a}	191.3^{a}	3.2075	0.0001
Met + Cys	132.6^{d}	149.3^{cd}	189.5^{b}	156.4^{c}	165.7^{c}	239.5^{a}	5.1492	0.0001
Phe + Tyr	127.6^{d}	141.2^{d}	189.8^{c}	181.0^{c}	217.5^{b}	243.3^{a}	3.1440	0.0001

[1]*n* = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-f]Means within a row with different superscripts are significantly different at $P < 0.05$

Table 5.7. Digestible indispensable amino acid score (DIAAS)-like[1] values for select novel proteins compared with NRC recommended allowances for adult dogs at maintenance

	Aquatic and Reptile Protein Sources							
Indispensable Amino Acids, %	**Salmon**	**Whitefish**	**Carp Slurry**	**Eel**	**Spirulina**	**Rattlesnake**	**SEM[2]**	**P-value**
Arginine	145.7^{c}	146.0^{c}	156.9^{c}	177.3^{b}	173.5^{b}	218.2^{a}	2.5020	0.0001
Histidine	78.0^{c}	67.5^{d}	122.3^{b}	135.9^{a}	76.6^{cd}	82.5^{c}	2.2005	0.0001
Isoleucine	65.7^{f}	77.8^{e}	118.6^{c}	94.1^{d}	131.8^{b}	155.8^{a}	1.6898	0.0001
Leucine	59.7^{e}	73.0^{d}	106.9^{b}	85.4^{c}	107.0^{b}	252.0^{a}	1.6256	0.0001
Lysine	134.8^{d}	144.0^{d}	236.0^{b}	198.6^{c}	119.3^{e}	301.4^{a}	2.7101	0.0001
Methionine	57.7^{e}	63.9^{d}	79.5^{b}	70.1^{c}	63.3^{d}	85.9^{a}	0.5481	0.0001
Phenylalanine	54.3^{e}	65.1^{d}	84.8^{c}	79.7^{c}	91.1^{b}	132.4^{a}	1.2435	0.0001
Threonine	62.2^{e}	70.7^{d}	90.1^{b}	81.5^{c}	93.8^{b}	138.9^{a}	1.6364	0.0001
Tryptophan	44.7^{d}	42.4^{d}	78.2^{a}	54.9^{c}	69.5^{b}	31.7^{e}	0.5657	0.0001
Valine	59.5^{e}	70.6^{d}	97.0^{b}	79.0^{c}	106.3^{a}	106.7^{a}	1.7892	0.0001
Met + Cys	73.9^{d}	83.2^{cd}	105.5^{b}	87.2^{c}	92.3^{c}	133.4^{a}	2.8696	0.0001
Phe + Tyr	124.5^{d}	137.8^{d}	185.2^{c}	176.7^{c}	212.3^{b}	237.4^{a}	3.0688	0.0001

[1]*n* = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-f]Means within a row with different superscripts are significantly different at $P < 0.05$

Table 5.8. Digestible indispensable amino acid score (DIAAS)-like[1] values for select novel proteins compared with AAFCO nutrient profile for adult cats at maintenance

	Aquatic and Reptile Protein Sources							
Indispensable Amino Acids, %	**Salmon**	**Whitefish**	**Carp Slurry**	**Eel**	**Spirulina**	**Rattlesnake**	**SEM[2]**	**P-value**
Arginine	127.5^{c}	127.8^{c}	137.3^{c}	155.1^{b}	151.88^{b}	190.9^{a}	2.1892	0.0001
Histidine	123.5^{c}	106.8^{d}	193.7^{b}	215.2^{a}	121.4^{cd}	72.2^{e}	3.4495	0.0001
Isoleucine	124.8^{e}	147.9^{d}	225.3^{b}	178.8^{c}	250.5^{a}	136.37^{de}	3.1274	0.0001
Leucine	85.2^{e}	104.1^{d}	152.5^{b}	121.8^{c}	152.6^{b}	220.5^{a}	2.1636	0.0001
Lysine	147.4^{c}	157.5^{c}	258.2^{a}	217.2^{b}	130.5^{d}	263.8^{a}	2.9498	0.0001
Methionine	247.9^{d}	274.2^{c}	341.1^{a}	301.0^{b}	271.8^{c}	75.2^{e}	2.2619	0.0001
Phenylalanine	151.3^{d}	181.4^{c}	236.4^{b}	222.1^{b}	253.9^{a}	115.9^{e}	3.2236	0.0001
Threonine	95.1^{d}	108.0^{c}	137.7^{a}	124.5^{b}	143.3^{a}	121.6^{b}	2.3889	0.0001
Tryptophan	101.8^{d}	96.6^{d}	177.9^{a}	125.0^{c}	158.1^{b}	27.7^{e}	1.2457	0.0001
Valine	122.4^{e}	145.2^{d}	199.4^{b}	162.3^{c}	218.5^{a}	219.3^{a}	3.6765	0.0001
Met + Cys	113.5^{d}	127.8^{cd}	162.2^{b}	133.9^{c}	141.8^{c}	205.0^{a}	4.4081	0.0001
Phe + Tyr	124.8^{d}	138.0^{d}	185.6^{c}	177.1^{c}	212.7^{b}	237.9^{a}	3.0749	0.0001

