Evaluation of bioprocessed soybean meal as a protein ingredient in extruded dog and cat diets

by

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B.S., China Agricultural University, 2018
M.S., China Agricultural University, 2020

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
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Abstract

The utilization of traditional soybean meal in pet food has been limited by its low methionine content and flatulence-causing oligosaccharides. Microbially enhanced soybean protein (MEP) and Aspergillus oryzae fermented soybean meal (F-SBM) are bioprocessed soybean meal products with improved nutritional value. The objectives of this thesis were to determine: 1) the optimal inclusion level of MEP for extruded pet food to enhance nutrient utilization and palatability, 2) the optimal A. oryzae fermentation process to improve soybean meal protein composition, and 3) the performance of F-SBM compared to traditional soybean protein products in extruded dog food. The effects of MEP and F-SBM on extrusion processing, kibble characteristics, palatability, nutrient digestibility, and stool quality were assessed in dogs and cats. The first experiment evaluated dog and cat diets with increasing levels of MEP (0, 5, 10, and 15% substitution for soybean meal). The second experiment optimized inoculation dosage and fermentation duration to enhance protein content in soybean meal and compared a diet with 30% F-SBM to diets with either 30% traditional soybean meal (SBM), 30% traditional soybean meal and 1% Amaferm (AMF), or 18% soybean protein isolate (SPI). Diets were fed to 12 dogs or 12 cats in a 4x4 replicated Latin square design, with a 9-day adaptation period followed by a 5-day total fecal collection. Data were analyzed using a generalized linear mixed model (GLIMMIX, SAS version 9.4, SAS Institute, Inc., Cary, NC) with treatment as a fixed effect and animal and period as random effects. In the first study, the replacement of traditional soybean meal with MEP increased ($P < 0.05$) kibble expansion in both dog and cat foods. Fecal dry matter output decreased ($P < 0.05$) in a linear manner as MEP increased in dog diets. Fecal quality in cats were not affected by MEP inclusion. Overall, nutrient digestibility was maintained with increased levels of MEP. Fecal ammonia showed a linear increase ($P < 0.05$) as MEP increased in cat diets. For palatability, dogs preferred ($P < 0.05$) diet with 15% soybean meal to diets with 5 or 10% MEP, while cats preferred ($P < 0.05$) diet with 10% MEP to a diet with 15% soybean meal. For the second experiment, an *Aspergillus oryzae* spore inoculation dosage of 1×10^4 spores/g and fermentation duration of 36 hours were found to be optimal for increasing protein content and avoiding increasing soluble dietary fiber in the soybean meal. The F-SBM had improved ($P < 0.05$) nutrient profile and increased ($P < 0.05$) specific mechanical energy and kibble expansion compared to traditional soybean meal during extrusion. Fermentation also decreased ($P < 0.05$) content of sucrose and oligosaccharides in soybean meal. Fecal quality and nutrient digestibility were similar among dogs fed on the SBM, the FSBM and the AMF, while those of dogs on SPI had less ($P < 0.05$) fecal output and greater ($P < 0.05$) nutrient digestibility. Colonic fermentation was the greatest ($P < 0.05$) in dogs on the AMF and the least ($P < 0.05$) in dogs on SPI, with dogs on SBM and FSBM intermediate. Dogs preferred SBM to FSBM but did not show preference between SBM and SPI. Overall, the bioprocessed soybean meals did not affect fecal quality, general nutrient digestibility when fed to cats and dogs compared to diets with traditional soybean meal. To maintain palatability, the inclusion level should not exceed 15% of the diet. Further research is needed to explore health benefits and more processing characteristics of bioprocessed soybean meal as a protein source in pet foods.

Keywords: soybean meal; extrusion; fermentation; dogs; cats; nutrient digestibility; palatability

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Keywords: soybean meal; extrusion; fermentation; dogs; cats; nutrient digestibility; palatability

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Chapter 1 - Literature Review: The utilization of soybean as a protein source in pet food

Overview of soybean

Soybean (*Glycine max* [L.] Merrill) is believed to have originated from eastern Asia, particularly in China, with the earliest written record dates back to more than 2000 years BC (Liu, 2012; Murphy, 2008). Soybeans were initially grown as a forage crop and for its seeds that provided protein in human food and animal feed. Today, soybeans are one of the most widely cultivated crops and the principal source of protein for the feed industry in the world with a worldwide production of 378 million metric tons in 2022/2023 (USDA FAS, 2024a; Willis, 2003). At least 70% of soybean meal is used for animal feed as a source of protein for poultry, swine and cattle to support livestock industry, with the rest of them serve as aquatic feed, cats and dogs food (Stein et al., 2008). The rest of them are used for human food, biodiesel and plastics, etc. The top soybean- producing countries are Brazil (39%), the United State (29%), Argentina (13%), China (5 %), India (3 %), Paraguay (3 %) and Canada (2 %) (USDA FAS, 2024b).

In the United State, the USDA classify soybeans into Yellow soybeans and Mixed soybeans (USDA, 2023). The Yellow soybeans include soybeans with yellow or green seed coats with less than 10% of soybeans with other colors. Soybeans with black or bicolored (brown and black) seed coats also belongs to this class. Mixed soybeans refer to soybeans that do not meet the Yellow soybeans standards. Foreign materials are commonly found in soybeans and are defined by the USDA as all matter that pass through a $^1/_8$-inch sieve and all materials other than soybeans that remain on the sieve after sieving. Apart from this, damaged kernels, animal filth, splits and soybeans of other colors are frequently encountered materials that compromise soybean grade in trading (Liu, 2012). Among all, soybeans with yellow seed coats are the most commonly used type in food and feed industry.

Two major anatomic structures of soybean, the hull (8%) and the embryo , are separated by the embryo sac (Thorne, 1981). The embryo consists of two cotyledons and one embryonic axis (Figure 1.1). The former one provides nutrition during germination while the latter one grows into the sprout, the stem and the roots (Liu, 2012). The cotyledons are of the highest commercial values as they comprise about 90% weight of the whole seed and contains mostly proteins (43%) and oil (29%; Kawamura, 1967).

Soybean Protein

Discrete spherical protein bodies are distributed in the cytoplasm of palisade-like cells of cotyledons and surrounded by numerous oil bodies (Bair and Snyder, 1980; Tombs, 1967). The diameter of protein bodies ranges from 2-20μm, while that of oil bodies are less than 1μm. When soaked in water, protein bodies can swell and eventually rupture to release smaller protein granules with a diameter less than 0.5μm. However, this protein body membrane is able to maintain the structure during oil extraction with hexane (Tombs, 1967).

Soybean seeds have average of 40% protein on dry mater basis, of which the storage proteins comprises 65-80% while metabolic enzyme, structural proteins and membrane proteins comprise the rest of it (Grieshop et al., 2003; Krishnan, 2000; Liu, 2012; Medic et al., 2014; Murphy, 2008). Soybean proteins can also be classified into 2S, 7S, 11S and 15S proteins by sedimentation coefficient (Vanelli et al., 2021). Glycinin and β-conglycinin are the major storage protein in soybeans and account for 40% and 25 % of the total embryo protein (Zarkadas et al., 2007). Glycinin (11S) is a hexamer that belongs to legumin family and it consists of two proglycinin trimers with acidic and basic polypeptides connected by disulfide bonds (Adachi et al., 2003; Nishinari et al., 2018). The β-conglycinin (7S) is a trimer that belongs to the vicilin family and the three subunits- α, α’, and β-are connected through strong hydrophobic, hydrogen and di-sulfide bonds (Murphy, 2008; Wadahama et al., 2012). Soybeans also contain a small amount of 2S albumin as storage protein that potentially contribute to allergenicity (Lin et al., 2006; Moreno and Clemente, 2008). Other 2S soybean proteins consist of trypsin inhibitors and other small molecular weight enzymes (Murphy, 2008). As for the 15S fraction, Wolf & Nelson (1996) reported them as dimers of glycinin.

From a companion animal nutrition perspective, soybean protein has a lysine/total protein ratio comparable to animal proteins even though lysine is the common limiting amino acid in plant proteins (Donadelli et al., 2019; Gorissen et al., 2018). Methionine/sulfur- containing amino acids (SAA) are the limiting amino acids (LAA) in soybean protein for monogastric animals which makes soy protein a good complementary ingredient among plant proteins (Berry et al., 1962; Hee S. Kim et al., 2023b; Zarkadas et al., 2007). Specifically, the glycinin in soybeans contains more SAA than β-conglycinin. Each subunit of glycinin has higher SAA residues percentage than that of the α and α’ subunit of β-conglycinin, the β-subunit has no SAA residue at all (Utsumi et al., 1997). However, cats are strict carnivores that are not able to synthesize adequate taurine, a non-proteinogenic amino acid, by themselves. Lack of dietary taurine can cause serious clinical conditions in cats including myocardial failure, retinal degeneration, leukopenia, infertility, etc. (Hayes and Trautwein, 1989; Schuller-Levis et al., 1990). In this sense, taurine is also a LAA in soybean protein as it is not detectable in soybean proteins.

Soybean Protein Products

Soybean Meal

Soybean meal is the by-product of the extraction of soybean oil from whole soybeans. Several processes exist in soybean meal production resulting in different subtypes. Soybean meal is usually classified for marketing by its crude protein content. High-protein types are obtained from dehulled seeds and contain 47-49% protein and 3% crude fiber (as-is basis). Other types of soybean meal include the hulls or part of the hulls and contain less than 47% protein and more than 6% crude fiber. In solvent-extracted soybean meals, the oil content is typically lower than 2% while it exceeds 3% in mechanically-extracted meals (Cromwell, 2012). All types of soybean meal have lower crude fiber content than most other oilseed meals (Willis, 2003). The full-fat soybean meal is produced by cooking or roasting whole soybeans. Such soybean product has high fat and energy content and is a good source of fatty acids. The soybean meal that is more commonly referred to is the by-product (usually dehulled) of the extraction of soybean oil and is widely used as a protein supplement in animal feeds. The most common approach for separating oil from whole soybeans is solvent extraction which involves major steps of dehulling, grinding, conditioning, flaking, solvent extraction (hexane/acetone/ethanol/ isopropyl alcohol), desolventization and heating (Hee S. Kim et al., 2023b; Willis, 2003). Mechanical expression also plays a minor role in this industry with the advantage of soybean meal free from chemical residues. According to the USDA database (2018), soybean meal typically has around 93% dry matter (DM), 53% crude protein (DM basis), 3 19% carbohydrate (DM basis), 6.0% ash (DM basis) and 2.6% crude fat (DM basis). Among all the non-structural carbohydrates, sucrose (4-7%), stachyose (3-5%) and raffinose (0.5-1%) are the most abundant sugars (Grieshop et al., 2003; Hee S. Kim et al., 2023b).

Soybean Protein Concentrate and Soybean Protein Isolate

Both soybean protein concentrate (SPC) and soybean Protein Isolate (SPI) are processed from defatted soybean meal (Figure 1.2) (Hee S. Kim et al., 2023b; Wang et al., 2004). Proteins in soybean meal are precipitated through either acid wash at pH around 4.5 or 60% alcohol wash. Centrifugation or filtration separates the protein from soluble carbohydrates and other non-protein materials in the supernatant. The concentrated protein fraction is then dried to become final SPC product. Protein isolate is also prepared from defatted soybean meal/flake/flour but with alkaline (pH at 8.0) extraction at the beginning followed by centrifugation, precipitation, and lyophilization (Petruccelli and Anon, 1995; Wang et al., 2004).

Soybean protein concentrate and soybean protein isolate have as least 65% and 90% crude protein on DM basis, respectively. There is still about 20% carbohydrates, 1-3% crude fat and 3-5% dietary fiber in soybean protein concentrate while those in the isolate are very low in the isolate.

Soybean Utilization in Pet Food

The majority of world leaders are aware of the climate-change issue as it has become the most challenging concern of this century. Pet foods with sustainable and environment friendly claims may gain consumer preference as well. Meat takes 75 times more energy to produce than that of corn, 4 to 5 times more water than rice, and about 8 to 10 times more land to nourish one person (Mendoza, 2023). Producing plant-based proteins directly from crops bypasses the inefficiencies of converting plant matter into animal feed, which is then used to grow livestock. This direct use of crops for protein production improves overall efficiency and reduces pressure on agricultural land (Langyan et al., 2022). It is obvious that plant-based high-quality proteins will play a greater role in pet food industry in the future.

In 2019, the total amount of soybean ingredients—including soybean flour, soybean meal, soybean protein concentrates, soybean oil, and other variations—used in U.S. pet food accounted for approximately 6.18% of the total pet food ingredients. Among plant-based pet food ingredients, soybean meal ranked as the third largest component behind corn and corn gluten meal (IFEEDER, 2020). Cats are carnivores while dogs are omnivores, and dog food has higher production volume than cat food, thus, soybean-based ingredients are usually more common in dog food than in cat food in respect to volume, especially soybean meal (3 times more in dogs than in cats) and soy oil (4 times more in dogs than in cats) based on the report of IFEEDER (2020).

Nutritional Value of Soybeans as Pet Food Ingredient

The pet food industry is becoming more aware of product sustainability, which aligns with the government's broader goal of reducing global carbon emission (Dierenfeld, 2023; Swanson et al., 2013)n. Soybean protein has a history as a plant protein source in animal feed that dates back to the 1920-30s (Ruiz et al., 2020). The widespread adoption of soybean meal in pet food formulations grew significantly after World War II, as the demand for high-quality, cost-effective protein sources increased (Cromwell, 2012). Many studies have been conducted to understand the influence of soybean meal containing pet foods on nutrient digestibility and animal health. In summary, people have not found serious negative effects on nutrient digestibility and general health parameters from soy protein in pet foods as long as properly formulated (Hee S. Kim et al., 2023b; Vanelli et al., 2021). Plant-based ingredients also have less variable nutritional composition than animal-based ingredients which is a favorable feature for pet food quality control (Dust et al., 2005; Grieshop et al., 2003).

Protein Digestibility

The study of Kendall and Holme (1982) reported that two-third replacement of commercial dry dog food with either textured soybean protein, soybean meal or full fat soybean flour increased crude protein digestibility (83-85%) in dogs compared to DD (73%). However, the dry dog food (control) used in this study was a low-protein (14.4% DM basis) low-fat (7.4%) diet compared to diets partially replaced the control with soybean products (> 40% crude protein content on DM basis). The increased crude protein digestibility could be a result of increased crude protein content in the diet.

The uncertainty brought by differences in crude protein content in diets was addressed in following studies. Zuo et al. (1996) evaluated experimental diets with similar macro-nutrients (organic matter, crude protein, total dietary fiber and gross energy) contents and found that replacing corn and poultry meal with SBM at levels up to 37.1% increased both ileal and total tract crude protein digestibility in dogs. The ileal digestibility increased from 65.1% to 78.3% in 37.1% low oligosaccharide SBM diet and total tract digestibility increased from 77.2% to up to 84.6 in 37.1% conventional SBM. This study suggests that in some instances, greater quantities of SBM can promote higher protein and amino acid digestibility than poultry meal. Such results were reinforced by Clapper et al. (2001) who found that dogs fed a diet with 44% (as-is) SBM had higher crude protein digestibility (both apparent ileal digestibility and ATTD) than those fed a similar nutrient content diet that relied on poultry meal as the protein source. Diets with soybean flour (45% inclusion level) or soybean protein concentrate (inclusion level at around 34%) had the same influence. In both studies mentioned above, apparent ileal digestibility of SAA (methionine and cystine) was higher in dogs fed the soybean protein-containing diets, and such increase was even greater with higher soybean protein inclusion level. These studies were conducted more than 20 years ago when more byproducts from human food industry were used to produce. For instance, poultry meal may contain incidental levels of feathers, feet, heads, and connective tissue that would compromise its nutritional value (Bednar et al., 2000; Clapper et al., 2001). In the work reported by Bednar et al. (2000), SBM (30%)-containing diets fed to dogs had numerically but not statistically higher apparent ileal digestibility of crude protein than those fed a poultry meal diet but lower ATTD of crude protein. The latter outcome may be attributed to higher total dietary fiber content in the SBM-containing diet. The idea that soybean protein has higher protein digestibility than poultry meal in dogs was to some extent supported by studies conducted later (Maria et al., 2017; Nery et al., 2010; Yamka et al., 2003; Zanatta et al., 2013).

Additional studies in which animal protein was replaced by soybean protein and crude protein digestibility was measured have been published. Many of these experiments found that crude protein, crude fat and gross energy digestibility were similar among diets containing soybean protein and animal protein (Carciofi et al., 2009, 2006; Menniti et al., 2014; Tortola et al., 2013). In comparison, others have found that soybean protein had negative effects on nutrient digestibility compared to poultry meal, corn gluten meal, or rice protein concentrate when inclusion levels were high (over 50%) (Félix et al., 2012; Hee S. Kim et al., 2023a; Nery et al., 2010; Yamka et al., 2003). Food intake and dry matter intake were not affected by soybean protein inclusion in most of the studies. In some palatability studies, diets with moderate inclusion levels (12-30%) of soybean products even had greater first choice frequency in dogs than control diet (Beloshapka et al., 2016; Hee S. Kim et al., 2023a). Such results indicate that soybean protein does not cause diet palatability problems in dogs.

Fecal Quality

There are other effects apart from nutrient digestibility to consider for inclusion of soybean protein in the diet increased wet fecal output (Bednar et al., 2000; Beloshapka et al., 2016; Clapper et al., 2001; Hee S. Kim et al., 2023a; Maria et al., 2017; Yamka et al., 2006); whereas, Carciofi et al. (2009) reported a decrease. Menniti et al. (2014) and Beloshapka et al. (2016) both observed decreased fecal dry matter percentage in dogs fed with soybean meal containing diets. The increased fecal wet output can be a consequence of the non-structural carbohydrates in soybean products that generated greater osmotic effect in the colon (products from hind-gut fermentation of soluble fiber) and mechanical stimulation (insoluble fiber) to intestinal wall to secrete more liquid and mucus (McRorie and McKeown, 2017). However, in these studies the fecal score did not differ among diets in spite of their fecal wet output and/or dry matter output. Thus, consumers would notice little impact if soy were included in the diet.

Other than factors mentioned above, inconsistencies in selection of different soybean protein products, study design, treatment design (comparing soybean protein to single or multiple animal protein sources), and inclusion of other plant protein sources among studies all entail cautious interpretation of the conclusions.