[1]*n* = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-e]Means within a row with different superscripts are significantly different at $P < 0.05$

Table 5.9. Digestible indispensable amino acid score (DIAAS)-like[1] values for select novel proteins compared with NRC recommended allowances for adult cats at maintenance

	Aquatic and Reptile Protein Sources							
Indispensable Amino Acids, %	**Salmon**	**Whitefish**	**Carp Slurry**	**Eel**	**Spirulina**	**Rattlesnake**	**SEM[2]**	**P-value**
Arginine	132.5^{cd}	127.8^{d}	142.7^{c}	161.2^{b}	157.7^{b}	198.4^{a}	2.2713	0.0001
Histidine	114.0^{c}	98.6	178.8^{b}	198.6^{a}	112.0^{cd}	75.0^{e}	3.1879	0.0001
Isoleucine	116.1^{e}	137.6^{d}	209.6^{b}	166.3^{c}	233.0^{a}	141.6^{d}	2.9144	0.0001
Leucine	79.6^{e}	97.3^{d}	142.6^{b}	113.9^{c}	142.7^{b}	229.1^{a}	2.0443	0.0001
Lysine	277.6^{c}	296.5^{c}	486.0^{a}	409.0^{b}	245.6^{d}	274.0^{c}	5.5190	0.0001
Methionine	224.3^{d}	248.1^{c}	308.73^{a}	272.4^{b}	245.9^{c}	78.1^{e}	2.0481	0.0001
Phenylalanine	122.2^{d}	146.5^{c}	191.0^{b}	179.4^{b}	205.1^{a}	120.4^{d}	2.6180	0.0001
Threonine	103.0^{d}	117.0^{c}	149.1^{a}	134.8^{b}	155.2^{a}	126.3^{cb}	2.5821	0.0001
Tryptophan	96.4^{d}	91.4^{d}	168.4^{a}	118.4^{c}	149.7^{b}	28.8^{e}	1.1807	0.0001
Valine	114.5^{e}	135.7^{d}	186.5^{b}	151.8^{c}	204.3^{a}	205.0^{a}	3.4381	0.0001
Met + Cys	282.6^{d}	318.2^{cd}	403.6^{b}	333.4^{c}	353.0^{c}	510.3^{a}	10.9718	0.0001
Phe + Tyr	120.5^{d}	133.3^{d}	179.2^{c}	170.9^{c}	205.4^{b}	229.7^{a}	2.9679	0.0001

[1]*n* = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-e]Means within a row with different superscripts are significantly different at $P < 0.05$

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CHAPTER 6:

EVALUATION OF INSECT AND PLANT-BASED PROTEINS AND CALCULATION OF DIGESTIBLE INDISPENSABLE AMINO ACID SCORES FOR COMPANION ANIMAL DIETS USING THE PRECISION-FED CECECTOMIZED ROOSTER ASSAY

Abstract

As the human and pet populations continue to grow, there is an increasing demand for dietary proteins. Consumers may choose alternative proteins (e.g., plant-based and insects) for various reasons. These may include nutritional quality and health benefits and concerns with environmental and animal welfare practices (Tso et al., 2021). The objectives of this study were to determine the chemical composition of novel proteins cricket meal, chocho powder, pumpkin protein powder (denoted as pumpkin powder), and hemp protein powder (referred as hemp powder) as well as using the precision-fed cecectomized rooster assay to determine the quality of novel proteins by measuring standardized amino acid (**AA)** digestibility and calculating digestible indispensable amino acid score (**DIAAS**)-like values. The DIAAS-like reference proteins were based on the nutrient profiles of the Association of American Feed Control officials (**AAFCO**) and the National Research Council (**NRC**) recommended allowances for adult dogs and cats at maintenance. Dry matter values were similar across the protein ingredients and ranged from 93.5% to 98.9%. Crude protein (**CP**) concentration for cricket meal, chocho powder, and hemp powder were 63.6%, 63.3%, and 61.4%, respectively, whereas pumpkin powder had the highest CP concentration at 75.5%. Acid hydrolyzed fat (**AHF**) concentration was highest in cricket meal (37.1%) and lowest in hemp powder (14.3%) on DM basis. Chocho powder and pumpkin powder were similar, 21.4% and 20.8%, respectively. These ingredients

were also analyzed for total dietary fiber (**TDF**) and it was determined that chocho powder contained the highest concentration (28.7%) with 21.4% and 7.2% for insoluble and soluble portions, respectively. Total dietary fiber of pumpkin and hemp powders produced similar values, both 24.3%; however, hemp powder contained a higher portion of insoluble fiber compared to pumpkin powder (18.3% and 11.4%, respectively). Cricket meal had the lowest TDF concentration at 19.6%, being mostly insoluble (16.8%). Standardized AA digestibility values for all proteins were highly digestible with the exception of lysine in pumpkin powder (77.2%). The most often first-limiting AA was observed to be methionine for dogs based on NRC recommended allowances and AAFCO nutrient profile. For cats, cricket meal contained no first-limiting AA. Arginine and threonine were the first-limiting AA for chocho and pumpkin powders, respectively, using both AAFCO and NRC reference values.