Limitations of Soybean as Pet Food Ingredient

The current limitations of promoting soybean protein utilization mainly come from two aspects: 1) negative consumer/pet owner perception, 2) decreased nutritional value when added at high level. Consumer attitudes towards soybean in pet food varies among individual preferences and perceptions of health benefits, sustainability, and ethical considerations (Possidónio et al., 2021). Pet owners' major concerns about soybean in pet food include allergies, relatively poor protein quality, genetically modified organisms (GMO), poor digestibility and anti-nutritional factors (Laflamme et al., 2014). The GMO concern can be a bias for the influence of genetically modified plant. For the allergy concern, while some pets may have allergies to soy, it is not as prevalent as commonly believed. Allergies to soy are actually relatively rare in dogs and cats compared to beef and dairy products that are the top 2 allergens in both dogs and cats (Verlinden et al., 2006). Even for those actual soy-allergic pets, the glycinin and β-conglycinin are implicated as major allergens in soy protein. Proper processing can diminish or even eradicate some negative effects associated with soybean meal/protein, but pet food customers/pet owners still hold a bias to soybean products in pet food. Organic and non-GMO soybean sources are available for pet food formulations but at a greater cost. As for the protein quality and digestibility, there are plenty of studies that show when properly processed and formulated, soybean meal can provide high-quality protein with comparable digestibility for pets.

Anti-nutritional Factors

Soybeans do contain anti-nutritional factors (ANFs) that can interfere with nutrient absorption. The major concern in ANFs include trypsin inhibitors (TIs), oligosaccharides, phytate and urease etc. About 2-6% of soybean proteins are protease inhibitors, of which the Kunitz trypsin inhibitor (KTI) takes the most part and is the most well-studied; Bowman-Birk inhibitor (BBI) is another (Medic et al., 2014; Saini, 1989). The Bowman-Birk inhibitor (BBI) is a smaller globulin compared to the Kunitz trypsin inhibitor (KTI), yet it possesses multiple reactive sites, enabling it to bind to both trypsin and chymotrypsin (Blow et al., 1974). Soybean TIs naturally exist to protect the plants from predation due to proteases secreted by pests and microorganisms and also to regulate inherent proteases in the plant. Trypsin inhibitors can compromise pancreatic enzymes in monogastric animals when soybean protein is used as food/feed. In other protease inhibitors, like those in Ehrlich ascites tumor cells, inhibition relies on thiol-disulfide exchanges that form or break disulfide bonds with the protease, creating inactive enzyme-inhibitor complexes (Steven and Podrazky, 1978). Soybean TIs, however, operate through structural complementarity and specific binding interactions due to their active sites that mimic the natural trypsin substrate (Blow et al., 1974). This allows them to bind directly to the trypsin active site and form complexes through hydrogen bonds, electrostatic forces, and hydrophobic interactions, rather than thiol-disulfide exchanges and inhibit the binding of actual protein or amino acid residues (Blow et al., 1974). Soybean TIs have also been found to cause pancreatic hypertrophy in rodents but not in larger animals including dogs and human being (Patten et al., 1971). In order to compensate for loss in trypsin functionality, increased protease secretion also demands more SAA which is unfortunately the limiting amino acids in soybean protein. Heat combined with moisture (e.g. extrusion processing) can help reduce TIs in pet food but usually there is still 10-20% TI activity remains which is believed to be the BBI as it is more resistant to heat due to greater portion of disulfide cross-linking compared to the KTI (Murphy, 2008).

Oligosaccharides

Soybean oligosaccharides (OS) are also considered anti-nutritional. In the early stages of soybean development, there are considerable amounts of monosaccharides (e.g., glucose, fructose and galactose). They decrease to a neglectable extent by the time of full maturation, when only sucrose remains as a significant monosaccharide in the seeds (Middelbos and Fahey, 2008). When processed into soybean meal, glucose, galactose, and fructose are not detectable anymore. On the contrary, stachyose and raffinose accumulate over time and serve as critical storage or transport carbohydrates in mature seeds. Among the non-structural carbohydrates in soybeans, sucrose, stachyose, raffinose, and verbascose are the most abundant soluble sugars that take 11- 25% of total seed weight. Raffinose, stachyose, and verbascose are formed with attachment of one, two, and three galactose residues, respectively, to sucrose via α-1,6 glycosidic bonds. Dogs and cats are not able to efficiently digest soy OS due to the lack of α-galactosidases in their intestinal mucosa - a shared characteristic in mammals (Medic et al., 2014). Undigested OS are highly fermentable. They pass to the large intestine and become substrate for microbial fermentation in the hind gut, which produce gas such as hydrogen, carbon dioxide and methane. Short chain fatty acids are also hind gut fermentation products that can be absorbed by colonocytes and utilized by animals. Up to 7% of the metabolic energy of dogs, and to a lesser extent in cats, is produced by microbial fermentation in the colon (Brosey et al., 2000; Herschel et al., 1981).

Excessive colonic fermentation due to improper diet intake can have a negative influence on host health. Highly fermentable fibers can lead to extra gas production and may induce flatulence and even abdominal pain (Silvio et al., 2000; Yamka et al., 2003); undigested proteins can also lead to increased production of toxic fermentation metabolites (e.g., ammonia, amines and hydrogen sulfide), and can result in drowsiness and nausea. Unbalanced diets with too much reliance on soluble fibers can results in disordered fermentation that leads to production of pro-inflammatory metabolites which may circulate in the system and impact lipid metabolism (Singh et al., 2018).

Phytate or phytic acid is not sensitive to heat or moisture treatment (Menniti et al., 2014). Phytate in soybeans serves as a storage form of phosphorus and minerals to ensure these nutrients availability when needed most: during germination. Phytase enzymes break down phytate during germination, releasing phosphorus and other minerals, providing a nutrient source for the developing seedling (Dong and Saneoka, 2020). However, the heat process inactivates phytase in soybean but leave phytate uncompromised. Phytate, minerals, protein and phytochemical naturally form a complex that hinder bioavailability of these nutrients through physically blockage and chemically binding (Nkhata et al., 2018). Feeding pig soybean-containing diet requires additional phosphorus supplement due to low availability of phytate phosphorus (Ilyas et al., 1995).

Urease is found in many oilseeds, but are especially prevalent in soybeans. In soybean, three urease isoforms have been described: the embryo-specific urease, the ubiquitous urease, and a third isoform (listed in an order of decreasing expression in mature seeds) (Rechenmacher et al., 2017). Urease catalyzes the hydrolysis of urea to ammonia and carbon dioxide. However, the urease in soybean itself is not able to cause any problem as feed, though it is very frequently mentioned in soybean meal quality evaluation as an indicator for proper heating (Cromwell, 2012). Heat is necessary to remove or inactivate ANFs such as trypsin inhibitors in soybean meal but excessive heat causes binding of amino acids with carbohydrates which reduces amino acid digestibility. Various studies have shown that both trypsin inhibitor and urease activities decrease with wet heat treatment, making urease a practical marker for monitoring the inactivation process of antinutritional factors in soybean meal (Baker and Mustakas, 1973; McNaughton and Reece, 1980).

Sulfur-containing Amino Acid Content

While published research has demonstrated that soybean protein can be an excellent nitrogen source in pet food if combined (or complimented) with other sources (e.g. animal proteins and other plant proteins) of SAA, soybean protein is still considered incomplete relative to animal proteins. This lower protein quality is somewhat driven by the method of evaluation. Protein efficiency ratio (PER) is one of several *in vivo* methods used to evaluate single protein ingredient. Donadelli et al. (2019) reported that soybean protein had similar PER with that of chicken meal and chicken by-product meal, but still much lower PER compared to spray-dried chicken, spray-dried eggs and (or) egg whites. Such data indicates that soybean protein alone may not be capable of meeting nutrition needs of animals and that it needs to be paired with complementary sources or supplemented with limiting amino acids. With a greater allowance of soybean protein in formulas to the extreme end for development of strictly vegan pet foods will require augmentation (or enhancements) to make them nutritionally viable for companion animals.

Approaches to Improve Soybean Nutritional Value

Reduction of Trypsin Inhibitor Activity and Oligosaccharides

Previously researchers and food industry professionals have employed various methods to inactivate soybean trypsin inhibitors. Some of the traditional approaches include thermal treatments alone and in combination with chemical treatments. Thermal treatment such as conventional forced air drying and roasting, have been commonly used to eliminate trypsin inhibitors (Žilić et al., 2012). These methods involve heating soybean samples to specific temperatures for a certain duration to denature the TI proteins, which results in easier enzyme digestion. However, these conventional methods may have drawbacks such as non-uniform cooking and drying which can lead to uneven temperature distribution and potential loss of essential nutrients (Jasti et al., 2015). The combination of thermal and chemical treatments has been explored for inactivating trypsin inhibitors in soybean products as well. For example, the addition of sodium carbonate, sodium bicarbonate or diluted hydrochloride during cooking has been shown to reduce trypsin inhibitor activity (Che Man et al., 1991; Guerrero-Beltrán et al., 2009; Wallace et al., 1971). Cooking soybean flour with sodium hydroxide at 90°C for 15 minutes (Baker and Mustakas, 1973) or with ascorbic acid and cupric sulfate at 65 ℃ for 1 h (Sessa et al., 1990) have been shown to be effective.

Novel methods have been explored to improve inactivation of soybean trypsin inhibitors without compromising nutritional quality of soy products in other aspects. For example, only two min of microwave at 2,450 MHz was needed to decrease trypsin inhibitors t0 13.3% in soybeans (Barać and Stanojević, 2005). Presoaking can even decrease the amount of time necessary to microwave reduce TI (Yoshida and Kajimoto, 1988). Radiofrequency treatment, infrared treatment, gamma irradiation, ultrasound, high pressure processing, instant controlled pressure drop and molecular modelling are other methods that have shown somewhat effective on soybean trypsin inhibitor inactivation without significantly compromising other essential nutritional values of soybeans (Huang et al., 2008; Vagadia et al., 2017).

While these traditional methods have shown some success in reducing trypsin inhibitor activity in soybeans, they may have limitations in terms of uniformity, nutrient retention, and overall effectiveness. Novel methods represent promising avenues for enhancing the inactivation of soybean trypsin inhibitors, thereby improving the nutritional quality and digestibility of soy products. Continued research and development in this area are essential to optimize these techniques for practical application in the food industry.

In regard to oligosaccharides in soybeans and soybean meal/flour, Sugimoto and Van Buren (1970) managed to reach complete hydrolysis of galacto-oligosaccharides with an exogenous enzyme isolated from *Aspergillus saitoi*. However, such enzymes are considerably inhibited by inherent organic acids in soybeans, which makes it less cost-effective. With a combination of soaking, dehulling, washing, and cooking more than half of the total oligosaccharides can be removed (Egounlety and Aworh, 2003). In the same study, fermentation with *Rhizopus oligosporus* after a physical-thermal process decreased stachyose by 83.9% in soybeans while raffinose stayed constant and trypsin inhibitors had a slight increase during fermentation. Another study using α-hin from germinating soybean seeds reached a 72.3% and 89.2% decrease for stachyose and raffinose in soybean flour, respectively (de Fátima Viana et al., 2005). A later study suggests a complete removal of oligosaccharides in soybean milk by synergistic actions of different forms (maximal activities at differed pH) of extracellular α-galactosidases from *Aspergillus terreus* (Ferreira et al., 2011). Sprouting followed by α-galactosidase from *Aspergillus niger* also reached 84%, 99%, 80% reduction of raffinose, stachyose and verbascose, respectively in soybean flour (Raman et al., 2019). Co-fermentation of tempeh with *Rhizopus oligosporus* and *Actinomucor elegans* has been found to yield extensive hydrolysis of both trypsin inhibitors and flatulence-causing oligosaccharides (Huang et al., 2019). In summary, the reduction or removal of soybean oligosaccharides was possible with 1) traditional heat and moist processing; 2) addition of enzyme products from either plants or microbiomes; 3) microbial fermentation; and 4) combination of microbial fermentation and exogenous enzyme hydrolysis. These approaches remain to be improved in regard to enzyme stability, excessive heat treatment, cost control of commercialization and side effects on other nutrients or AFNs in soybeans.

Increasing Sulfur-containing Amino Acids

In regard to increasing SAA content in soybean protein, Krishnan (2005) outlined multiple approaches such as traditional plant breeding, expression of sulfur-rich heterologous seed proteins, modification of abundant endogenous proteins, enhancing accumulation of low abundance endogenous methionine-rich proteins and expression of synthetic proteins with balanced amino acid profile.

Traditional breeding has managed to increase protein content in soybean, though the SAA percentage of total protein remained proportionally the same (Wilcox and Shibles, 2001). Imsande (2001) used ethyl methane sulfonate to mutate soybean seeds and identified a few lines of 20% higher methionine content than that of parental line. Higher glycinin/β-conglycinin ratio led to a higher methionine content. Previous research found that both spontaneous and induced mutations can achieve modification of β-conglycinin. However, plants with these mutations are not able to develop or reproduce normally (Kitagawa, 1991). Transgenic soybean lines have also been developed to incorporate Brazil nut 2S albumin which contains 18% methionine and 8.5% cysteine (Townsend and Thomas, 1994). The methionine content in such plant lines increased by 15-40%. The Brazil nut albumin, however, has been identified as a major allergen (Nordlee Julie A. et al., 1996) so it does not represent an easy solution. A *de novo* synthetic protein that was originally developed for expression in rumen bacteria has also been successfully transformed into soybean plants and led to 16.2% and 65.9% increase in methionine and cysteine, respectively compared to parent line (Zhang et al., 2014). The challenge in this study was the drastic decrease of foreign proteins in mature soybean seeds. Wang et al. (2024) reported that drought stress combined with sulfur fertilization and SAA precursors can promote higher SAA yield in soybeans. An animal-derived hypoallergenic milk protein, bovine β-casein, has also been successfully incorporated into soybean plants to promote better balanced amino acid profile (Maughan et al., 1999); but it is unknown if the bovine β-casein is able to increase general protein quality of soybeans. As glycinin is the major contributor to SAA in soybean protein, researchers have also tried to increase glycinin expression via genetic engineering. There are at least five genes that encode for glycinin (Nielsen et al., 1989). Nielson et al. (1995) tried to insert oligonucleotides encoding methionine residues into one of the glycinin encoding genes; unfortunately, no protein accumulation was observed in spite of transcription detection for the modified gene. Though other researchers managed to insert amino acid residues without compromising protein configurations in another glycinin encoding gene to enrich methionine content in potato tubers, tobacco seeds and soybean (Gidamis et al., 1995; Utsumi et al., 1994), such increase in methionine in soybean proteins is very possible but still not enough to meet animals' need since only one of the five genes is modified (Krishnan, 2005). Other methionine-rich proteins in soybean are the 2S albumin (12.1% methionine) and 2S globulins (e.g. trypsin-inhibitors). They were either low accumulation or were major ANFs in soybeans, thus are not proper target for increasing overall methionine content in soybean proteins (Lin et al., 2006; Moreno and Clemente, 2008).

Soybean Fermentation

As mentioned in the previous section, hydrolysis provides new opportunities for soybean products in pet food. Fermentation is a natural process of hydrolysis, during which microorganism produces bioactive enzymes to metabolize the substrates and convert them into different chemicals. Fermentation can happen naturally as humans have utilized it for thousands of years; initially to preserve perishable food such as milk, grains, fruits and vegetables (Farnworth, 2003). Fermentation of grains by certain microorganisms not only inhibits growth of pathogenic bacteria, but also decomposes aflatoxins from fungi (Lou et al., 2024). Fermentation of legumes increases absolute content of nutrients and/or increases bioavailability of nutrients through various mechanisms (Figure 1.3). For example, plant structural components (e.g. cellulose, hemicellulose and lignin) that come into contact with fermentative enzymes can be decomposed; anti-nutritional factors can be degraded/inactivated;

and large protein molecules can be hydrolyzed into amino acids and small peptides(Adebo et al., 2022). The fermentation conditions (e.g. duration, solid/liquid phase, temperature and humidity) vary among microorganism species, substrate characteristics and nutrient modification goals. For soybean fermentation, *Lactobacillus* spp. and *Bacillus* spp. are the most popular bacteria and *Aspergillus* spp. are the most popular fungal species (Adebo et al., 2022; Yang et al., 2007). Fermented soybean products also bring benefits to animals including better growth performance, nutrient digestibility, intestinal enzyme activities, stress alleviation and stronger immunity in weaning piglets, avians, dairy cows and dogs (Beloshapka et al., 2016; Cervantes-Pahm and Stein, 2010; Feng et al., 2007b; Kim et al., 2012; Sumi et al., 1990; Teng et al., 2012; Yuan et al., 2017),

Nutrient Modification in Soybean Fermentation

Microbial Metabolism

The nutrient modifications depend on the metabolism characteristics of certain microorganism used in the fermentation process. The most commonly found microorganisms in soybean fermentation belong to the lactic acid bacteria (LAB), which refers to a group of gram-positive bacteria that produce lactic acid as end product of carbohydrate metabolism (George et al., 2018). Polysaccharides can be broken down to oligo- and monosaccharides by α- and β- amylase and amylo-pullulanase excreted from bacteria (Wang et al., 2021). Mono-/oligo-saccharides absorbed by bacteria have to go through glycolysis to create pyruvate. Through either homo-lactic or hetero-lactic fermentation pathway, pyruvate will be reduced to create lactate, the conjugated form of lactic acid (Figure 1.4). Meanwhile, monosaccharides can also be utilized by the bacteria to biosynthesize polysaccharides and released into the substrates as exo-polysaccharides.

Protein degradation, peptide transport, peptide degradation and AA catabolism roughly describe the steps in protein metabolism in LAB fermentation. Extracellular proteolytic enzymes initiate the metabolism of proteins. A distinctive difference between LABs and animal enterocytes is that the former can absorb oligopeptides while the latter only absorbs di- and tri-peptides (Sanz et al., 2003). Consequently, there is greater variety among intracellular exo- and endo-peptidases in LABs to process protein derivatives compared to enterocytes (Vesanto et al., 1996). Deamination and decarboxylation are major approaches for LABs to convert AAs into other bioactive chemicals (Figure 1.5). Organic acids derived from AAs are one of the major flavor compounds in fermented food. Additonally, alcohols, ketones and esters also affect flavors. The biosynthesis of flavor substances in LABs mainly relies on the citric acid metabolism pathway and the AA metabolism pathway (Smit et al., 2005; Wang et al., 2021). Nevertheless, metabolism of LABs differs among strains, some of which are not desirable for flavor and safety concern. Fermentation with multiple strains allow complementary LABs to cooperate to minimize unwanted chemical production.

Some toxic chemicals and ANFs can also be degraded by LABs. For example, incorporation of LABs has been shown to inhibit mycotoxins accumulation in cereal product and aflatoxin B1 and B2 in *Aspergillus flavus* infected almonds (Ben Taheur et al., 2019). Some LABs can also synthesize protease and phytase to decompose trypsin inhibitor and phytic acid, respectively (Gao et al., 2013; Teng et al., 2012).

Fermented soybean foods for humans such as miso, natto, tempeh, and soy sauce are widely available especially in east Asia. Filamentous fungi, such as *Aspergillus*, *Rhizopus, Actinomucor*, and *Penicillium* are the predominant fungi used in soybean fermentation (Tian et al., 2022). Koji mold is more common name for *Aspergillus oryzae* in Japan that is plays a major in production of miso and tempeh. Such species is a high producer of alpha-amylase and also a major source of commercial protease production in the food industry (Gomi, 2014). Taking *A. oryzae* as an example, it has metabolism pathways for proteins, carbohydrates and lipids (Figure 1.6) similar to LABs. In solid state fermentation where oxygen supply is sufficient, enzyme expression of the glycolytic pathway is decreased (Zhao et al., 2019). Proteinases and glycoside hydrolases biosynthesis is upregulated in solid state compared to submerged state fermentation.

The expression of α-amylase that cleaves α-1,4 linkages in linear amylose chain was not different between solid and submerged soybean meal fermentation with *A. oryzae;* while the expression of glucoamylase that hydrolyzes α-1,6 glycosidic linkages increased in solid compared to submerged condition. Peptidases can be divided into exopeptidase and endopeptidase; the former release one free amino acid from a peptide and the latter cut peptides into two smaller peptides. Optimal pH also distinguishes peptidases. In early stage of solid-state fermentation of soybean with A. oryzae, neutral protease cut proteins in to peptides, other neutral peptidases assist at the same time (e.g. tripeptidyl peptidase), followed by other tripeptidase, dipeptidase, aminopeptidase for further breakdown of small peptides (Zhao et al., 2019).

Fermentation with *Aspergillus. oryzae*

Many factors like culture condition, substrate particle size, ventilation, initial pH and fermentation temperature affect metabolism of the microorganisms during fermentation, which alters nutritional composition of the final products (Battaglino et al., 1991). Initial moisture contents of the substrate are known to critically influence mold growth and enzyme production in solid state fermentations as water can serve as solvent of nutrients and make them more accessible to fungi. However, excessive water will impede oxygen penetration and mold growth. Chutmanop et al. (2018) found that 50% is the ideal substrate moisture content for *A. oryzae* to produce protease. In the same study, they also found that initial pH of 7.5 yielded about 30% greater protease activity than initial pH of 5.5. This is consistent with the finding of Zhao et al (2019) that protein metabolism in A. oryzae starts with a neutral protease. Jiang et al. (2022) observed that mycelial growth of *A. oryzae* was inhibited at low (22 ℃) and high (42 ℃) temperatures. Low temperature stress increased starch metabolism and intracellular trehalose synthesis while high temperature stress inhibited catabolism of monosaccharides, leading to accumulation of sugar *in A. oryzae* biomass. Amylase activity increased in the early stages of solid fermentation with A. Oryzae and decreased drastically while protease activity kept increasing during the first 60 h of fermentation (Chutmanop et al., 2008). Hoa & Hung (2013) also reported that carbon source, nitrogen source and metal source in different substrates significantly affect cellulase activity during fermentation with *A. oryzae* (Hoa and Hung, 2013).

With the potent protease from *A. oryzae*, it is believed that major protein-based ANFs in soybeans such as trypsin-inhibitor and lectin can be reduced or inactivated (Teng et al., 2012). Amino acid profile in fermented soybean meal can be modified by fermentation but it does not always increase content of methionine or cysteine (Frias et al., 2008; Hong et al., 2004). As for oligosaccharides (OSs) that limit inclusion level of soybean meal in pet food , the *A. oryzae* has the ability to digest OS with its α-galactosidase (de Fátima Viana et al., 2005; Shankar and Mulimani, 2007) and therefore relieve flatulence. Soybean fermentation with *Aspergillus* spp. can also eliminates phytate or release minerals bound to phytic acid, making them more available for absorption by animals (Ilyas et al., 1995). The advantages of fermentation on nutritional value of soybean products reside in potential improvement of protein quality and decrease of ANFs. With fermentation, excessive heat may be avoided to maintain heat-sensitive nutrients. Additional studies evaluating content of protein, SAA, trypsin inhibitors and OS of soybean products before and after fermentation with *Aspergillus* spp. are summarized in Table 1.1.

Fermented Soybean Products in Pet Food

Fermented soybean has an academic history that dates back to early 1960s. The PubMed open-access database returned more than 1,000 publications for "fermented" or "fermentation" and "soybean" with the first paper published in 1961. However, when "dogs" or "cats" or "pet" are added to previous key words, there are only 15 returns that date back to 1968 and 2/3 (10/15) were published after 2012. After reviewing abstracts from those papers, there were merely 3 publications related to feeding fermented soybean foods to dogs (Beloshapka et al., 2016; Mikawa et al., 2021; Sumi et al., 1990) and none published regarding nourishment of cats.

A group of Japan scientists reported that feeding natto (soybean products fermented by *Bacillus subtilis*) or nattokinase to mongrel dogs with experimentally induced thrombosis induced thrombolysis indicating that such soybean products may serve as a treatment and preventative option for embolism (Sumi et al., 1990). In the study of Beloshapka et al. (2016), they incorporated a bioprocessed dehulled soybean meal product in dog foods. The product highlighted the characteristic of low ANFs. They found Beagle dogs had comparable stool quality, nutrient digestibility and hind gut fermentation level when poultry by-product meal was replaced by this soybean in the diet at levels up to 24%. A more recent study of Mikawa et al. (2021) reported that feeding a freeze-dried natto product to six miniature dogs in addition to regular dry food augmented cellular immune activity as they observed increased natural killer cell proportion in peripheral blood lymphocytes, cytotoxic effect of the peripheral blood mononuclear cells on canine tumor cells and expression of TNF-α in peripheral blood mononuclear cells following an antigen-stimulation.

The pet food industry holds a negative impression for fungi since they usually bring food safety risk in grain-containing pet foods (Atungulu et al., 2018; Kazimierska et al., 2021; Witaszak et al., 2020). *Aspergillus* is the prevalent genera (65–89%) of filamentous molds found in grains. *Aspergillus flavu*, is the most prevalent species under this genera, followed by *Aspergillus sydowii*, *Aspergillus fumigatus* and *Aspergillus versicolor* (Campos et al., 2008; Martínez-Martínez et al., 2021).*Aspergillus oryzae* is widely used in human food industry as a potent producer of α-amylase and proteases. It is not able to produce aflatoxins due to lack of relevant genes. Moreover, fermentation endows the potential of improved palatability to animals and acceptability to both animals and pet owners in regard to soybean proteins.

Conclusion

Previous studies have demonstrated that soybean products can be an excellent protein source in pet food if properly formulated with other complementary ingredients. There are some unacceptable negative effects on fecal quality, nutrient digestibility and palatability observed due to inclusion of soybeans in pet food; but these can be overcome. However, some pet owners still hold a negative bias against soybean products. To promote utilization of soybean products in pet food in order to improve the prospects of long-term sustainability of this industry, we need to present more specific nutritional values to soybean products and target differentiated attributes that mesh with consumer profiles that appeal to new users. Fermentation is a common and long understood food processing technique that is considered natural and healthy. The history of fermented soybean foods is long and rich with many examples in everyday foods (e.g., beer, yogurt, tempe, etc.). An application of this technology to soy might provide benefit. There is obviously a gap in scientific research on fermented soybean products as a pet food ingredient in regard to effects on processing, stool quality, nutritional value, palatability and so on. Therefore, the objectives of this work were to explore: 1) the proper inclusion level of fermented soybean meal for use in extruded pet food and 2) the proper fermentation protocol to produced targeted nutrient modification in soybean meal. To achieve these objectives, the effects of fermented soybean meal on extrusion processing, palatability, nutrient digestibility, stool quality and hind gut microbiome were assessed when fed to both dogs and cats.

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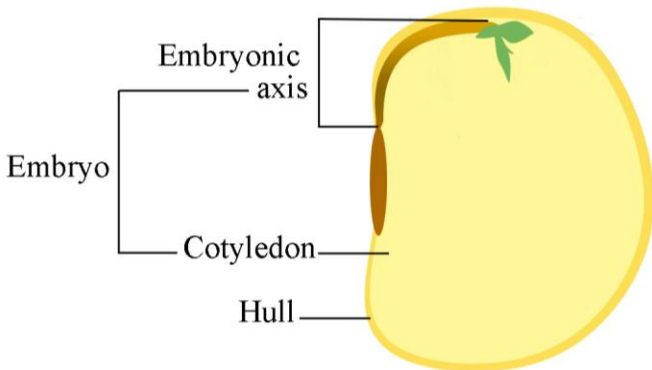

Figure 1.1. Schematic drawing of a soybean seed.

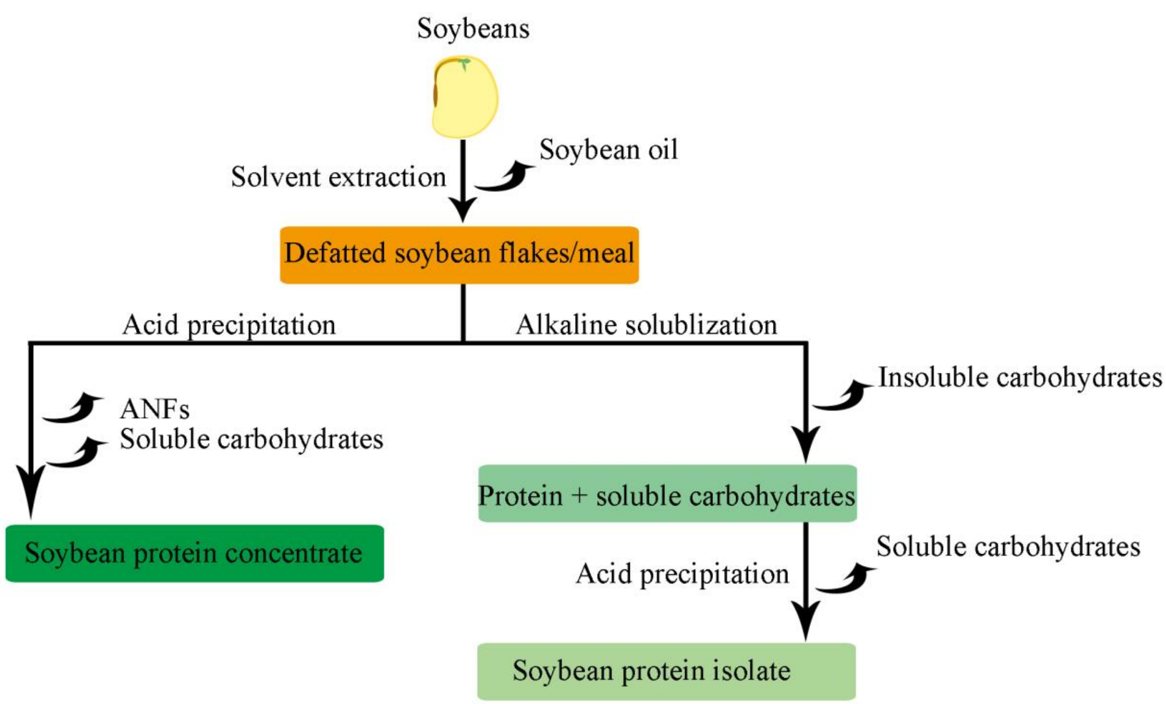

Figure 1.2. Production of soybean meal, soybean protein concentrate and soybean protein isolate.

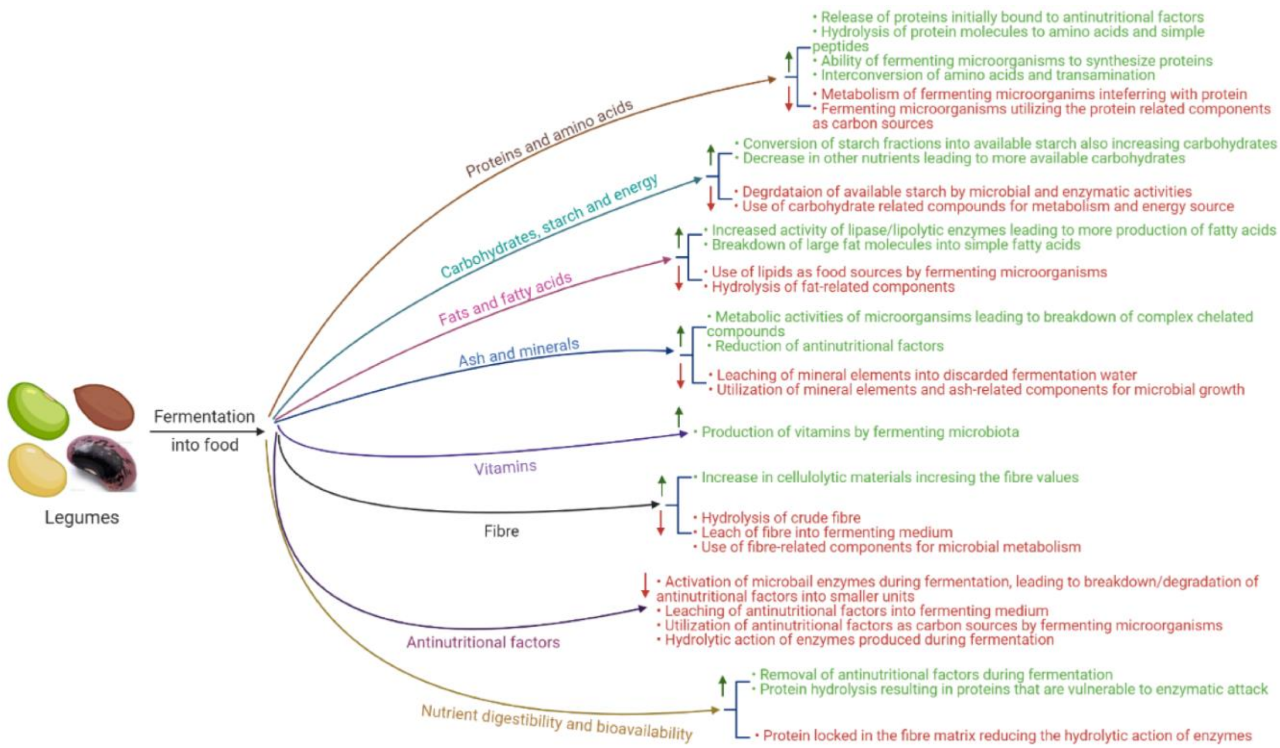

Figure 1.3. Mechanisms of nutrient modifications in fermented legumes (Adebo et al., 2022).

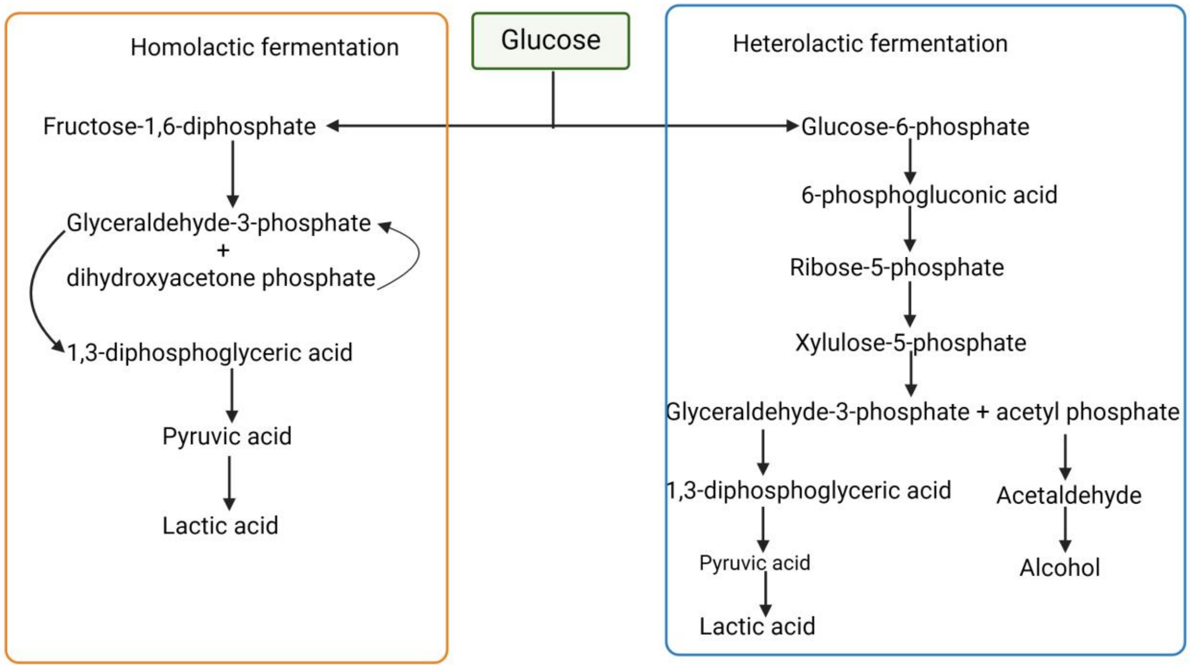

Figure 1.4. Homolactic fermentation (*Lactococcus* spp.) and heterolactic fermentation (*Lactobacillus* and Leuconostoc spp.) (Gänzle, 2015; Wang et al., 2021).