Introduction

Plant based and insect proteins have gained popularity in recent years in the pet food market as a response to what is in demand within the human food industry. Moreover, pet owners are generally making more health conscious decisions when purchasing food for themselves and thereby following the same priorities when selecting pet food products. Although the interest is high for protein alternatives, these ingredients are still novel and there is a need for information regarding chemical composition and amino acid (**AA**) digestibility in the context of companion animal nutrition. Entomophagy is the act of eating insects, which is already common practice in several non-Western communities (Tao, Li, 2018). Of the various edible insects, house crickets and black soldier fly are most commonly eaten (Ortiz et al., 2016) and have been accompanied by research conducted in canine and feline nutrition that evaluated the

incorporation of these ingredients in complete diets with results that are comparable to more traditional meat-based proteins (Kilburn et al., 2020; Do et al., 2021; Penazzi, 2021). The attitudes towards consuming insects have shifted in the U.S. with more Americans being willing to consume black soldier flies themselves as well as feeding to their dogs (Higa et al., 2021).

Plant-based proteins are incorporated in the diets of people from various parts of the world. Soybean products, such as tofu, are common meat alternatives and are readily consumed (He et al., 2020). According to a questionnaire organized by Dodd et al. (2019), owners that identify as vegans were more likely to feed their pets a plant-based diet. The most common concern among participants was the lack of nutritional information on plant proteins, underscoring the need for this research. Some plant-based diets have been found to be deficient in nutrients such as arachidonic acid, methionine, and calcium, and excessive in copper and zinc (Zafalon et al., 2020) which amplifies this concern among pet owners. Alternatively, research conducted by Reilly et al. (2020), determined that pulse ingredients have the nutritional potential to be a protein source in companion animal diets as long as the pulse ingredients are paired with complimentary proteins to compensate for limiting AA.

The objectives of this study were to characterize the macronutrient composition of cricket meal, chocho powder, pumpkin powder, and hemp powder and evaluate protein quality by calculating standardized AA digestibility from the precision-fed cecectomized rooster assay as well as calculation of digestible indispensable AA scores (DIAAS-like).

Materials and methods

Select novel protein sources and sample preparation

The select novel proteins evaluated in this study were cricket meal (*Acheta domesticus*; sourced from Chippin Inc., Silver Spring, MD, USA), chocho powder *(Lupinus mutabilis*, Mikuna Foods LLC, St. Barbara, CA, USA), organic pumpkin protein powder made from pumpkin seeds *(Cucurbita pepo L*; Z Natural Foods, USA), and organic hemp protein powder *(Cannabis sativa ssp. Sativa*; Z Natural Foods, USA*).* Further processing was not needed as these ingredients were already dry and in powder form.

Chemical analyses

All ingredients were sub-sampled in duplicate for each analysis. Dry matter (**DM**) and ash concentrations were determined by following methods 934.01 and 942.05 of AOAC (2006). Crude protein (**CP**) concentration was analyzed by measuring total N with LECO (TruMac N, LECO Corporation, St. Joseph, MI) and gross energy (**GE**) was determined by bomb calorimetry (Model 6200, Parr Instruments Co., Moline, IL). Acid hydrolyzed fat (**AHF**) method was used to extract total fat of the ingredients and was performed according to Budde (1952) and AACC (1983). Total dietary fiber (**TDF**) was measured according to Prosky et al. (1992). Complete amino acid (**AA**) profiles were determined as described by AOAC (2007).

Precision-fed cecectomized rooster assay

The precision-fed cecectomized rooster assay has been validated as a method to estimate standardized AA digestibility in companion animal nutrition because of high correlation reported

of AA ileal digestibility between ileal cannulated dogs and cecectomized roosters (Johnson et al., 1998). The cecectomized rooster assay was not validated comparing with AA digestibility of cats, but it has been used as a way to screen and rank protein quality of various dietary protein sources, prior to incorporating these ingredients in pet food products for cats. This assay was performed as described by Parsons et al. (1982). Each novel protein was fed to 4 Single Comb White Leghorn roosters. The roosters were fasted for 26 hours prior to the start of the assay. The trial began after birds were crop intubated and fed a 1:1 mixture of sample and ground corn. Over 48 hours excreta were collected, freeze dried, and ground for analysis. This assay provides the coefficients to calculate standardized AA digestibility to allow estimation of protein digestibility (Sibbald, 1979).