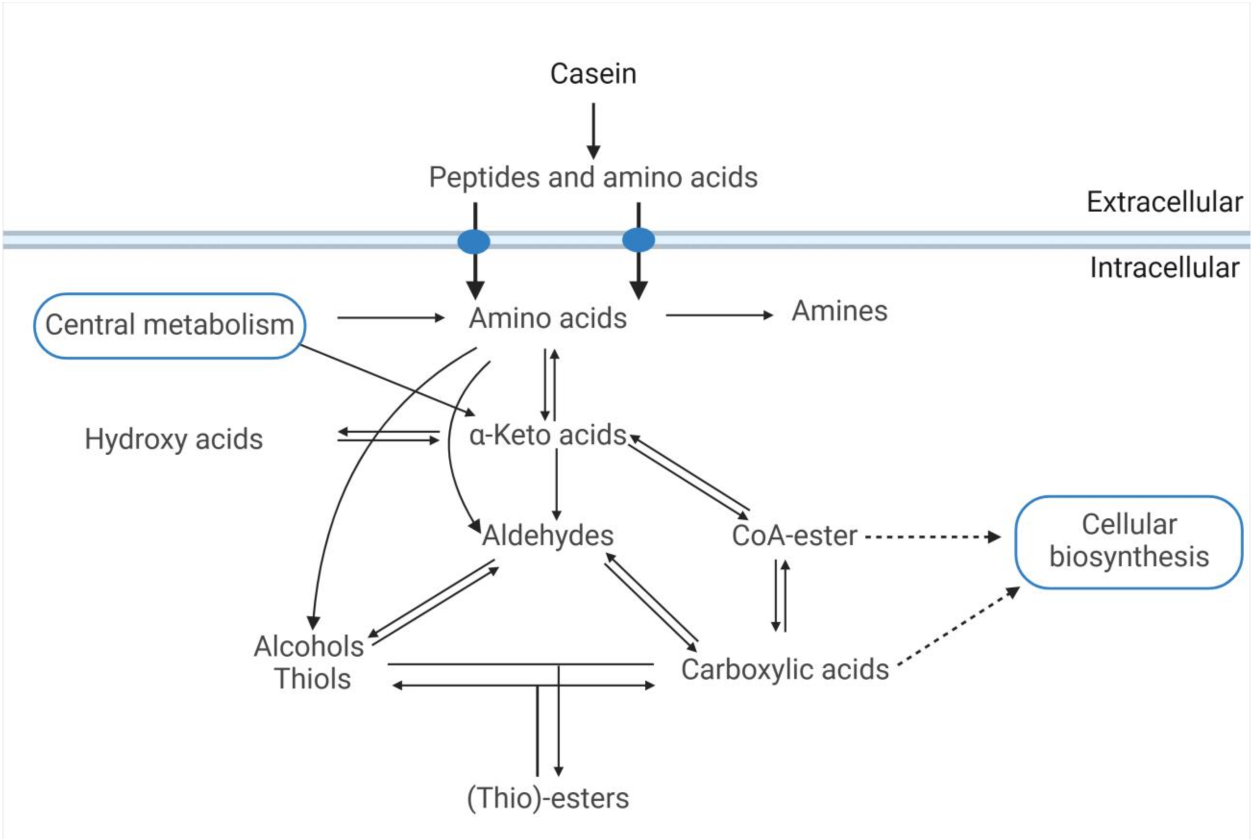

Figure 1.5. General protein conversion pathways relevant for flavor formation in dairy fermentations (revised from Smit et al., 2005).

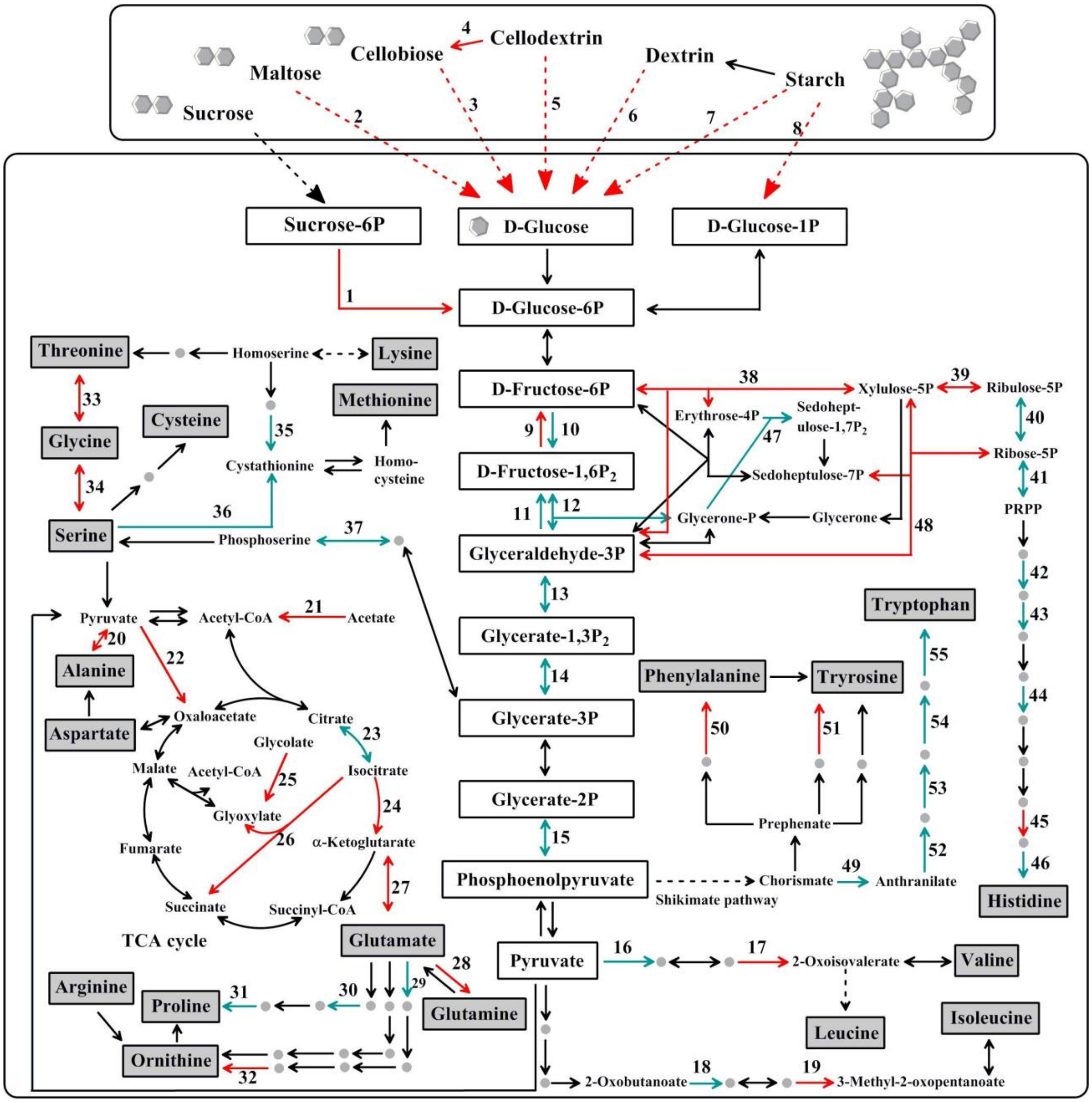

Figure 1.6. Metabolism of carbohydrates and amino acids in *Aspergillus. oryzae* in solid state fermentation compared to submerged fermentation. Red arrows represent enzymes with increased expression levels, and green arrows represent the decreased ones (Zhao et al., 2019).

Table 1.1. Nutritional modifications in soybean products through fermentation with *Aspergillus* spp under different conditions.

Publications[1]	Soybean products	Fermentation conditions	Inoculation dosage	Nutritional modifications[2]				
				Crude protein	Methionine	Cysteine	TI	OS
Yang, et al., 2020	SBM	89% IM, 30°C, 24 h, Alcalase assisted	10^8 spore/g	↓ 2.7%	ND	↓ 7.09%	NE	NE
Gao et al., (2013）	SBM	45% IM, 120h	4% (W/W)	↑ 13.11%	ND	↑ 3.80%	↓ 89.2%	NE
Chen et al. (2013)	SBM	50% IM, 30℃, 36h	$4*10^6$ spores/g	↑ 16.76%	↑ 11.11%	↑ 28.57%	Eliminated	Eliminated
Kim et al. (2012)	SBM	35% IM, 48 h	10^4 spore/g	↑ 10.85%	↑ 10.93%	NE	↓ 80.6%	NE
Teng et al. (2012)	SBM+WB (3:1, W/W)	67% IM, 28℃, 72 h	10^5 spore/g	↑ 9.99%	↑ 4.0%	↓ 11.29%	↓ 82%	NE
Frias et al. (2008)	SB	90% RH, 30℃, 48h	$5*10^3$ spores/g	NE	↑ 17.54%	↑ 11.76%	↓ data not shown	NE
Feng et al. (2007)	SBM	35% IM, 48 h	10^4 spore/g	↑ 5.95%	NE	NE	Eliminated	NE
Hong et al. (2004)	SB	90% RH，30℃, 48h	10^4 spore/g	↑ 4.36%	↑ 5.45%	↑ 3.70%	↓ 91.7%	NE
Hong et al. (2004)	SBM	35% IM， 48 h	10^4 spore/g	↑ 9.94%	↑ 6.49%	↑ 2.06%	↓ 84.4%	Eliminated[3]
Illyas et al. (1995) *Aspergillus.* usami	SBM	48h followed by 12 h of 50% IM	$2*10^4$ spore/g	↑ 13.19%	↑ 6.21%	NE	NE	NE

Zamora & Veum (1988)	SB	37℃, 18-24h	NE	↑ 1.11%	↑ 20%	ND	↓ 74.4%	NE

[1]*Aspergillus oryzae* was used in fermentation unless otherwise stated.

[2]Change of percentage calculated by ([nutrient in fermented product% / nutrient in unfermented product%]-1) × 100. Only numerical difference was considered here. Nutrient content was converted to dry matter if adequate information was provided in the publication.

[3]In the study of Cervantes-Pahm and Stein (2010) that used same fermentation protocol, stachyose and raffinose were found undetectable in fermented soybean meal.

TI-trypsin inhibitor; OS-oligosaccharides; SBM-soybean meal; SB-soybean; SBF-soybean flour; WB-wheat bran; NE-not evaluated; ND-not different numerically; IM-initial moisture content of substrate; RH-relative humidity of incubator.

Chapter 2 - Evaluation of Graded Levels of Microbially Enhanced Soybean Protein (MEP) on Extrusion Processing and Diet Utilization in Adult Dogs

Abstract

Microbially enhanced soybean meal (MEP; Me-Pro, Prairie AquaTech, Brookings, SD) is a product with high protein digestibility and palatability for terrestrial animals. Though no work has previously been published describing this for pets. The objectives of this study were to evaluate the nutritional value of MEP, performance of MEP in extrusion processing, and effects of graded MEP inclusion levels on diet utilization in dogs. The MEP samples were analyzed for their amino acid profile, protein digestibility corrected amino acid score (PDCAAS), mineral content and long-chain fatty acids. Four experimental diets were extruded on a single-screw extruder with processing data and samples collected at 15-min intervals during production. The control diet without MEP contained 15% soybean meal (SBM-D); soybean meal was replaced by MEP at 5, 10 and 15% to create three diets with graded level of MEP (5MEP-D, 10MEP-D and 15MEP-D). The experimental diets were fed to 12 adult dogs in a 4 x 4 replicated Latin square design. Dogs were adapted to diets for 9 days followed by a 5-d total fecal collection. Fresh fecal samples were collected for hind-gut fermentation analysis. Apparent total tract digestibility was calculated by total fecal collection (TFC) and titanium dioxide marker methods. Data were analyzed using the GLIMMIX procedure in SAS (version 9.4, SAS Institute, Inc., Cary, NC) with treatment as a fixed effect and dog and period as random effects. Least-square means were analyzed with a single degree of freedom contrasts at significance level of $\alpha = 0.05$.

The MEP had a PDCAAS of 0.41 with methionine/sulfur-containing amino acids as the limiting amino acid(s). Phosphorus and calcium were the most abundant minerals in MEP. Almost 50% of crude fat in MEP was linolenic acid. The preconditioner steam injection rate showed a linear decrease ($P<0.05$) as MEP increased in dog diets. The sectional expansion index (SEI) was greater ($P<0.05$) in kibbles of 5MEP-D and 15MEP-D compared to SBM-D. Food intake and dog fecal score were not impacted by dietary MEP inclusion. Dogs fed 15MEP-D had greater ($P<0.05$) crude protein digestibility (TCF method) than those fed SBM-D, with no significant effects on fecal pH, ammonia, or short-chain fatty acid production. For palatability trial, dogs preferred SBM-D over 5MEP-D and10MEP-D but did not show difference between SBM-D and 15MEP-D. Overall, including up to 15% MEP in extruded dog diets promoted kibble expansion without negatively affecting animal acceptability, stool quality, nutrient digestibility, or hind-gut fermentation.

Introduction

There are 63% of U.S. households that own pets, and the pet food industry in the U.S. commands a significant market worth more than $90 billion (APPA(American Pet Products Association), 2024). The pet food industry is a sophisticated consumer-oriented industry providing nutrition to companion animals which may be fed chronically for the life of the animal. Ingredients that bring extra nutrition and health benefits are much better accepted by pet owners than those which are simply an economic choice by the manufacturer (Schleicher et al., 2019). Animal-based proteins are generally considered to provide a better balance of essential amino acids for dogs and cats compared to plant-based proteins commonly used in pet foods (GAPFA, 2022). However, the impacts of animal agriculture and animal slaughtering for consumption on climate change and animal welfare have become a concern of the public

(Koneswaran and Nierenberg, 2008), thus, plant proteins have the potential to play a greater role in the future pet food industry.

Defatted soybean meal (SBM) is a byproduct of the soybean oil extraction process, primarily used as a protein supplement in feed for poultry, swine and cattle (Stein et al., 2008). The SBM is also a common complementary plan protein source in pet food for its high protein content and metabolizable energy (Stein et al., 2008). Amino acid profile and the bioavailability of limiting amino acids are critical for protein quality of a feed ingredient. However, the traditional SBM has low methionine content, which is an essential amino acid to dogs and cats (Berry et al., 1962; Zarkadas et al., 2007). Anti-nutritional factors (ANF) such as trypsin inhibitors, flatulence-causing oligosaccharides (OSs) and phytic acid also limited utilization of SBM in pet food (Hee S. Kim et al., 2023b; Yamka et al., 2006).

Enzyme coated soybean-based foods have been compared to poultry-based diets in dogs and showed that the former led to increased gut fermentation activity and improved the SBM protein value though amino acid absorption but was not as good as a poultry-based diet (Tortola et al., 2013). Fermentation is a natural process of microorganism producing bioactive enzymes to metabolize the substrates and convert them into different chemicals. Fermented or bioprocessed soybean meal has showed benefits in calves, piglets, broilers and dogs (Beloshapka et al., 2016; Kim et al., 2012; Mathivanan et al., n.d.; Yang et al., 2007). The microbially enhanced soybean meal (MEP) is an enzyme assisted fungal fermentation product from SBM (Gibbons and Brown, 2016). Its high protein digestibility, palatability and capability to promote greater fecal quality have been proved in weaned pigs and calves (Senevirathne et al., 2017; Sinn et al., 2017). However, fermented soybean meal products vary in physical-chemical properties and nutrient composition depending on the production protocols; this may have impacts on extrusion processing and diet utilization when fed to animals. Our hypothesis is that inclusion of MEP in dog diets will not negatively influence food production, nutrient utilization and palatability. Therefore, the objective of this study was to evaluate extrusion performance of MEP and effects of graded levels of MEP in extruded food on stool quality, nutrient digestibility, hind-gut fermentation and palatability in dogs.

Materials and Methods

Diet Formulation and Production

The MEP evaluated is a commercial product derived from soybean meal through a proprietary fermentation process (Prairie AquaTech, Brookings, SD, USA). Samples of MEP were analyzed in duplicate for dry matter (AOAC 934.01), organic matter and ash (AOAC 942.05).

Four experimental dog diets were formulated to be have similar nutritional composition and to be consistent with a premium dog food with high-protein and moderate level of fat (>25% CP and >10% CF). . The soybean meal in the control diet (SBM-D;) was replaced by MEP at 5, 10, and 15% (5MEP-D, 10MEP-D, and 15MEP-D, respectively). All diets included titanium dioxide (TiO_2; 0.4%) as an indigestible marker for determination of apparent total tract digestibility (ATTD) of dietary nutrients. As predicted by the formulation software, the concentrations of minerals (calcium, phosphorous, potassium, magnesium, sodium, sulfur, manganese, copper iron, and zinc) were similar among diets and met AAFCO nutrient profile recommendations for maintenance of adult dogs (Table 2.1).

The four dog foods were produced using a single screw extruder (model X115, Wenger Manufacturing, Sabetha, KS, USA). The preconditioner (model HIP 150, Wenger Manufacturing, Sabetha, KS, USA) was operated at a constant speed (20% mix intensity) and welded paddles. The extruder had a defined profile and barrel temperatures based on a typical commercial pet food configuration. At the end of the extruder barrel there were 2 inserts with 3 holes (6.7 mm diameter) per insert. Fixed input parameters were kept constant throughout each individual food production and included dry blends feed rate (739.5±2.5 kg/h), preconditioner water (132 ± 2.0 kg/h), extruder screw speed (350 rpm, except for 5MEP-D which was 325 rpm), and extruder knife speed (1300 ±50 rpm).

During extrusion process, preconditioner and extruder parameters were all collected from sensor readouts every 15 min to estimate the effects of different inclusion levels of MEP on extrusion. Output variables were those parameters resulting from the input variables, and included extruder die temperature, extruder motor load and specific mechanical energy (SME). Measurements were collected every 15 min during the production for a total of at least 2,000 lb per experimental diet and were considered treatment replicates.

Wet extrudates were conveyed pneumatically through an air hood system and deposited onto an oscillating belt spreader that spread the kibbles evenly across the dryer bed. The kibbles were dried on a belt passing two zones of a three-zone dryer (Airflow II, Wenger Manufacturing, Sabetha, KS, USA) to achieve the moisture content of kibbles below 10%. The dryer zones had varied temperatures and retention time as 19 minutes in zone 1, 10 minutes in zone 2 and 29 minutes in zone 3. Dried kibbles were coated with chicken fat and dry digest (Manx, AFB International, St. Charles, MO) in a drum mixer. Coated kibbles were stored in room temperature in poly-lined paper bags until fed.

SME was calculated using the following formula:

$$\text{SME (kJ/kg)} = \frac{\frac{(\tau - \tau_0)}{100} \times \frac{N}{Nr} \times Pr}{m}$$

where, τ is the extruder motor load, τ_0 is the extruder no load % torque, N is the extruder screw speed (rpm), Nr is the rated extruder screw speed, Pr is the rated extruder motor power, and m is dry feed rate (kg/s).

Kibble diameter and thickness (length) of 10 randomly selected kibbles from each collection point of each diet production off the extruder and off the dryer were measured with a digital caliper. Sectional expansion index (SEI) was determined by comparing the squared diameter of the dried extruded kibbles by the squared die diameter of the extruder:

$$\text{SEI}=\frac{D^2}{d^2}$$

where D is the extrudate diameter and d is the extruder die diameter.

Off the extruder kibble bulk density was measured manually off the extruder in each data collection point during each treatment processing using a 1 L cup and leveling the kibbles with a metal ruler and weighing on a digital scale with 1.0 g sensitivity.

Feeding Trials

The dog digestibility trial was conducted at the Kansas State University Large Animal Research Center (LARC) under the Institutional Animal Care and Use Committee (IACUC) protocol #4348. Dogs were housed in USDA approved kennels (Large Animal Research Center at Kansas State University; Manhattan, KS, USA). Twelve healthy adult beagles (6 neutered male and 6 spayed female) of similar age (1.0 ± 0.3 year) and similar body weights (8.4 ± 2.5 kg) were individually housed in metabolic pens (1.83m × 1.20m) equipped with an acrylic-mesh floor to allow for the separation of urine and feces. The dogs were housed on a 12 h light cycle with lights off from 1900 to 0700.The dogs were randomly assigned and fed a specific diet. Initial dietary intake on day 0 was determined by calculating daily metabolizable energy (ME) requirements for laboratory kennel dogs [$130 \times BW_{kg}^{0.75}$; NRC (2006)].