Mixed AA digestibility = [(FAA – EAA + End AA) / FedAA] x 100

Standardized AA digestibility = $AAD_{corn} – [(AAD_{corn} - AAD_{mixed}) / FAA\ ratio] \times 100$

Abbreviations: **FAA** = Fed AA; **EAA** = Excreted AA; **EndAA** = Endogenous AA; **AAD** = AA Digestibility

Digestibile indispensable amino acid score (DIAAS-like) values

The quality of each novel protein was evaluated by calculation of DIAAS-like values as described by Reilly et al. (2021). The reference proteins that were used for this calculation were obtained from the nutrient profiles for adult dogs and cats at maintenance from the Association of American Feed Control Officials (**AAFCO**) and National Research Council (**NRC**) recommended allowances. These DIAAS-like values are calculated as follows: mg of digestible indispensable AA in 1 of dietary protein divided by mg of the same indispensable AA in 1g of

reference protein and multiplied by 100 to be expressed as a percent. The first-limiting AA is the lowest DIAAS-like value and is used to characterize the overall quality of a protein. Scores that are >100% indicate a high quality protein, scores <100% but >50% are moderate quality, and scores <50% are low quality proteins. If there are no DIAAS-like values below 100%, then there is no first-limiting AA.

Statistical Analysis

All data were analyzed in SAS (SAS Institute Inc., version 9.4, Cary NC). Mixed Models procedure was applied, and statistical significance was established at $P < 0.05$. The procedure was conducted with random effect of roosters and fixed effect of treatment for protein sources. The Fisher-protected least significant difference test was used to determine the differences among treatments. Tukey adjustment was used to control experiment wise error.

Results and discussion

Chemical composition of select novel proteins

The chemical composition of each novel protein is summarized in **Table 6.1** on a DM basis and expressed as a percent. Dry matter values were similar across each protein ranging from 92.8% to 98.9%. Organic matter was also similar with cricket meal and chocho powder being numerically closer (95.6% and 97.8%, respectively). Pumpkin powder contained the highest amount of CP at 75.5% while cricket meal (63.6%), chocho powder (63.3%), and hemp powder (61.4%) had similar values. Results for CP concentration in chocho are greater than what has been previously reported by Berru et al. (2021) where CP was determined to be 40.8%;

however, these values were from raw seeds that were ground as a whole meal. The chocho ingredient evaluated in the current study was a commercial protein powder which contains a higher CP concentration because it is a protein concentrate. When chocho protein concentrates were evaluated by Curty et al. (2022), CP concentration (69% DM) was more similar to what was obtained in our study. CP concentration of pumpkin powder was also higher than what is reported in the literature, 27% in ground pumpkin seeds (Elinge et al., 2012). The pumpkin powder that was analyzed in our study was processed by cold pressing pumpkin seeds. Cold pressing is an oil extraction method by which oil is removed from pressing seeds under hydraulic screws (Ahlström et al., 2022). The CP concentration of cold pressed pumpkin has been reported to be approximately 54% (Sobczak et al., 2020) and 65.7% (Sinkovic and Kolmanic, 2020). Hemp powder was also processed by cold pressing and other studies that have applied this method have reported CP values of 30% to 53% (House et al., 2010; Potin et al., 2019). These previously reported values are lower than what was obtained in the present study which may be an impact of different pumpkin cultivars used, and different processing methods. Other cricket meals have been evaluated for protein and their concentrations ranged from 60% to 71% CP on DM basis (Kipkoech et al., 2017; Udomsil et al., 2019; Matin et al. 2021), which are align with the value obtained in our study, 63.6%. The AHF concentration for hemp powder was the lowest among ingredients at 14.3%. Chocho and pumpkin powders had similar AHF values, 28.7% and 24.3%, respectively, and cricket meal had the highest, 37.1%. Cricket meal had the lowest TDF value (19.6%) and a higher amount of insoluble fiber (16.8%) compared to soluble (6.2%). A contributing factor to the insoluble dietary fiber portion of cricket meal may be chitin, a polysaccharide composed of β(1,4)-N-acetylglucosamine units that is present in shells of insects (Song et al., 2012) and can elevate fiber content. Chocho powder contained the highest TDF

concentration (28.7%), being mostly insoluble (21.4%). Pumpkin powder had had the greatest amount of soluble fiber at 12.9%, comprising about 53% of the TDF concentration. Values for GE ranged from 5.3% to 6.2%, being highest for cricket meal that also had the highest concentration of AHF.

Amino acid composition was analyzed for each novel protein and results are presented in **Table 6.2** as a percent by weight of protein ingredient on DM basis. In general, the concentrations of indispensable AA and dispensable AA were similar across all proteins. Elevated amounts of alanine (5%) and tyrosine (3.7%) were found in cricket meal with similar amounts from various cricket species reported by Rumpold and Schluter (2013) and Kilburn et al. (2020). Chocho and pumpkin powders contained the greatest amounts of glutamic acid, 11.2% and 11.1%, respectively. Chocho powder also had the greatest amount of arginine at 9.5%. Hemp powder did not surpass the other protein sources in AA content; however, it contained the lowest amount of lysine, 1.9%. Legumes typically have methionine and cysteine as limiting amino acids (Agarwal, 2017) which is reflected in the lower concentration of these AA in chocho, 0.3% and 0.8%, respectively.

Precision-fed cecectomized rooster assay

Results for standardized AA digestibility of indispensable AA and dispensable AA of novel proteins are reported on an as fed basis (**Table 6.3** and **Table 6.4**, respectively). Generally, hemp powder had lower indispensable AA digestibility values overall, but was most similar to cricket meal. These similarities were observed in histidine (81.7% and 83.8%, respectively), isoleucine (89.4% and 89.7%, respectively), and valine (85.7% and 87.9%, respectively).

Pumpkin powder had lowest (77.2%; $P < 0.05$) lysine digestibility when compared to cricket meal (88.4%), chocho powder (95.2%), and hemp powder (80.8%). Chocho and pumpkin powder also had greater ($P < 0.05$) dispensable AA digestibility values compared to cricket meal and hemp powder. Proline digestibility in chocho powder was 102.5%; however, standardized AA digestibility is an estimate which permits this result to be interpreted as being highly digestible by the cecectomized rooster model. The lowest standardized AA digestibilities were most often found in cricket meal, but no values falling below 82% (cysteine) were observed. Soybean meal has been analyzed for standardized AA digestibility using the precision fed cecectomzied rooster by Reilly et al. (2021). Results for soybean meal are most similar to chocho powder; however, soybean meal produced greater histidine (94.8%) and lysine (92.1%) digestibility values compared to cricket meal (83.8% and 88.4%, respectively) pumpkin powder (86.2% and 77.2%, respectively), and hemp powder (81.7% and 80.8%, respectively).

DIAAS-like values

The data for DIAAS-like values calculated using AAFCO nutrient profile and NRC recommended allowances for adult dogs at maintenance are presented in **Tables 6.5 and 6.6** and results for DIAAS like values using the same reference protein for adult cats are presented in **Tables 6.7 and 6.8**. Based on the scoring criteria of the DIAAS-like method, the first-limiting AA determines the overall quality of a protein source. Chocho powder was the lowest scoring novel protein for both cats and dogs based on AAFCO and NRC reference protein values. Using AAFCO and NRC values the first-limiting AA associated with chocho powder was methionine for dogs and arginine for cats. Based on AAFCO recommended values for dogs, cricket meal contained methionine as the first-limiting AA (81.1%), lysine was first-limiting in pumpkin

powder (75.5%) as well as hemp powder (66.2%), designating these ingredients as moderate quality proteins. The lowest DIAAS-like value was found in chocho powder, 28.5% for methionine based on AAFCO nutrient profiles for adult dogs at maintenance. According to NRC recommended allowances for dogs, methionine was first-limiting for all proteins with DIAAS-like values of 45.3% in cricket meal, 15.9% in chocho powder, 51.4% in pumpkin powder, and 47.6% in hemp powder. Based on these coefficients, cricket meal, chocho powder, and hemp powder are characterized as being low quality while pumpkin powder is considered moderate. Chocho powder contained the greatest amount of individual AA that received lower ($P < 0.05$) DIAAS-like values when compared to cricket meal, pumpkin powder, and hemp powder. This was observed in methionine (15.9%), phenylalanine (66.8%), tryptophan (45.8%), valine (64.4%), and methionine + cysteine (51.8%). Using AAFCO and NRC recommended values for cats, cricket meal was determined to be high quality as it contained no first-limiting AA. Chocho powder was found to be a low quality protein according to AAFCO and NRC comparisons, the lowest DIAAS-like coefficient was in arginine, 36.5% (AAFCO) and 38% (NRC). Pumpkin powder and hemp powder were characterized as a moderate quality proteins and had threonine as the first-limiting AA. The associated DIAAS-like value for pumpkin powder was 75.3% for both AAFCO and NRC reference values, and for hemp powder, DIAAS-like values were 73.8% (AAFCO) and 79.9% (NRC). DIAAS-like calculations were made for soybean meal, a common plant protein ingredient in companion animal diets, by Reilly et al. (2021). The authors found that methionine was the first-limiting AA for dogs when using AAFCO and NRC references for adult dogs, 70.5% and 39.4%, respectively; however, soybean meal contained no scores <100% using AAFCO and NRC comparisons for adult cats. Other studies have applied DIAAS-like calculations to evaluate the quality of insect and plant protein sources in recent years. Do et al.