The ME of experimental dog diets was calculated using the prediction equation in pet food [(8.5$\times$ CF) +(3.5 $\times$ CP) + (3.5 $\times$ NFE)]. The daily food allowance was calculated using the daily ME requirement divided by predicted ME of diets. Dogs were fed twice daily (8:00 AM and 4:00 PM) in equal ration to meet daily food allowance. The body weights of dogs were measured at the beginning, middle, and end of each period and their food allowance was adjusted by 5 or 10% for the following week to maintain their BW. Water was provided for *ad libitum* consumption.

The feeding trial used a replicated Latin square design, where each animal served as its own control. Each of the four periods composed 9 d for adaptation followed by 5 d of collection. During collection period, all feces and orts were collected daily. Every fecal sample was weighed and scored on a 5-point scale where 1 = liquid diarrhea, 2 = very soft consistency, unformed stool; 3 = soft stool that retains shape; 4 = well-formed firm stool that does not leave residue when picked up; and 5 = very hard, dry stool. Fecal samples were stored at -20℃ until the end of trial and then thawed at room temperature, pooled by dog, weighed, and dried in a forced air oven at 55◦C for up to 48h until the moisture level was below 10%. The partially dried fecal samples were also weighed, and the values were used when calculating the DM (dry matter) of the fecal samples. Diet samples and partially dried fecal samples were ground through a 1-mm screen by a laboratory fixed blade impact mill (Retsch, type ZM200, Haan, Germany) and stored in Mason jars at room temperature for further chemical analysis.

In addition, the pH of one fresh fecal sample per dog per period (within 15 min of defecation) was recorded in triplicate with a calibrated glass-electrode pH probe (FC240B, Hanna Instruments, Smithfield, RI) immediately after collection. Fresh fecal samples were stored at -80℃ until further analysis.

Chemical Analysis

The MEP ingredient, ground experimental diet samples (after coating) and fecal samples were analyzed for dry matter (DM), organic matter (OM), and ash according to methods in AOAC 934.01 and 942.05. Crude protein was determined (AOAC 990.03) using a nitrogen analyzer (FP928, LECO Corporation, Saint Joseph, MI). Crude fat was determined by acid hydrolysis followed by hexane extraction (ISO 11085:2008) using semi-automated equipment (Hydrotec 8000 and ST 255 Soxtec, Foss, Denmark). Gross energy was measured as total heat of sample combustion by calorimetry (Parr 6200 Calorimeter, Parr Instrument Company, Moline, IL). The titanium dioxide content in diet and fecal samples was analyzed with a colorimetric method (Myers et al., 2004). The soluble dietary fiber (SDF), insoluble dietary fiber (IDF) and total dietary fiber (TDF) content of diet samples was measured (AOAC 991.43) by an ANKOM Dietary Fiber Analyzer (ANKOM Technology, Macedon, NY, USA).

Additional nutrient analysis was conducted for the MEP ingredient including long-chain fatty acids (LCFA), minerals and amino acids at the University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO). The LCFAs were analyzed (AOAC 996.06) by detection of fatty acid methyl esters (FAME) via gas chromatography. Elements were analyzed [AOAC 985.01(A, B, D)] with inductively coupled plasma-optical emission spectroscopy. All amino acids, except methionine, cysteine, and tryptophan, were digested with 6 N HCl for 24 h at 110°C. The amino acids were then separated by ion-exchange chromatography and the concentration was determined with a Beckman 6300 amino acid analyzer (Beckman, Palo Alto, CA). Methionine and cysteine were first oxidized by performic acid to methionine sulfone and cysteic acid, respectively, prior to acid hydrolysis. Tryptophan was hydrolyzed in 3 M mercaptoethanesulfonic acid before analysis. Available lysine was determined (AOAC 975.44) and lysine availability (%) was calculated as the ratio of available lysine to total lysine.

Ammonia concentration in the fresh fecal samples was analyzed through colorimetric method described by Chaney and Marbach (1962). The fresh fecal samples were thawed and diluted with deionized water and 0.1N HCl and homogenized. The homogenized samples were centrifuged at 3,000 g for 20 min to separate the suspended solids. The 1 mL of the supernatant of the centrifuged samples was collected and kept frozen at −20 ℃ for at least 24 h to complete deproteinization. The acidified samples were thawed, centrifuged at 20,000 g for 15min, and plated.

Fecal SCFA including BCFA contents were analyzed on a gas chromatography (GC; Agilent 7890, Agilent Technologies, Santa Clara, CA). The fresh fecal samples were thawed and diluted with deionized water and homogenized, followed by centrifugation at 3,000 g for 20 min to separate the suspended solids. The 1mL of the supernatant of the centrifuged samples was collected acidified with 0.25mL of 25% m-phosphoric acid was before deproteinization at −20 ℃ for at least 24 h. The GC is equipped with flame ionization detector (FID) and a capillary column (DB-WAX, Agilent 127-7012, 10m × 0.1mm × 0.1µm, Agilent Technologies, Santa Clara, CA). Hydrogen was used as a carrier gas with a flow rate of 35 mL/min, and the split ratio was 100:1 with injection volume of 0.2 µL. Nitrogen was used as the makeup gas with a flow rate of 25 mL/min. The detector and injector temperatures were set at 300℃, and the initial oven temperature was set to 40℃ with a ramp rate of 20℃/min to 180℃ for a total run time of 8 min. The peak area of chromatograms was determined using an integrative software (OpenLab CDS, Agilent Technologies, Santa Clara, CA). The concentrations of SCFA (acetate, propionate, and butyrate) and BCFA (isobutyrate, isovalerate, and valerate) in samples were quantified by comparing the sample peak area to three standards with known concentrations of each volatile free acid and correcting for the fecal dry matter content.

Digestibility Calculation

Two methods were utilized to estimate apparent total tract nutrient digestibility. The total fecal collection (TFC) method requires the collection of all feces excreted by the experimental animals. The marker method uses TiO_2 as an indigestible dietary marker. In current study, apparent total tract digestibility (ATTD) of DM, OM, CP, CF and GE were calculated according to the TFC and marker methods by following equations:

1. TFC method:

$$Nutrient\ digestiblity\ (\%)= \frac{(nutrient\ consumed\ (g) - nutrient\ excreted\ (g))}{nutrient\ consumed\ (g)} \times 100\%$$

2. Marker method:

$$Nutrient\ digestiblity\ (\%)= [1- \frac{(nutrient\ in\ feces\ \% \times TiO2\ in\ food\ \%)}{(nutrient\ in\ food\ \% \times TiO2\ in\ feces\ \%)}] \times 100\%$$

In-vitro Protein Digestibility

The *in vitro* pepsin-pancreatin protein digestibility method was determined in duplicates as 1 gram of sample was weighed into 50 mL centrifuge tubes, followed by addition of 15 mL of 0.1N HCL-pepsin (porcine; Merck Millipore 516360-2.5GM) solution to each tube and placed in a shaking water bath for 6 hours at 37°C. After pepsin incubation, the tubes were removed and 7.5 mL of 0.5 N NaOH was added to neutralize the sample and stop pepsin hydrolysis. Next, porcine pancreatin (4 mg; Sigma Aldrich P1750-100G) was added to 7.5 mL of phosphate buffer (pH 8), followed by 1 mL of sodium azide (for microbial control). Tubes were then placed in the shaking water bath for 18 additional hours at 37°C.

After 18 hours of incubation, 1 mL of 10% TCA was added to each tube to help with protein precipitation. Vials were then centrifuged at 3,700 g for 20 minutes. The supernatant was discarded by filtration (Whatman 541 filter paper). Then the samples were washed with distilled water, centrifuged and filtered again for 3 times. Final sediments were oven-dried overnight at 105°C and then analyzed for nitrogen utilizing the Dumas combustion method (AOAC 990.03). *In-vitro* protein digestibility was calculated using the following equations:

$$\text{Crude protein (CP) (\%)} = \text{N\%} \times 6.25$$

$$\text{Residue CP} = \text{residue weight (g)} \times \text{residue CP (\%)}$$

$$\text{CP digestibility (\%)} = \frac{Sample\ CP\ (g) - Residue\ CP(g)}{Sample\ CP(g)} \times 100\%$$

Protein corrected amino acid scores (PDCAAS) was calculated using the Food and Agricultural Organization suggested equation (FAO, 1991) :

$$\text{Amino acid score (AAS)} = \frac{Amino\ acid\ content\ of\ the\ test\ protein,\%}{Reference\ amino\ acid\ pattern,\%}$$

$$\text{PDCAAS} = \text{AAS of LAA} \times \text{CP digestibility}$$

Where reference amino acid pattern is dog or cat maintenance recommended values by NRC (2006). The LAA refers to limiting amino acid, which has the lowest AAS. Values for PDCAAS greater than 1.00 were treated as 1.00.

Palatability Trial

Palatability trial for dog diets were conducted at a commercial research kennel (Summit Ridge Farms; Susquehanna, PA, USA). Each MEP containing dog diets (5MEP-D, 10MEP-D and 15MEP-D) were compared to the control diet (SBM-D) where each comparison was determined in 30 beagle dogs with a two-bowl forced choice test over two days with bowl position switched for each day to remove side-bias.

Statistical analysis

Least square means of data were estimated by 1-way ANOVA using the GLIMMIX procedure in SAS (version 9.4, SAS Institute INC, Cary, NC, USA). Pairwise treatment comparisons were conducted using a Tukey's post hoc test. Contrasts comparing control (SBM-D) vs treatments (5MEP-D, 10MEP-D, and 15MEP-D), and linear, quadratic, and cubic relationships among all diets were considered significant at $P < 0.05$. For each diet production, sampling was conducted at evenly spaced intervals which were considered replicates. For digestibility trial analysis, dog and period were considered random effects in the model for analysis of data. In the palatability experiments, the intake ratio was analyzed using a t-test in a 2-way ANOVA and the first-choice preference was analyzed using a Chi^2 test.

Results

Chemical Analysis of MEP and Diets

Dry matter (DM), crude protein and crude fat content of MEP samples were 95.5%, 77.03% (DM basis) and 0.66% (DM basis), respectively. The *in vitro* protein digestibility of MEP was 98.46±0.12%. The essential amino acid analysis and PDCAAS (0.41) of MEP showed that methionine was the limiting amino acid for cats can dogs (Table 2.2). Total non-essential amino acids comprised 52.63% of total amino acids in MEP with aspartic acid (112.1 mg/g CP) and glutamic acid (172.2 mg/g CP) the most abundant (Table 2.3). Phosphorus (3150 ppm, as-is basis) and calcium (2260 ppm, as-is basis) and had the highest concentration in MEP among the 12 minerals analyzed (Table 2.4). Long-chain fatty acids comprised over 90% of the total fatty acids in CF in MEP (Table 2.4). Among all the long-chain fatty acids, over 54.5% was linolenic acid (18:3n3), almost 0.2% was docosahexaenoic acid (DHA, 22:6n3), and no eicosapentaenoic acid (EPA, 20:5n3) was detected. All diets were similar in DM, CP, CF and GE content (DM basis). The IDF content increased numerically as MEP increased while SDF was highest in SBM-D and 15MEP-D.

Extrusion Process

A linear decrease ($P < 0.05$) of preconditioner steam was observed as MEP inclusion increased (Table 2.5). A cubic relationship ($P < 0.05$) between extruder shaft speed and MEP inclusion was observed while this parameter was not different among diets. Neither did specific mechanical energy (SME) differ among diets, but a cubic relationship ($P < 0.05$) between SME and MEP inclusion was observed. The 15MEP-D required 59.67 kg/h extruder water injection which was greater ($P < 0.001$) than that of all other dog diets (22.00 kg/h for both SBM-D and 5MEP-D, 21.83 kg/h for 10MEP). Extrudates of 5MEP-D had lower ($P < 0.05$) bulk density (374.0 kg/m^3) than that of the SBM-D (408.6 kg/m^3). A cubic relationship ($P < 0.05$) between bulk density and MEP inclusion was observed. Extrudates of 5MEP-D and 15MEP-D had similar diameter (11.89 and 11.59 mm, respectively) but were lower ($P < 0.001$) than that of the other two diets (11.08 mm for SBM-D and 11.28 mm for 10MEP-D). Such influence was consistently reflected on sectional expansion index (SEI) that also showed a quadratic relationship ($P < 0.001$) with MEP inclusion.

Feed Intake and Fecal Characteristics

Food intake was similar among diets and no refusal to the food was observed during this feeding trial. Fecal wet output in dogs fed with 15MEP-D (113.3 g/d) was lower than that of dogs fed with SBM-D (128.8 g/d) and a linear relationship ($P < 0.05$) was observed between fecal wet output and MEP inclusion (Table 2.6). Fecal dry matter output also showed a linear relationship ($P < 0.05$) with MEP inclusion but was maintained at an average of 36.8 g/d among dogs fed with diets with variable percentages of MEP. Food intake, fecal moisture, defecation frequency and fecal score all remained similar among dogs fed with different experimental diets.

Apparent Total Trat Digestibility

With total fecal collection method (TFC), the ATTD of dry matter increased linearly ($P < 0.05$) as MEP inclusion increased in dog diets, indicating dogs fed with 15MEP-D (82.12%) had greater dry matter ATTD than dogs fed with SBM-D (80.61%) (Table 2.7). Same pattern applied to ATTD of crude protein ($P < 0.05$). There was a cubic relationship in ATTD of organic matter ($P < 0.05$), crude protein ($P < 0.05$) and crude fat ($P < 0.001$) among diets with different MEP inclusion levels. The ATTD of crude fat was the greatest ($P < 0.05$) in dogs fed with 15MEP-D (98.10%) and the lowest in dogs fed with 10MEP-D and SBM-D (96.91% and 96.49% respectively) while that of dogs fed with 5MEP-D (97.55%) was intermediate. In comparison, the ATTD calculated with titanium dioxide marker method only revealed a cubic relationship $P < 0.001$) between crude fat digestibility and MEP inclusion level, indicating that the ATTD of crude fat in dogs fed with 15MEP-D (97.88%) was the greatest compared to dogs fed with SBM-D (96.55%).

Hind Gut Fermentation

The pH of fresh fecal samples all fell into the range of 5.0-6.0 and did not differ among dogs fed with different diets (Table 2.8). The ammonia concentration in fresh fecal samples was numerically the greatest in dogs fed with the 5MEP-D (103.2 μmol/g DM feces) while that from dogs fed with other three diets were similar to each other (at an average of 98.6 μmol/g DM feces). None of the fatty acid concentrations in fresh feces differ among dogs fed with different diets. The propionate concentration in fecal samples showed a tendency of quadratic relationship (P=0.0804) with MEP inclusion.

Palatability

When comparing SBM-D to 5MEP-D or 10MEP-D, dogs preferred ($P < 0.05$) SBM-D as indicated by both first choice and intake ratio (Table 2.9). The SBM-D was chosen firstly over 5MEP-D for 45 out of 60 times and over 10MEP-D for 46 out of 60 times by 30 dogs in two days, respectively. However, such preference for SBM-D to MEP-containing diet did not last to 15MEP-D where two diets had similar palatability.

Discussion

Chemical Analysis of MEP and Diets

The crude protein content of MEP (77.03% on DM basis) was much higher than that of soybean meal (44-50%) and intermediate between that of soybean protein concentrate (over 65%) and soybean protein isolate (over 90%; Murphy, 2008). This could be a result of fungal protein accumulation and decrease of carbohydrates of during production of MEP (Mukherjee et al., 2015). A cross-sectional study evaluated amino acid composition and 14 soybean cultivars in Canada, the average methionine and cysteine content were 20.92 (16.14-22.22) and 20.93 (18.49-23.96) mg/g total protein (Zarkadas et al., 2007). Another study involving soybean meal produced in the USA reported a methionine and (methionine + cysteine) content of 14.2 and 29.2 mg/g CP, respectively (Reilly et al., 2021). Grieshop et al. (2003) reported that the mean methionine content in soybean meals from 10 U.S. processing plants were 13.47 (10.93-16.33) mg/g CP. In comparison, methionine (13.73 mg/g CP) and (methionine + cystine) content (27.59 mg/g CP) in MEP did not provide advantages over regular soybean meal. Surprisingly, the *in-vitro* protein digestibility of MEP was as high as over 98% while Ou et al. (2004) found that of soybean protein isolate powder was around 86.4%. Another study found the *in vitro* protein digestibility of soybean protein increased from 83% to 93% (Amadou et al., 2010). Note that in the study of Amadou, only trypsin was used to determine protein digestibility while pepsin and pancreatin were used in our study; but the effect of fermentation on increasing *in-vitro* protein digestibility was consistent. Despite the ultra-high *in vitro* protein digestibility, the PDCAAS score of MEP was relatively low (0.41) with methionine or SAAs the limiting amino acids just like in traditional soybean protein (Reilly et al., 2021; Zarkadas et al., 2007). The mineral analysis provided valuable information to use when incorporating MEP into certain formulas. Considering the low crude fat content of MEP, it would not be considered as a major lipid source in the diet or for essential fatty acids.

The maintenance of moisture content among dietary treatments was expected as drying conditions were controlled during processing. The 15MEP-D had slightly higher CP content compared to SBM-D despite the much lower chicken meal content in the former was expected as the CP content in MEP (77.03% DM basis) was much higher than soybean meal (53.9% DM basis) used in this study. Even though then chicken meal was replaced with brewers' rice as MEP increased, it was still hard to delicately balance CP content while keeping other macro-nutrient similar among diets. The increase in IDF content when soybean meal was replaced by MEP was also in observed in a study evaluating MEP in calf feed (Senevirathne et al., 2017) and a study evaluating enzyme treated soybean meal in extruded dog diet (Beloshapka et al., 2016). The decrease in SDF in MEP-containing diets are expected as MEP is low in OSs which are considered soluble fiber (Gibbons and Brown, 2016).

Extrusion Process

The differences in dry blend feed rate, preconditioner water, and extruder knife speed were minimal and interpreted to be of no practical importance. The decreased preconditioner steam for 10MEP-D compared to SBM-D and 5MEP-D was due to the extruder operator and not the dietary matrix. There is rare research on influence of microbially enhanced soybean protein on pet food extrusion. Despite the fact that not only fiber but also protein were the major variants in diet formulas in the current study, some of the concepts from studies of fiber sources on extruded pet food shall be valuable to the interpretation of results from this study.