(2020) assessed the quality of black soldier fly larvae of different ages using the precision-fed cecectomized rooster assay and DIAAS-like calculations with AAFCO nutrient profiles and NRC recommended values as comparisons. Methionine was determined to be the first-limiting AA for dogs and cats using both AAFCO and NRC comparisons with DIAAS-like values that ranged from 79.3% to 92.8% for AAFCO and 40.3% to 51.8% for NRC. Black soldier fly larvae was determined to be moderate quality for dogs using AAFCO nutrient profiles as all methionine values were less than 100%. Using NRC recommended values for adult dogs, younger larvae (0 days, 11 days, 14 days, and 18 days of age) were low quality with values <50%. Quality increased to moderate quality in 23 days and 29 days old larvae, with DIAAS-like values of 51.2% and 51.5%, respectively. For cats, however, there were no first-limiting AA present for either AAFCO or NRC comparisons, designating this ingredient to be high quality. For feline diets, Do et al. (2021) evaluated the protein quality of black soldier fly larvae that were fed various concentrations of calcium by applying the DIAAS-like method. It was found that methionine + cysteine and phenylalanine + tyrosine were the first-limiting AA for dogs and cats using both AAFCO and NRC recommended values. The authors concluded that black soldier larvae would be low quality according to AAFCO and NRC reference proteins for adult dogs due to methionine + cysteine being the first-limiting AA with DIAAS-like values ranging from 42.9% to 45.8%. These ingredients were determined to be moderate quality for adult cats based on NRC comparisons where DIAAS-like values ranged from 91.3% to 97.4% for methionine + cysteine.

Conclusion

The pet food industry has seen an increase in demand for high quality proteins as pet owners are becoming more conscious of what they and their pets are consuming. The market interest in utilizing novel proteins has underscored the lack of information regarding the composition and AA digestibility of alternative protein sources. The data acquired in the current study indicate that the select novel proteins evaluated herein may be beneficial as protein sources in dog and cat diets with the proper complimentary proteins. Data from precision-fed cecectomized rooster assay suggest that, in general, all AA were highly digestible based on standardized AA digestibility values that are >80%. The AA concentrations were found to be lower in methionine and cysteine, which is characteristic of plant-derived (e.g., pulses) proteins. After the calculation of DIAAS-like values, methionine was the first-limiting AA for adult dogs when compared to AAFCO and NRC recommended values, while arginine and threonine were first-limiting for cats. Chocho powder was determined to be the lowest quality protein for dogs and cats as the first-limiting AA were <50%. Future research may evaluate the inclusion of these ingredients in formulations of pet food products and determine the effects of heat processing on these proteins due to normal chemical Maillard reactions as well as diet acceptability, macronutrient digestibility, and fecal metabolites and microbiota.

Tables

Table 6.1. Chemical composition of select novel protein sources

	Plant and Insect Protein Sources			
Item %, DM basis	**Cricket Meal**	**Chocho Powder**	**Pumpkin Powder**	**Hemp Powder**
Dry matter, %	97.8	92.8	98.9	93.5
		%, DMB[1]		
Organic matter, %	95.6	97.8	97.9	90.3
Crude protein, %	63.6	63.3	75.5	61.4
Acid hydrolyzed fat, %	22.7	21.4	13.1	14.5
Total dietary fiber, %	19.6	28.7	24.3	24.3
Insoluble	16.8	21.4	11.4	18.3
Soluble	2.8	7.2	12.9	6.0
Gross energy, kcal/g	6.2	6.1	5.3	5.3

Table 6.2. Amino acid concentrations of select novel protein sources as a percentage of the total crude protein

% by weight, DM basis	Plant and Insect Protein Sources			
	Cricket Meal	**Chocho Powder**	**Pumpkin Powder**	**Hemp Powder**
Indispensable AA				
Arginine	4.2	5.2	9.5	6.0
Histidine	1.5	1.4	1.4	1.3
Isoleucine	2.7	2.6	2.5	2.1
Leucine	4.4	3.7	4.4	3.3
Lysine	3.7	3.2	2.6	1.9
Methionine	1.0	0.3	1.3	1.1
Phenylalanine	2.3	2.1	3.0	2.2
Threonine	2.4	1.9	1.7	1.6
Tryptophan	0.7	0.4	1.1	0.5
Valine	3.8	2.1	3.3	2.6
Dispensable AA				
Alanine	5.0	1.8	2.7	2.1
Aspartic acid	5.0	5.2	5.3	5.0
Cysteine	0.5	0.8	0.8	0.8
Glutamic acid	6.7	11.2	11.1	8.4
Proline	3.4	2.1	2.2	1.9
Serine	2.2	2.4	2.5	2.0
Tyrosine	3.7	2.0	2.0	1.6

Table 6.3. Standardized amino acid digestibility of select novel proteins calculated using cecectomized rooster assay[1]

	Plant and Insect Protein Sources					
Indispensable Amino Acids, %	**Cricket Meal**	**Chocho Powder**	**Pumpkin Powder**	**Hemp Powder**	**SEM[2]**	**P-value**
Arginine	94.9^{ab}	98.5^{a}	91.3^{b}	92.9^{b}	1.1590	0.0050
Histidine	83.8^{b}	90.7^{a}	86.2^{ab}	81.7^{b}	1.5293	0.0079
Isoleucine	89.7^{b}	97.1^{a}	97.0^{a}	89.4^{b}	1.2425	0.0006
Leucine	91.9^{bc}	98.0^{a}	96.9^{ab}	87.5^{c}	1.3495	0.0005
Lysine	88.4^{ab}	95.2^{a}	77.2^{c}	80.8^{bc}	2.0672	0.0002
Methionine	96.1	95.2	96.4	91.2	1.2898	0.0745
Phenylalanine	92.1^{ab}	97.6^{a}	96.8^{a}	89.0^{b}	1.3688	0.0024
Threonine	90.2^{ab}	97.5^{a}	93.7^{a}	83.4^{b}	1.9659	0.0018
Tryptophan	92.1^{c}	99.0^{a}	97.4^{ab}	95.0^{bc}	0.8936	0.0008
Valine	87.9^{b}	98.8^{a}	96.8^{a}	85.7^{b}	1.5478	0.0001

[1]n = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-c] means within a row with different superscripts are significantly different at $P < 0.05$

Table 6.4. Standardized amino acid digestibility of select novel proteins calculated using cecectomized rooster assay[1]

	Plant and Insect Protein Sources					
Dispensable Amino Acids, %	**Cricket Meal**	**Chocho Powder**	**Pumpkin Powder**	**Hemp Powder**	**SEM[2]**	**P-value**
Alanine	88.0[b]	97.2[a]	94.9[a]	84.9[b]	1.4242	0.0002
Aspartic acid	88.8[b]	95.9[a]	95.6[a]	89.9[b]	1.0327	0.0005
Cysteine	82.2[ab]	98.6[a]	96.5[a]	78.1[b]	4.1059	0.0088
Glutamic acid	91.4[b]	98.4[a]	96.5[a]	92.2[b]	0.8544	0.0002
Proline	86.9[bc]	102.5[a]	95.8[ab]	82.0[c]	2.4130	0.0003
Serine	89.4[b]	97.3[a]	97.1[a]	86.9[b]	1.5807	0.0008
Tyrosine	90.7[b]	98.9[a]	96.2[a]	89.8[b]	1.3014	0.0009

[1]n = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-c] means within a row with different superscripts are significantly different at $P < 0.05$

Table 6.5. Digestible indispensable amino acid score (DIAAS)-like[1] values for select novel proteins compared with AAFCO nutrient profile for adult dogs at maintenance

	Plant and Insect Protein Sources					
Indispensable Amino Acids, %	**Cricket Meal**	**Chocho Powder**	**Pumpkin Powder**	**Hemp Powder**	**SEM[2]**	**P-value**
Arginine	217.9[c]	51.4[d]	400.5[a]	302.2[b]	4.3059	0.0001
Histidine	192.8[a]	181.4[a]	151.0[b]	158.5[b]	3.2107	0.0001
Isoleucine	179.0[a]	176.6[a]	150.3[b]	137.2[c]	2.2331	0.0001
Leucine	167.2[a]	142.7[b]	148.9[b]	117.0[c]	2.1269	0.0001
Lysine	146.1[a]	127.2[b]	75.5[c]	66.2[c]	2.2963	0.0001
Methionine	81.1[c]	28.5[d]	92.1[a]	85.1[b]	0.7404	0.0001
Phenylalanine	135.6[b]	119.8[c]	155.7[a]	120.7[c]	1.6130	0.0001
Threonine	127.6[a]	102.3[b]	79.5[c]	77.9[c]	2.0394	0.0001
Tryptophan	120.5[b]	72.1[d]	158.3[a]	81.8[c]	1.0175	0.0001
Valine	188.7[a]	115.4[d]	153.4[b]	127.2[c]	2.6321	0.0001
Met + Cys	121.5[b]	93.0[c]	151.0[a]	143.2[a]	3.4163	0.0001
Phe + Tyr	238.0[a]	211.5[b]	199.6[b]	157.8[c]	2.9124	0.0001

[1]n = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-d] means within a row with different superscripts are significantly different at $P < 0.05$

Table 6.6. Digestible indispensable amino acid score (DIAAS)-like[1] values for select novel proteins compared with NRC recommended allowances for adult dogs at maintenance