Usually, increased insoluble fiber and decreased soluble fiber lead to less sectional expansion (Robin et al., 2012). This seemed to be true in this study when switching production from SBM-D to 5MEP-D with all input parameters unchanged. To achieve an extrudate bulk density close to the SBM-D, extruder shaft speed was then intentionally decreased in 5MEP-D. The consequently increased expansion in 5MEP-D could be explained by extended retention time for cooking due to decrease shaft speed. The decreased motor power and SME regardless of increased barrel fill indicated that the melt 5MEP-D had good flowability. The SME is usually positively related to expansion while other factors such as fiber content, fiber particle size, retention time and nutrient composition also play a role (Riaz, 2000; Robin et al., 2012). It has been reported that insoluble fibers provide additional friction in the extruder barrel (Donadelli et al., 2021) and dietary fiber water solubility can be significantly increased when increasing SME during extrusion (Redgwell et al., 2011). It seems that the 15MEP-D had the greatest SME due to abundant insoluble fiber content. With the increase of SME, fiber solubility increased and competed with starch for water as both insoluble and soluble fiber are considered to compete with starch for water (Donadelli et al., 2021). Therefore, the high extruder water requirement for 15MEP-D to achieve target extrudate bulk density was probably due to its high dietary fiber content and starch content compared to other diets. The increased fiber solubility in 15MEP-D may also have contributed to viscosity of the melt, thus greater kibble expansion.

It was reported that even though soluble fiber mix with the melt material in the extruder, it does not always provide structural forming like gelatinized starch, which lead to less expansion (Parada et al., 2011). This may be the case for SBM-D diet as it had the greatest soluble fiber content among all diets and had the least expanded kibbles. Fiber seemed to have acted differently in SBM-D and 15MEP-D. This may be the results of different chemical-physical properties between fibers in soybean meal and in MEP, which could be the next study topic. Also, the 15MEP-D had more fiber from brewers rice. Fat also has negative impacts in creating mechanical energy in extrusion since it acts like lubricant between the shaft and melt material (Riaz, 2000). The SBM-D had the greatest chicken meal content which was the only variation in fat source in the extrusion raw mix. This may have caused higher fat content in the melt and also let to less expansion of SBM-D extrudates.

In summary, we did not observe a pattern of changes in extrusion conditions and kibble expansion as MEP increased in diets. With extrusion input parameters slightly adjusted, it did not seem to be challenging to replace soybean meal with MEP at up to 15% of the formula. Additionally, replacing soybean meal with MEP benefited kibble expansion.

Feed Intake and Fecal Characteristics

Since the diets had similar gross energy content (4.7-5.0 kcal/g), the food DM intake was expected to be close among treatments. Dog fecal contamination is a major social conflict about dog ownership and poses zoonotic risks (Mori et al., 2023). Diets that promote decreased fecal output and defecation are appreciated by dog owners and would encourage cleaning up. The linear decrease in wet fecal output as MEP increased in the diet provided such advantages. Though fecal dry matter output, fecal moisture content and fecal score were not statistically different among treatments, we did see a consistent numerical change to the desired direction of these parameters as MEP increased in diets. Another study on piglet observed reduced diarrhea when regular soybean meal was replaced by MEP (Sinn et al., 2017). It will be valuable to further investigate if MEP would bring benefit in dogs with diarrhea-causing gastric-intestinal conditions. Yamka et al. (2006) reported decrease in wet fecal output in dogs fed with diet of 30% low-OS low-phytate soybean meal (LLM) compared to that of dogs fed with diet of 30% regular soybean meal. As OSs are almost removed from MEP used in this study (Sinn et al., 2017), both our study and Yamka's study supported that removal of soybean OSs contributed to decreased dog fecal wet output compared to regular soybean meal. Beloshapka et al. (2016) found that when gradually replacing poultry byproduct meal with bioprocessed soybean meal in extrude dog diets, wet fecal output did not change until replacing level reached over 24%. Yamka et al. (2006) also observed that diets with 30% LLM or 30% regular soybean meal both increased dog wet fecal output compared to diet with 22% poultry meal. In the current study, chicken meal inclusion also decreased as MEP increased in diets. The wet fecal output did no differ among three MEP-containing diets, which agreed with results from study of Beloshapka et al. (2016). It would be interesting to evaluate fecal output in the future when replacing chicken meal with MEP at over 30% to determine if MEP is comparable to chicken meal in regard to fecal output characteristics.

Apparent Total Trat Digestibility

Many studies have discussed influences on nutrient digestibility when soybean products were included in dog diets, among which soybean meal was the most studied one (Bednar et al., 2000; Carciofi et al., 2009; Félix et al., 2012; Maria et al., 2017; Menniti et al., 2014; Tortola et al., 2013; Vanelli et al., 2021; Yamka et al., 2003; Zuo et al., 1996). The soybean meal inclusion level in previous studies ranged from 9 to 47% with variable results on change of nutrient digestibility. The ATTDs for dry matter ranged from 73 to 87%, for protein were over 80%, for crude fat were around 90% and for gross energy were close 85%. However, when looking at ileal protein digestibility, Yamka et al. (2003) found that inclusion of soybean meal decreased dry matter and crude protein digestibility. More studies found that soybean meal increased ileal protein digestibility in dogs compared to animal protein ingredients (Bednar et al., 2000; Clapper et al., 2001; Zuo et al., 1996). The variation of results and non-drastic difference in nutrient digestibility between soybean meal and common animal ingredients do not seem adequate to make a solid statement that soybean meal provides more digestion benefits to dogs than animal proteins. To promote utilization of soybean meal products in pet food, enzyme assisted fermentation technique has come in light for it is believed to be able to promote nutrient bioavailability in soybeans. Numerous studies have proved that microbial fermentation can increase protein content, decrease protein molecular weight, reduce antinutritional factors and bring health benefits to monogastric animals (Amadou et al., 2010; Hoa and Hung, 2013; Mikawa et al., 2021; Mukherjee et al., 2015; Nkhata et al., 2018; Yuan et al., 2017). There are also studies on feeding fermented soybean meal or enzyme-treated soybean meal to dogs and pigs (Beloshapka et al., 2016; Cervantes-Pahm and Stein, 2010; Yamka et al., 2006). To our knowledge, this is the first study evaluating the MEP, an enzyme-assisted fungal fermentation product from soybean meal, as extruded dog food ingredient.

In this study, dogs were healthy and intentionally maintained bodyweight throughout the study by adjusting food allowance based on weekly weight check. Stable body weight ensures consistent metabolic state, body composition and energy requirements to minimize confounding factors on nutrient digestibility (Rosenbaum et al., 2000). The linear increase in ATTD of dry matter calculated by TFC method was consistent with the linearly decreased wet fecal output and dry fecal output while fecal moisture content remained similar. In the study of Yamka et al. (2006), low-OS low-phytate soybean meal also increased ATTD of dry matter in dogs compared to regular soybean meal. The MEP used in this study was reported to have 0% raffinose and 0.02% stachyose (Sinn et al., 2017), which may be the reason of increased ATTD of DM. Zuo et al. (1996) and Yamka et al. (2003, 2006) both indicated that diets containing stachyose less than 27-30 g /kg and raffinose less than 2 g/kg did not decrease nutrient digestibility in dogs. Such observations well explained that the differences in ATTD of DM, CP and CF between MEP-containing diets and SBM diet did not happen until MEP inclusion increased to 15%: only when soybean meal was replaced by MEP at adequately high level (15% in this study), could the negative influence on nutrient digestibility from OSs be removed.

The study of Beloshapka et al. (2016) evaluated a similar product (HP-300) is only enzyme treated (low ANFs) but not fermented soybean meal, they found that ATTD of DM, OM, CF and energy did not differ until poultry meal was replaced by HP-300 at as high as 48% in dog diets while ATTD of CP did not differ among treatments at all. In our study, the MEP derives from soybean meal through a combination of enzyme hydrolysis and fungal fermentation. It is supposed to have greater protein/AA digestibility than what is only enzyme hydrolyzed. The SBM-D had the highest level of soybean meal and chicken meal but no MEP, while the 15MEP-D had the highest level of MEP and chicken meal but no soybean meal. The improved CP digestibility in 15MEP-D could be a combined effect of replacing regular soybean meal protein and chicken meal protein by the MEP protein. Additionally, Zuo et al. (1996) also found that both soybean meal and low-OS soybean meal increased ileal digestibility and ATTD of CP in dogs compared to poultry meal. A reasonable next step would be to compare MEP and poultry meal at different replacement levels in dog diets.

Hind Gut Fermentation

Mammals are not able to efficiently digest soybean OSs due to the lack of α-galactosidases in their intestinal mucosa to break down α-1,6-glycosidic bonds (Medic et al., 2014). Undigested OSs pass to the large intestine, where they become substrates for microbial fermentation, producing short-chain fatty acids (SCFA; mostly acetate, propionate and butyrate) and gases such as hydrogen, carbon dioxide, and methane (Sunvold et al., 1995b). Malabsorbed dietary proteins/peptides can also be fermented in the colon to produce branched-chain fatty acids (BCFA), gases, ammonia, amines and phenolic and indolic compounds (Macfarlane et al., 1986). Proper colonic fermentation benefits the host by providing nutrients (SCFAs) and competitively (e.g. competition for oxygen, nutrients, mucosal adhesion sites, and creating an unfavorable environment for non-commensal species by lowering colonic pH, etc.) excluding potential pathogenic bacterial in the gut (Suchodolski, 2011). Though colonic fermentation of dietary fiber is generally beneficial, the fast gas production from excessive soluble OSs (i.e. soluble dietary fiber) in soybean meal are believed to cause flatulence and altered gut mobility (Suarez et al., 1999; Yamka et al., 2006, 2003). Research also reported that a diet low in fermentable oligosaccharides, disaccharides, monosaccharides, and polyols considerably relieved irritated bowl syndrome in human patients (Staudacher et al., 2012).

Soybean meal dietary fiber consists of both non-starch polysaccharide (NSP) and OSs where the OS only contribute to around 20% of it (Choct et al., 2010; Middelbos and Fahey, 2008; Opazo et al., 2012). The similar SCFA concentrations observed in this study was possibly due to close NSP content among diets that overweighed the difference in OSs. In the study of Beloshapka et al. (2016), SCFA content did not differ until ＞24% of HP300 included compared to poultry by-product meal (PBPM). Considering the facts that MEP inclusion level is only up to

15% in our study and dietary fiber profile in MEP is bound to be closer to soybean meal than to PBPM, it should require much higher inclusion level of MEP and soybean meal to induce changes in SCFA content if any.

The similar ammonia, individual BCFAs and total BCFAs contents observed in this study was unexpected as the MEP was supposed to decrease colonic fermentation of proteins/peptides. The results indicate that though ATTD of CP were statistically different among diets, the difference was too minimal to have practical significance. The dog study of Nery et al. (2011) indicated that highly digestible plant proteins are superior to mediumly digestible poultry proteins in regard to reduced concentrations of protein-based fermentation products in feces together with improved fecal quality in dogs. Based on our results, the MEP has the potential to be a cost-efficient protein source in pet food compared to moderately digestibility animal protein sources. Further research to compare MEP with various animal ingredients would be justified.

Palatability

The two-bowl forced choice method is commonly used in pet food palatability evaluation. The trial consists of two parts of results: first choice (FC) and intake ratio (IR) as FC reflects e olfactory characteristics and IR is considered to better reflect an overall preference (Aldrich and Koppel, 2015).

In this study, both FC and IR indicated that dogs preferred SBM-D to either 5MEP-D or 10MEP-D, but such preference did not exist between SBM-D and 15MEP-D. That means as MEP inclusion level increased, some contributing factors overweighed negative factors in regard to palatability. Félix et al. (2012) suggested that low molecular sugars contribute to palatability of dog diets. This might also be true in our study as the oligosaccharides were decomposed into smaller sugar molecules by added enzymes and/or fungal glycosidases during production of

MEP. It is reported that dogs always preferred control diet with no fiber to diet with fiber from sugar cane or wheat bran (Koppel et al., 2015). However, the total dietary fiber (TDF) in their treatment diets were around twice the amount in control diet in that study. In our study, it seemed to be contradictory as diets with higher TDF content were preferred to diets with lower TDF. This gives rise to two indications: a) diet palatability in this study was determined by other factor(s) instead of minor differences in TDF content; or b) source, solubility and other characteristics of dietary fiber are more influential on palatability than merely the amount (Koppel et al., 2015). In summary, results in this study indicated that 15% inclusion level of MEP was better than lower levels in regard to dog food palatability.

Conclusion

Results of this study indicated that even though MEP has an imbalanced amino acid profile, it is an acceptable plant protein source in extruded dog foods. Compared to traditional soybean meal, the MEP seems to be able to decrease fecal output and improve total tract protein digestibility at 15% inclusion level while maintaining practical extrusion and process comparable palatability. The potential influences of MEP on colonic fermentation may require more specific diet designs to manifest. It is possible that MEP can be preferable to not only regular soybean meal but even some animal protein sources in regard to fecal quality, digestion and colonic fermentation, which requires further research to verify.

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Table 2.1. Dog diet formulars with nutrient compositions of the experimental diets with increasing levels of MEP (SBM-D, 0%; 5MEP-D, 5%; 10MEP-D, 10%; and 15MEP-D, 15%).

Ingredient, %	SBM-D	5MEP-D	10MEP-D	15MEP-D
Soybean meal, Hi-Prot	15.00	10.00	5.00	0.00
MEP	0.00	5.00	10.00	15.00
Corn	38.00	38.00	38.00	38.00
Rice, Brewers	11.59	13.33	14.78	15.76
Beet pulp	4.00	4.00	4.00	4.00
Chicken meal	17.66	15.61	13.61	11.68
Fish meal	3.00	3.00	3.00	3.00
Salt	0.65	0.65	0.65	0.65
Potassium chloride	0.25	0.25	0.25	0.39
Dicalcium phosphate	0.25	0.25	0.50	1.00
Choline chloride, 60% dry	0.20	0.20	0.20	0.20
Natural AOX, dry	0.03	0.03	0.03	0.03
Natural AOX, liquid	0.03	0.03	0.03	0.03
Titanium dioxide	0.40	0.40	0.40	0.40
Vitamin premix[1]	0.15	0.15	0.15	0.15
Trace Mineral Premix[2]	0.10	0.10	0.10	0.10
Chicken fat (topical)	7.68	7.99	8.30	8.61
Digest-dry dog flavor (topical)	1.00	1.00	1.00	1.00
Analyzed nutrient composition				
Dry matter, %	91.88	94.12	92.64	91.07
	Dry matter basis			
Crude protein, %	27.68	27.53	27.38	28.50
Crude fat, %	10.47	12.76	10.87	13.51
Ash, % DM	8.12	7.31	6.60	6.60
Total dietary fiber, %	17.31	16.36	16.19	21.19
Soluble dietary fiber, %	4.66	2.32	1.73	3.29
Insoluble dietary fiber, %	12.65	14.05	14.46	17.90

Gross energy, kcal/kg	4752.9	4840.3	4718.9	4831.9

[1]Vitamin premix: 5.51% moisture, 4.02% crude protein, 34.5% ash, 13.4% calcium, 17,162,999 IU/kg Vitamin A, 920,000 IU/kg Vitamin D, 79,887 IU/kg Vitamin E, 14,252 mg/kg thiamine, 4,719 mg/kg riboflavin, 12,186 mg/kg pantothenic acid, 64,736 mg/kg Niacin, 5,537 mg/kg pyridoxine, 720 mg/kg Folic acid, 70 mg/kg biotin, 22 mg/kg vitamin B_{12}.
[2]Trace mineral premix: 0.66% moisture, 21.5% calcium, 0.02% sodium, 0.57% magnesium, 38,910 mg/kg iron, 11,234 mg/kg copper, 5,842 mg/kg manganese, 88,000 mg/kg zinc, 1,584 mg/kg iodine, 310 mg/kg selenium, 19% carbohydrate, 1% crude fat.

Table 2.2. Content of essential amino acids (for dogs and cats) in MEP.

Amino acid	MEP, mg/g crude protein	NRC references, mg/g crude protein	AAS
Arginine	66.06	35.00	1.89
Histidine	26.40	18.75	1.41
Isoleucine	50.60	37.50	1.35
Leucine	79.70	67.50	1.18
Lysine	58.70	35.00	1.68
Phenylalanine	53.15	45.00	1.18
Phenylalanine + Tyrosine	83.46	73.75	1.13
Methionine	13.73	32.50	0.42
Methionine + Cystine	27.59	65.00	0.42
Threonine	38.60	42.50	0.91
Tryptophan	17.40	13.75	1.27
Valine	50.30	48.75	1.03
Available Lysine	54.60	-	-
Hydroxylysine	0.68	-	-
PDCAAS calculation			
Limiting amino acid	Methionine and SAA[1]		
In-vitro protein digestibility, %	98.46		
PDCAAS	0.41		

AAS-amino acid score; SAA, sulfur amino acids (methionine and cystine); PDCAAS-protein digestibility corrected amino acid score.

Table 2.3. Content of non-essential amino acids (for dogs and cats) in MEP.

Amino acid	Amino acid content	
	As-is percentage, %	mg/g crude protein
Aspartic Acid	8.25	112.14
Serine	3.12	42.41
Glutamic Acid	12.67	172.22
Proline	3.69	50.16
Hydroxyproline	0.06	0.82
Glycine	3.05	41.46
Alanine	3.13	42.54
Cystine	1.02	13.86
Tyrosine	2.23	30.31
Taurine[1]	0.03	0.41
Lanthionine[1]	0.00	0.00
Ornithine[1]	0.09	1.22

[1]Non-proteinogenic amino acids.

Table 2.4. Content of minerals and fatty acids in MEP (as-is basis).

Minerals	Concentration	Fatty acids	Concentration, % of total fat
Selenium, ppm	0.751	Linolenic (18:3n3)	49.21
Calcium, ppm	2260	Palmitic (16:0)	16.07
Phosphorus, ppm	3150	Oleic (9c-18:1)[1]	15.37
Sodium, ppm	16.4	Stearic (18:0)	5.28
Magnesium, ppm	409	Vaccenic (11c-18:1)	1.71
Potassium, ppm	788	Behenoic (22:0)	0.41
Manganese, ppm	22.2	Arachidic (20:0)	0.30
Iron, ppm	98.9	Nervonic (24:1n9)	0.30
Copper, ppm	20.9	Lignoceric (24:0)	0.21
Zinc, ppm	43.4	C14:0	0.18
Chloride, W/W%	<0.1	DHA (22:6n3)	0.18
Iodine, ppm	<100	Margaric (17:0)	0.17
		Palmitoleic (9c-16:1)	0.16
		Gonodic (20:1n9)	0.13
		Elaidic (9t-18:1)[2]	0.12
		C20:2	0.12
		Arachidonic [20:4n6]	0.10
		Stearidonic (18:4n3)	0.07
		C15:0	0.06
		10c-17:1	0.06
		g-Linolenic [C18:3n6]	0.05
		C21:0	0.03

The MEP sample has zero content of fatty acids listed below: Linoelaidic (18:2t), Linoleic (18:2n6), C15:1n5, Myristoleic (9c-14:1), 3n-Arachidonic (20:4n3), EPA (20:5n3), Homo-g-linolenic (C20:3n6), Homo-a-linolenic(20:3n3), Erucic (22:1n9], C22:2n6, Adrenic (C22:4n6), C23:0, Clupanodonic (22:5n3).

[1]The "c" indicates cis configuration of the fatty acid.
[2]The "t" indicates trans configuration of the fatty acid.

Table 2.5. Extrusion processing data and post-extrusion kibble measurements for experimental dog diets.

Parameter	Treatment[1]				MSE	*P*-value			
	SBM-D	5MEP-D	10MEP-D	15MEP-D		SBM vs. T	L	Q	C
Sample size	3	2	6	3					
Feed rate, kg/h	744.33	742.50	744.83	746.33	4.76	0.8673	0.6381	0.9528	0.4330
PC steam[2], kg/h	41.00 [a]	41.00 [a]	35.67 [b]	37.00 [ab]	1.98	0.0041	0.0014	0.5733	0.2612
EX shaft speed[3], rpm	350.00	322.50	344.17	350.00	10.17	0.5603	0.6792	0.0947	0.0118
Motor load, %	74.00	74.00	76.83	77.67	3.39	0.4042	0.1521	0.8361	0.4154
Motor power, kW	32.57	30.055	33.90	35.13	2.71	0.3931	0.1773	0.6904	0.0995
SME, kJ/kg	43.76	38.43	45.47	47.04	3.80	0.2741	0.1481	0.4115	0.0484
EX water, kg/h	22.00 [b]	22.00 [b]	21.83 [b]	59.67 [a]	0.39	<0.001	<0.001	<0.001	<0.001
OE[4] Bulk density, kg/m^3	408.67 [a]	374.00 [b]	387.67 [ab]	392.67 [ab]	10.92	0.5049	0.1093	0.0405	0.0318
Sample size	30	20	60	30					
Diameter, mm	11.08 [b]	11.89 [a]	11.28 [b]	11.59 [a]	0.49	0.0044	0.3826	<0.001	0.0088
Thickness, mm	6.23	5.98	6.06	6.12	0.39	0.4380	0.1949	0.1759	0.0707
SEI	2.74 [b]	3.16 [a]	2.84 [b]	3.00 [a]	0.25	0.0039	0.4152	<0.001	0.0069

[1]Control (SBM-D), no microbially enhanced protein added; and 5%, 10% and 15% (5MEP-D, 10MEP-D and 15MEP-D) microbially enhanced protein added to offset soybean meal in the diet, respectively.
[ab]Means within a row with different superscripts differ ($P < 0.05$).
PC-preconditioner; EX-extruder; OE-off the extruder.

Table 2.6. Least square means and contrasts (SBM vs. MEP5-15 [T]; linear [L]; quadratic [Q]; cubic [C]) for food intake, fecal output, fecal score, and defecation frequency of dogs fed diets containing increasing levels of MEP.

	Treatment[1]					*P*-value			
Parameter	SBM-D	5MEP-D	10MEP-D	15MEP-D	SEM	SBM vs. T	L	Q	C
Intake (DM), g/d	202.43	203.96	199.42	198.78	7.44	0.5736	0.3713	0.9001	0.4288
Fecal output (As-is), g/d	128.78[a]	116.59[ab]	116.01[ab]	113.38 [b]	6.09	0.3742	0.0111	0.0389	0.8393
Fecal output (DM), g/d	39.18	36.75	35.79	35.42	1.75	0.2514	0.0138	0.1649	0.8930
Fecal moisture, %	66.76	66.31	65.90	65.68	0.01	0.7150	0.5877	0.1178	0.6009
Defecation frequency, times/day	2.28	2.10	2.15	2.17	0.11	0.7780	0.4681	0.4177	0.4681
Fecal score[2]	3.72	3.77	3.78	3.78	0.08	0.6728	0.3820	0.5821	0.8115

[1]Control (SBM-D) no microbially enhanced protein added, and 5%, 10% and 15% (5MEP-D, 10MEP-D and 15MEP-D) microbially enhanced protein added to offset soybean meal in the diet, respectively.
[2]Subjective 1 to 5 scale with 1, runny; 2, soft; 3, firm and moist; 4, firm; 5, dry and hard.
[ab] Means within a row with different superscripts differ ($P < 0.05$).

Table 2.7. Least square means and contrasts (SBM vs. MEP5–15 [*T*]; linear [*L*]; quadratic [*Q*]; cubic [*C*]) for nutrient ATTD (dry matter basis) calculated using total fecal collection method (TFC) by dogs fed diets with increasing levels (0%, 5%, 10%, and 15%) of MEP.

	Treatment[1]					*P*-value			
Parameter	SBM-D	5MEP-D	10MEP-D	15MEP-D	SEM	SBM v.s. *T*	*L*	*Q*	*C*
Total fecal collection method									
Dry matter, %	80.6[b]	81.95[ab]	82.06[ab]	82.15[a]	0.005	0.2969	0.0162	0.0828	0.6369
Organic matter, %	84.55	85.77	85.45	85.68	0.004	0.7747	0.0928	0.0373	0.4402
Crude protein, %	79.63[b]	81.06[ab]	80.95 [ab]	81.94[a]	0.007	0.8971	0.0330	0.0181	0.5489
Gross energy, %	83.90	85.21	84.57	84.62	0.006	0.9952	0.4236	0.0961	0.1768
Crude Fat, %	96.49[c]	97.55[b]	96.91[c]	98.10[a]	0.002	0.0035	0.0044	$<$0.001	0.0409
TiO_2 marker method									
Dry matter, %	80.97	80.21	81.65	79.84	0.010	0.1733	0.6510	0.1239	0.6270
Organic matter, %	84.84	84.39	85.12	83.82	0.008	0.3294	0.9325	0.2022	0.5094
Crude protein, %	80.00	79.18	80.57	79.60	0.010	0.3505	0.5950	0.3247	0.8602
Gross energy, %	85.32	84.81	85.38	83.68	0.010	0.3612	0.7743	0.1389	0.2974
Crude Fat, %	96.55[c]	97.29[ab]	96.84[bc]	97.88[a]	0.002	0.0639	0.0834	$<$0.001	0.0771

[1]Control (SBM-D) no microbially enhanced protein added, and 5%, 10% and 15% (5MEP-D, 10MEP-D and 15MEP-D) microbially enhanced protein added to offset soybean meal in the diet, respectively.
[abc] Means within a row with different superscripts differ ($P < 0.05$).

Table 2.8. Least square means and contrasts (SBM vs. MEP10–30 [*T*]; linear [*L*]; quadratic [*Q*]; cubic [*C*]) for short-chain fatty acid (SCFA), branched-chain fatty acid (BCFA), and total fatty acids (SCFA + BCFA) production from the fresh fecal sample collected from the dogs fed diets with increasing levels (0%, 5%, 10%, and 15%) of MEP expressed in a µmol/g of feces in dry matter basis.

Parameter,	Treatment[1]					*P*-value			
	SBM-D	5MEP-D	10MEP-D	15MEP-D	SEM	SBM v.s. T	*L*	*Q*	*C*
Fecal pH	5.65	5.76	5.71	5.64	0.13	0.7481	0.8575	0.8767	0.2967
Ammonia, µmol/g DM feces	98.8	103.2	99.1	97.8	10.61	0.7553	0.8219	0.6933	0.7261
SCFA, µmol/g DM feces	331.6	312.4	351.2	332.8	22.94	0.2316	0.3369	0.3096	0.6135
Acetate	200.4	192.5	206.0	204.8	14.69	0.5965	0.5554	0.6836	0.5291
Propionate	98.3	89.3	107.1	90.9	8.32	0.0868	0.3797	0.0804	0.8932
Butyrate	32.9	30.5	38.1	37.1	5.51	0.2555	0.1576	0.6315	0.3537
BCFA, µmol/g DM feces	10.29	12.80	11.82	10.72	1.244	0.6910	0.6263	0.5435	0.1394
Isobutyrate	4.66	4.93	5.17	4.79	0.391	0.1861	0.2085	0.8151	0.3982
Isovalerate	3.57	5.86	4.20	3.95	1.005	0.8248	0.9978	0.3146	0.1628
Valerate	2.06	2.01	2.45	1.99	0.180	0.0344	0.1381	0.1420	0.5367
Total, µmol/g DM feces	341.9	325.2	363.0	343.5	23.23	0.2222	0.3223	0.3280	0.6814

[1]Control (SBM-D) no microbially enhanced protein added, and 5%, 10% and 15% (5MEP-D, 10MEP-D and 15MEP-D) microbially enhanced protein added to offset soybean meal in the diet, respectively.

Table 2.9. Palatability assessment of diets containing MEP relative to the control (SBM-D) by dogs.

Diet A vs. B	First choice, n[1]	Intake ratio of diet A[2]
SBM-D vs. 5MEP-D	45*	0.733*
SBM-D vs. 10MEP-D	46*	0.682*
SBM-D vs. 15MEP-D	27	0.551

[1]First choice number of first visits to bowl with diet B can be obtained by 60-n.
[2]Intake ratio of diet A = average of intake (g) of diet A/total intake (g) of diets A + B.
*P-value is < 0.05.

Chapter 3 - Evaluation of Graded Levels of Microbially Enhanced Soybean Protein (MEP) on Extrusion Processing and Diet Utilization in Adult Cats

Abstract

The study evaluated microbially enhanced soybean meal (MEP), known for high protein digestibility and palatability, for its extrusion processing performance and effects on diet utilization in young adult cats. Four experimental diets were produced with a single-screw extruder with processing data and samples collected at 15-min intervals. The control diet contained 15% soybean meal (SBM-C) which was replaced at 5, 10 and 15% by MEP (5MEP-C, 10MEP-C and 15MEP-C). The diets were fed to 12 cats in a 4 x 4 replicated Latin square experiment design. Cats were adapted to diets for 9 days followed by a 5-d total fecal collection. Fresh fecal samples were collected for colonic fermentation analysis. Apparent total tract digestibility was calculated by total fecal collection (TFC) and titanium dioxide marker methods. Data were analyzed using the generalized linear mixed model with statistical software (GLIMMIX; SAS version 9.4, SAS Institute, Inc., Cary, NC) with treatment as a fixed effect and cat and period as random effects. Least-square means were analyzed with a single degree of freedom contrasts at significance level of $\alpha = 0.05$. Processing effects of MEP inclusion were minimal. The SEI was the lowest and the greatest ($P < 0.05$) in kibbles of SBM-C (2.50) and 15MEP-C (2.82), respectively. Fecal moisture content was greater ($P < 0.05$) in cats fed with 15MEP-C (69.78%) compared to that of cats fed with other experimental diets (at an average of 67.29%). Crude protein digestibility calculated by either method were the greatest ($P < 0.05$) in cats fed with the 15MEP-C, compared to that of cats fed with the

SBM-C. Cat fecal ammonia concentration increased linearly ($P < 0.05$) as MEP increased. The isobutyrate and isovalerate concentrations showed a quadratic increase ($P = 0.0517$) as MEP increased. Total fatty acid concentration was lower ($P < 0.05$) in feces from cats fed with the 10MEP-C compared to that from cats fed with SBM-C. Cats did not have a preference for any of the experimental diets over another. Overall, the inclusion of MEP in cat diets at up to 15% did not negatively affecting processing parameters, stool quality, nutrient digestibility in cats; inclusion of MEP at 5-10% tended to decrease hind gut fermentation.

Introduction

Cats, as strict carnivores, have protein requirement that are 2 to 3 times higher than other non-carnivores for adult maintenance due to the inability to down regulate nitrogen loss through the urea cycle (Morris, 2002; Rogers et al., 1977). Cat owners prefer animal-based proteins to plant-based proteins, for the former provide a better balance of essential amino acids for cats (Schleicher et al., 2019). It has been forecasted that by 2030, 35% of USA household will own a cat (APPA(American Pet Products Association), 2024). Production of complete and balanced commercial cat foods demands a huge amount of protein, but this may have negative aspects due to animal slaughter of concern to portions of the public (Koneswaran and Nierenberg, 2008). Offsetting some of the animal-based proteins with vegetable sources might alleviate some of this demand pressure for long term sustainability. Presuming that the vegetable-based proteins contain appropriate levels of essential and bioavailable amino acids.

Soybean meal (SBM) is produced by grinding soybean cake after oil extraction (Cromwell, 2012). It is a complementary plant protein source in pet food due to its high protein content and distinctive amino acid composition: most plant proteins have limited lysine content but legumes such as soybean are limited by methionine content (Donadelli et al., 2019; Gorissen et al., 2018).

Such characteristic endows soybean protein an important role in developing nutritionally complete diets with complementary proteins that may also extend the quality of animal proteins in pet foods. However, traditional SBM is generally not appreciated by pet owners as an ingredient as it is considered to be inferior with a bias in marketing deploring “no soy” in some premium branded products. Reasons include a biased perception of soybean protein allergenicity, relatively poor protein quality, genetically modified organisms (GMO), poor digestibility, anti-nutritional factors and other health/nutrition related concerns (Laflamme et al., 2014; Kim et al., 1995).

It must be conceded that there are inherent issues with SBM as a cat food ingredient, but misunderstandings by consumers may overstate the potential for use of SBM in cat foods. Improvements in soybean may be a way to overcome some of these negative perceptions. Fermentation may play a role. There exists production of microbially enhanced soybean meal (MEP) through enzyme assisted fermentation from SBM (Gibbons and Brown, 2016) that has proven to be superior compared to SBM in farm animal species and it may overcome some of the pet food consumer acceptability issues since fermentation is generally considered beneficial for food processing(Banovic and Grunert, 2023). Inclusion of MEP in feed has been shown to enhance nutrient digestibility and overall feed efficiency in weaned piglets and calves (Senevirathne et al., 2017; Sinn et al., 2017). This same response could make it an attractive ingredient for pet food formulations. However, information in this regard is not readily available as it pertains to the potential impacts of MEP or any other fermented soybean products on stool quality, diet digestibility, and intestinal health when fed to cats. Our hypothesis is the addition of MEP in extruded food will not have negative effects on stool quality, nutrient digestibility and palatability in cats.

Materials and Methods

Diet Formulation and Production

The MEP used in this research is a commercial product derived from genetically-modified soybean meal through a proprietary fermentation process (Prairie AquaTech, Brookings, SD, USA). Four cat diets were formulated to be nutritionally balanced for adult cats according to AAFCO (Table 3.1). The soybean meal in the control diet (SBM-C) was replaced by MEP at 5, 10, and 15% (5MEP-C, 10MEP-C, and 15MEP-C, respectively). The experimental cat diets were formulated to have high-protein (＞35%) and moderate level of fat (>12%). Titanium dioxide (TiO_2; 0.4%) was included in each diet as an indigestible marker for determination of apparent total tract digestibility (ATTD) of dietary nutrients. As predicted by the formulation software, the concentrations of minerals (calcium, phosphorous, potassium, magnesium, sodium, sulfur, manganese, copper iron, and zinc) were similar among diets and met AAFCO nutrient profile recommendations for adult cats at maintenance.

The dry expanded pet foods were produced using a single screw extruder (model X115, Wenger Manufacturing, Sabetha, KS, USA). The pre-conditioner (PC; model HIP 150, Wenger Manufacturing, Sabetha, KS, USA) was operated at. a constant speed (20% mix intensity) and welded paddles. The extruder had a defined profile and barrel temperatures based on a typical commercial pet food configuration. At the end of the extruder barrel there were 2 inserts of 10 openings (5.00 mm diameter) per insert. Fixed input parameters were kept constant throughout each individual food production and included dry blends feed rate (596.8 ±5.1 kg/h), PC water (118±1.0 kg/h), PC steam (30.0 kg/h) and extruder shaft speed (350 rpm).

During extrusion process, PC and extruder output parameters were all collected from sensor readouts every 15 min to estimate the effects of different inclusion levels of MEP on extrusion.

Output variables were those parameters resulting from the input variables, and included extruder die temperature, extruder motor load and specific mechanical energy (SME). Measurements were collected every 15 min during the production for a total of at least 2,000 lb per experimental diet and were considered treatment replicates.

Wet extrudates were conveyed pneumatically through an air hood system and deposited onto an oscillating belt spreader that spread the kibbles evenly across the dryer bed. The kibbles were dried on a belt passing two zones of a three-zone dryer (Airflow II, Wenger Manufacturing, Sabetha, KS, USA) to achieve the moisture content of kibbles below 10%. The dryer zones had varied temperatures and retention time as 19 minutes in zone 1, 10 minutes in zone 2 and 29 minutes in zone 3. Dried kibbles were coated with chicken fat and dry digest (Manx, AFB International, St. Charles, MO) in a drum mixer. Coated kibbles were stored in room temperature in poly-lined paper bags until fed.

Specific mechanical energy (SME) was calculated using the following formula:

$$\text{SME (kJ/kg)} = \frac{\frac{(\tau - \tau_0)}{100} \times \frac{N}{Nr} \times Pr}{m}$$

where, τ is the extruder motor load, τ_0 is the extruder no load % torque, N is the extruder screw speed (rpm), Nr is the rated extruder screw speed, Pr is the rated extruder motor power, and m is dry feed rate (kg/s).

Kibble diameter and thickness (length) of 10 randomly selected kibbles from each collection point of each diet production off the extruder and off the dryer were measured with a digital caliper. Sectional expansion index (SEI) was determined by comparing the squared diameter of the dried extruded kibbles by the squared die diameter of the extruder:

$$\text{SEI} = \frac{D^2}{d^2}$$

where D is the extrudate diameter and d is the extruder die diameter.

Off the extruder kibble bulk density was measured manually off the extruder in each data collection point during each treatment processing using a 1 L cup and leveling the kibbles with a metal ruler and weighing on a digital scale with 1.0 g sensitivity.

Feeding Trials

The cat digestibility trial was conducted at the Kansas State University College of Veterinary Medicine under the Institutional Animal Care and Use Committee (IACUC) protocol #4348. Twelve healthy American shorthair cats (6 males and 6 female) of similar age (10 month ±5 days) were enrolled in this study. The cats had an average body weight of 4.6 ± 1.4 kg, and food allowance was controlled to maintain their weight throughout the study. The daily metabolizable energy (ME) requirement was calculated for laboratory cats [$100*BW_{kg}^{0.67}$ (NRC, 2006)]. The cats were housed on a 12 h light cycle with lights off from 1900 to 0700. In the adaption period, the cats were group-housed but fed individually. Whereas in the collection period, the cats were individually housed in stainless steel cages. The cats received two feedings per day at 0800 and 1600 h with access to food for 1 h. In case a cat refused to eat an experimental diet, an additional 0.5% to 1.0% flavor enhancer was added topically to the food.