	Plant and Insect Protein Sources					
Indispensable Amino Acids, %	**Cricket Meal**	**Chocho Powder**	**Pumpkin Powder**	**Hemp Powder**	**SEM[2]**	**P-value**
Arginine	177.1[c]	41.8[d]	325.4[a]	245.6[b]	3.4992	0.0001
Histidine	108.2[a]	101.8[a]	84.7[b]	89.0[b]	1.8030	0.0001
Isoleucine	99.4[a]	98.1[a]	83.5[b]	76.2[c]	1.2411	0.0001
Leucine	92.8[a]	79.2[b]	82.7[b]	65.0[c]	1.1821	0.0001
Lysine	146.6[a]	127.6[b]	75.7[c]	68.4[c]	2.3036	0.0001
Methionine	45.3[c]	15.9[d]	51.4[a]	47.6[b]	0.4131	0.0001
Phenylalanine	75.7[b]	66.8[c]	86.9[a]	67.3[c]	0.8998	0.0001
Threonine	79.1[a]	63.4[b]	79.5[a]	48.3[c]	1.4590	0.0001
Tryptophan	76.5[b]	45.8[d]	100.5[a]	51.9[c]	0.6458	0.0001
Valine	105.2[a]	64.4[d]	85.6[b]	71.0[c]	1.4681	0.0001
Met + Cys	67.7[b]	51.8[c]	84.1[a]	79.8[a]	1.9037	0.0001
Phe + Tyr	232.3[a]	206.4[b]	194.8[b]	154.0[c]	2.8422	0.0001

[1]n = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-c] means within a row with different superscripts are significantly different at $P < 0.05$

Table 6.7. Digestible indispensable amino acid score (DIAAS)-like[1] values for select novel proteins compared with AAFCO nutrient profile for adult cats at maintenance

	Plant and Insect Protein Sources					
Indispensable Amino Acids, %	**Cricket Meal**	**Chocho Powder**	**Pumpkin Powder**	**Hemp Powder**	**SEM[2]**	**P-value**
Arginine	154.9[c]	36.5[d]	284.8[a]	214.9[b]	3.0620	0.0001
Histidine	171.4[a]	161.2[a]	134.2[b]	140.9[b]	2.8549	0.0001
Isoleucine	189.0[a]	186.4[a]	158.6[b]	144.8[c]	2.3574	0.0001
Leucine	132.4[a]	113.0[b]	117.9[b]	92.7[c]	1.6855	0.0001
Lysine	160.3[a]	139.6[b]	82.8[c]	74.8[c]	2.5197	0.0001
Methionine	194.5[c]	68.4[d]	220.8[a]	204.2[b]	1.7760	0.0001
Phenylalanine	210.8[b]	186.2[c]	242.1[a]	187.7[c]	2.5071	0.0001
Threonine	120.9[a]	96.9[b]	75.3[c]	73.8[c]	1.9308	0.0001
Tryptophan	174.1[b]	104.2[d]	228.6[a]	118.2[c]	1.4692	0.0001
Valine	216.3[a]	132.3[d]	175.9[b]	145.8[c]	3.0161	0.0001
Met + Cys	104.0[b]	79.6[c]	129.2[a]	122.6[a]	2.9250	0.0001
Phe + Tyr	232.7[a]	206.9[b]	195.2[b]	154.33[c]	2.8481	0.0001

[1]n = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-c] means within a row with different superscripts are significantly different at $P < 0.05$

Table 6.8. Digestible indispensable amino acid score (DIAAS)-like[1] values for select novel proteins compared with NRC recommended allowances for adult cats at maintenance

	Plant and Insect Protein Sources					
Indispensable Amino Acids, %	**Cricket Meal**	**Chocho Powder**	**Pumpkin Powder**	**Hemp Powder**	**SEM[2]**	**P-value**
Arginine	161.0[c]	38.0[d]	295.0[a]	223.3[b]	3.1809	0.0001
Histidine	158.2[a]	148.8[a]	123.8[b]	130.0[b]	2.6353	0.0001
Isoleucine	175.8[a]	173.4[a]	147.6[b]	134.7[c]	2.1923	0.0001
Leucine	123.8[a]	105.7[b]	110.3[b]	86.7[c]	1.5751	0.0001
Lysine	301.8[a]	262.8[b]	155.9[c]	140.8[c]	4.7424	0.0001
Methionine	176.0[c]	61.9[d]	199.9[a]	184.8[b]	1.6078	0.0001
Phenylalanine	170.3[b]	150.4[c]	195.5[a]	151.6[c]	2.0257	0.0001
Threonine	130.9[a]	104.9[b]	75.3[c]	79.9[c]	2.0597	0.0001
Tryptophan	164.9[b]	98.6[d]	216.4[a]	111.9[c]	1.3899	0.0001
Valine	202.3[a]	123.7[d]	164.4[b]	136.4[c]	2.8209	0.0001
Met + Cys	258.9[b]	198.2[c]	321.7[a]	305.1[a]	7.2806	0.0001
Phe + Tyr	224.7[a]	199.7[b]	188.5[b]	148.9[c]	2.7499	0.0001

[1]n = 4 cecectomized roosters per select protein

[2]SEM = standard error of the mean

[a-d] means within a row with different superscripts are significantly different at $P < 0.05$

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