The ME of experimental diets was calculated using the prediction equation in pet food [$(8.5 \times CF) + (3.5 \times CP) + (3.5 \times NFE)$]. The daily food allowance was calculated using the daily ME requirement divided by predicted ME of diets. Cats were fed twice daily (8:00 AM and 4:00 PM) in equal ration to meet daily food allowance. The body weight of each cat was measured at the beginning, middle, and end of each period and their food allowance was adjusted by 5 or 10% for the following week to maintain their BW. Water was provided for *ad libitum* consumption.

The feeding trial used a replicated Latin square design, where each cat served as its own control. Each of the four periods composed of 9-day for adaptation followed by 5-day of collection. During collection period, all feces and orts were collected daily. Every fecal sample was weighed and scored on a 5-point scale where 1 = liquid diarrhea, 2 = very soft consistency, unformed stool; 3 = soft stool that retains shape; 4 = well-formed firm stool that does not leave residue when picked up; and 5 = very hard, dry stool. Fecal samples were stored at -20℃ until the end of trial and then thawed at room temperature, pooled by cat, weighed, and dried in a forced air oven at 55◦C for up to 48h until the moisture level was below 10%. The partially dried fecal samples were also weighed, and the values were used when calculating the DM (dry matter) of the fecal samples. Diet samples and partially dried fecal samples were ground through a 1-mm screen by a laboratory fixed blade impact mill (Retsch, type ZM200, Haan, Germany) and stored in Mason jars at room temperature for further chemical analysis.

In addition, the pH of one fresh fecal sample per cat per period (within 15 min of defecation) was recorded in triplicate with a calibrated glass-electrode pH probe (FC240B, Hanna Instruments, Smithfield, RI) immediately after collection. Fresh fecal samples were stored at -80℃ until further analysis.

Chemical Analysis

The amino acid composition of MEP sample was analyzed at the University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO; AOAC 982.30 and 988.15). All amino acids, except methionine, cysteine, and tryptophan, were digested with 6 N HCl for 24 h at 110°C. The amino acids were then separated by ion-exchange chromatography and the concentration was determined with a Beckman 6300 amino acid analyzer (Beckman, Palo Alto, CA). Methionine and cysteine were first oxidized by performic acid to methionine sulfone and cysteic acid, respectively, prior to acid hydrolysis. Tryptophan was hydrolyzed in 3 M mercaptoethanesulfonic acid before analysis. Available lysine was determined (AOAC 975.44) and lysine availability (%) was calculated as the ratio of available lysine to total lysine.

The ground experimental diet samples (after coating) and fecal samples were analyzed for dry matter (DM), organic matter (OM), and ash according to methods in AOAC 934.01 and 942.05. Crude protein was determined (AOAC 990.03) using a nitrogen analyzer (FP928, LECO Corporation, Saint Joseph, MI). Crude fat was determined by acid hydrolysis followed by hexane extraction (ISO 11085:2008) using semi-automated equipment (Hydrotec 8000 and ST 255 Soxtec, Foss, Denmark). Gross energy was measured as total heat of sample combustion by calorimetry (Parr 6200 Calorimeter, Parr Instrument Company, Moline, IL). The titanium dioxide content in diet and fecal samples was analyzed with a colorimetric method (Myers et al., 2004). The soluble dietary fiber (SDF), insoluble dietary fiber (IDF) and total dietary fiber (TDF) content of diet samples was measured (AOAC 991.43) by an ANKOM Dietary Fiber Analyzer (ANKOM Technology, Macedon, NY).

Fecal ammonia concentration in the fresh fecal samples was analyzed through colorimetric method described by Chaney and Marbach (1962). The fresh fecal samples were thawed and diluted with deionized water and 0.1N HCl and homogenized. The homogenized samples were centrifuged at 3,000 g for 20 min to separate the suspended solids. The 1 mL of the supernatant of the centrifuged samples was collected and kept frozen at −20 °C for at least 24 h to complete deproteinization. The acidified samples were thawed, centrifuged at 20,000 g for 15min, and plated.

Fecal SCFA and BCFA contents were analyzed on a gas chromatography (GC; Agilent 7890, Agilent Technologies, Santa Clara, CA). The fresh fecal samples were thawed and diluted with deionized water and homogenized, followed by centrifugation at 3,000 g for 20 min to separate the suspended solids. The 1mL of the supernatant of the centrifuged samples was collected acidified with 0.25mL of 25% m-phosphoric acid was before deproteinization at −20 ℃ for at least 24 h. The GC is equipped with flame ionization detector (FID) and a capillary column (DB-WAX, Agilent 127-7012, 10m × 0.1mm × 0.1μm, Agilent Technologies, Santa Clara, CA). Hydrogen was used as a carrier gas with a flow rate of 35 mL/min, and the split ratio was 100:1 with injection volume of 0.2 μL. Nitrogen was used as the makeup gas with a flow rate of 25 mL/min. The detector and injector temperatures were set at 300℃, and the initial oven temperature was set to 40℃ with a ramp rate of 20℃/min to 180℃ for a total run time of 8 min. The peak area of chromatograms was determined using an integrative software (OpenLab CDS, Agilent Technologies, Santa Clara, CA). The concentrations of SCFA (acetate, propionate, and butyrate) and BCFA (isobutyrate, isovalerate, and valerate) in samples were quantified by comparing the sample peak area to three standards with known concentrations of each volatile free acid and correcting for the fecal dry matter content.

Digestibility Calculation

Two methods were utilized to estimate apparent total tract nutrient digestibility. The total fecal collection (TFC) method requires the collection of all feces excreted by the experimental animals. The marker method uses TiO_2 as an indigestible dietary marker. In current study, apparent total tract digestibility (ATTD) of DM, OM, CP, CF and GE were calculated according to the TFC and marker methods by following equations:

TFC method:

$$Nutrient\ digestibility\ (\%) = \frac{(nutrient\ consumed\ (g) - nutrient\ excreted\ (g))}{nutrient\ consumed\ (g)} \times 100\%$$

Marker method:

$$Nubrient\ digestibility\ (\%) = [1- \frac{(nutrient\ in\ feces\ \% \times TiO2\ in\ food\ \%)}{(nutrient\ in\ food\ \% \times TiO2\ in\ feces\ \%)}] \times 100\%$$

Palatability Trial

The MEP-containing diets (5MEP-C, 10MEP-C and 15MEP-C) were evaluated for palatability versus the non-MEP diet (SBM-C) by a cat panel at a commercial facility (Summit Ridge Farms, Susquehanna, PA). Each experiment was conducted as a split-plate test, in which two stainless steel bowls containing 100 g of food were presented to animals for a total of 4 h. Each comparison trial was repeated for 2 d, with bowl position switched daily. Thirty cats were fed daily, providing 60 observations for each paired comparison test. Preference was determined based on animals' first choice and total food consumption. Data from consumption were represented as the following ratio:

$$\text{Intake ratio} = \frac{consumption\ of\ Diet\ A}{consumption\ of\ Diet\ A + Diet\ B} \times 100\%$$

Statistical analysis

Least square means of data were estimated by 1-way ANOVA using the GLIMMIX procedure in SAS (version 9.4, SAS Institute INC, Cary, NC, USA). Pairwise treatment comparisons were conducted using a Tukey's post hoc test. Contrasts comparing control (SBM-C) vs treatments (5MEP-C, 10MEP-C, and 15MEP-C), and linear, quadratic, and cubic relationships among all diets were considered significant at $P < 0.05$. For each diet production, sampling was conducted at evenly spaced intervals which were considered replicates. For digestibility trial analysis, cat and period were considered random effects in the analysis model. In the palatability experiment, the consumption ratio was analyzed using a t-test in a two-way ANOVA, and the first-choice preference was analyzed using a Chi^2 test.

Results and Discussion

Chemical Analysis of MEP and Diets

Total essential amino acids comprised 45.5% of total crude protein in MEP with methionine/sulfur-containing amino acids (SAAs) serve as the limiting amino acid(s) for cats (Table 3.2). All diets contained an average CP concentration of 37 .0% (range 35.6–37.9%), an average fat concentration of 13.3% (range 12.9–13.7%), an average TDF concentration of 17.2% (range 15.8–20.2%), and an average GE content of 4861 kcal/kg (range 4686–4999 kcal/kg) on DM basis (Table 3.1). However, the SBM-C had high TDF and IDF content compared to MEP containing diets.

As cats used to be a much smaller pet population than dogs and not as direct a model for human nutrition/medicine, there are far fewer research publications available regarding cat nutrition, foods and various ingredients used therein. Due to a shorter small intestine relative to body weight than that of dogs, cats generally have lower apparent digestibility of AAs for lower-quality proteins; but have similar values for high-quality proteins (e.g., those with 90% or higher apparent digestibility) (de-Oliveira et al., 2012). The MEP has lower protein/peptide molecular weights compared to regular soybean meal protein, which led to higher digestibility in terrestrial animals and verified through *in vitro* protein digestibility (98.5%) in this study. Cats also have a distinctive requirement for arginine to prevent ammonia toxicity (Morris and Rogers, 1978), the MEP had an amino acid score (AAS) for arginine greater than 1.0 for cats even after taking protein digestibility into account. The phenylalanine plus tyrosine are responsible for maintaining black hair color of cats (Yu et al., 2001), the AAS for phenylalanine plus tyrosine in MEP was also over 1.0 with protein digestibility accounted. Additionally, low level of taurine (0.41 g/mg CP), a non-proteinogenic but essential dietary nutrient for cats (Morris, 2002), was found in measurable quantities in MEP. Based on the above, the MEP appears to be a decent plant protein for consideration in cat diets if formulated as a complement to animal proteins or with judicious use of purified amino acid additives.

The experimental diets in this study met the targeted moisture content of around 5% (4.28-6.27%) well below the 10% threshold that might be considered to permit mold growth (Gautam et al., 2018). The slight increase of crude protein (CP) content from SBM-C to MEP-containing diets was anticipated based on the analysis of the experimental sample of MEP (77.03% DM basis) which had a much higher protein level than the soybean meal (53.8% DM basis) used. The decrease in CP in 15MEP-C compared to the other two MEP-containing diets was most likely due to sampling or analytical variations. Low ash content in 15MEP-C was also anticipated as it had the most low-ash chicken meal included. Considering that fiber sources in all experiment diets were similar except soybean meal and MEP, the decreased total dietary fiber and insoluble fiber (IDF) in MEP-containing diets indicated that the MEP had much lower content of these two types of fiber compared to the soybean meal used in this study. The similar soluble dietary fiber (SDF) among diets reflected less difference in SDF than in IDF between MEP and soybean meal used in this diet. The slight increase in SDF in 5MEP-C was possibly due to analytical variation.

Extrusion Process

Input parameters remained similar among diets except knife speed that was manually decreased for 0MEP-C and 15MEP-C compared to SBM-C and 5MEP-C (Table 3.3). A cubic relationship ($P < 0.05$) between PC steam and MEP inclusion was observed. A linear relationship between knife speed and MEP inclusion was observed ($P < 0.001$). The diameter, thickness and SEI of extrudates increased linearly ($P < 0.001$) as MEP inclusion increased. The extrudates of MEP-containing diets were more expanded ($P < 0.001$) than that of SBM-C.

Generally speaking, replacement of soybean meal with MEP in this study at up to 15% did not entail drastic changes in extrusion processing conditions to reach a proper extrudate expansion and bulk density. The inclusion of MEP at 15% did not require more water injection in cat diets compared to dog diets discussed in Chapter 2. This might be because the MEP did not contribute to the general protein composition in cat diets as much as in dog diets (cat diets had around 10% more total CP content than dog diets) while dietary MEP inclusion levels were same for both foods. Thus, in order to see whether MEP would impact extrusion of similar cat diets used in this study, higher inclusion level might be necessary.

The SBM-C had the least kibble expansion versus any of the MEP-D containing diets; possibly due to the high IDF content as inclusion of IDF usually decreases sectional expansion (Donadelli et al., 2021; Robin et al., 2012). The increased longitudinal expansion of SBM-C kibbles compared to that of 5MEP-C kibbles were consistent with other studies in it has been observed that decreased sectional expansion occurs when increasing IDF content which often leads to increased longitudinal expansion (Robin et al., 2011; Stojceska et al., 2008). The increased kibble thickness in 10MEP-C and 15MEP-C were expected as the knife speed was manually decreased during production. Before adjustment of knife speed, the kibbles from these two diets were too thin indicating decreased longitudinal expansion. This agreed with previous observations of negative relationship between sectional and longitudinal expansion of Robin et al. (2011) and Stojceska et al. (2008). The possible mechanisms are decreased sectional elastic recovery at the die exit facilitated energy impact on increased longitudinal expansion (Bouzaza et al., 1996) and addition of fiber as nucleation agents decreased energy requirement for bubble nucleation and led to earlier nucleation in the die and favored stretching of the bubbles in the direction of the melt flow, thus greater longitudinal expansion (Lee, 2014). If we compare dog diets with MEP, the cat kibbles had greater SME and less expansion compared to dog kibbles, which can be explained by less starch in cat diets to create viscosity and elasticity. In summary, replacement of soybean meal with MEP at up to 15% promoted expansion and did not pose any challenges in extrusion techniques. The contribution to expansion seemed to derive from the great dietary fiber composition between soybean meal and MEP used in this study.

Feed Intake and Fecal Characteristics

Food intake, and wet and dry fecal output remained similar among cats fed with experimental diets. Fecal moisture content in cats fed 15MEP-C (69.78%) was greater ($P < 0.05$) than that of cats fed with other experimental diets (67.70%, 67.04% and 67.12% for SBM-C, 5MEP-C and 15MEP-C) and showed a cubic relationship ($P < 0.001$) with MEP inclusion (Table 3.4). Fecal score and defecation frequency (at an average of 3.85 and 1.1 time/day, respectively) of cats were also not affected by dietary inclusion of MEP.

Energy is the major driving factor for food intake of cats and dogs (Morris et al., 2006). The similar food intake was expected as all diets had similar gross energy content. Though statistically different, the increase in fecal moisture content in cats fed with 15MEP-C was of limited practical influence as fecal scores and wet fecal output were maintained among cats fed with each experimental diet. However, the fecal wet and dry matter output were numerically lower in cats fed with MEP-containing diets compared to that of cats fed with the non-MEP diet. As a stretch, this may indicate a possible increase in nutrient digestibility with inclusion of MEP which is discussed later in this article. It has been reported that IDF decreased apparent digestibility and increased fecal dry matter content and fecal bulk in monogastric animals (Schedle et al., 2008; Wichert et al., 2002). The SBM-C diet had more IDF than other experimental diets, which could explain the higher fecal dry matter percentage/lower moisture percentage in feces from cats fed with the SBM-C.

Apparent Total Tract Digestibility

The apparent total tract digestibility (ATTD) of nutrients calculated with both total fecal collection method (TFC) and marker method (Titanium dioxide) had similar standard error of mean (SEM) values and generally agreed with each other in regards to rankings (Table 3.5). Although statistically significant differences in ATTD were more frequent with the marker method. With TFC method, the ATTD of dry matter, organic matter, crude protein and gross energy were not different among cats fed with experimental diets (Table 3.5). The ATTD of crude fat of cats fed with 5MEP-C (92.17%) was lower ($P < 0.05$) than that of cats fed with diets with 10% and 15% MEP (94.56% and 94.98%, respectively). A quadratic, a linear and a cubic model was found ($P < 0.05$) in prediction ATTD of crude protein, gross energy and crude fat, respectively, in cats fed with diets with varied MEP inclusion levels. On the other hand, the ATTD of dry matter, organic matter, crude protein and gross energy calculated with titanium dioxide marker method showed cubic relationships ($P < 0.05$) with MEP inclusion (Table 3.5). The ATTD of crude protein was the greater in cats fed with 15MEP-C (72.5 %) than that of cats fed with SBM-C and 10MEP-C (67.3% and 67.8%. respectively). There was a cubic relationship ($P < 0.05$) between the ATTD of crude fat and MEP inclusion, but cats fed with 0% MEP (91.3%) had an intermediate crude fat digestibility among cats fed all experimental cat diets (from 89.7 to 93.1%).

As mentioned previously, the increased ATTD of dry matter in cats fed with the 15MEP-C was consistent with the lower fecal dry matter content compared to that of cats fed with the other three diets. The ATTD of all nutrients in cats fed with MEP-containing diets were comparable or even greater than those of cats fed with the control diet. Such results can be attributed to three major aspects: different dietary fiber content among diets, high protein digestibility of MEP and low oligosaccharides in MEP. The high IDF and TDF content in the control diet compared to MEP-containing diets may have impacted nutrient digestibility. Previous studies have reported such decrease in nutrient digestibility in cats as fiber increased as well (Fischer et al., 2012; Kilburn-Kappeler et al., 2022; Sunvold et al., 1995a). The mechanisms by which fiber decreases nutrient digestibility include shorter gastrointestinal tract transit time, nutrient binding, physical blockage to enzyme access, increased endogenous loss and reduction in brush border enzyme activity, etc. (Adams et al., 2018; Eggum, 1995; Roura, 2019). The high protein digestibility is one of major advantages in MEP since fungal fermentation and enzyme addition have degraded large protein molecules into smaller peptides to facilitate digestion (Gibbons and Brown, 2016). The MEP is free of soybean oligosaccharides (OS) that are considered as an anti-nutritional factor. Highly fermentable soybean OS may decrease nutrient digestibility by stimulating faster transition and forming complexes with other nutrients(Eastwood, 1992). Yamka et al. (2006), low-OS low-phytate soybean meal increased ATTD of dry matter in dogs compared to regular soybean meal. Sinn et al. (2017) reported that MEP increased standardized ileal digestibility of protein and some amino acids in post-weaning piglets when compared to fish meal.

However, the ATTD of crude protein in this study seemed to be low regardless of treatments. In the study of Kilburn-Kappeler et al. (2022), the treatment/diet design was very similar to what was in this study. The control diets in both studies had 15% soybean meal; the soybean meal was replaced by another ingredient of interest at 5, 10 and 15% to create treatment diets; the analyzed crude protein content in all diets were close to 36%. The ATTDs of crude protein in their study were all above 85% when fed to cats and calculated by TiO_2 marker method. In contrast, the dietary crude protein contents in this study were similar to theirs while the ATTD of crude protein were only around 70% calculated by marker method and no more than 80% calculated by TFC method. Similar situations were noted when comparing this study to another study of Kilburn-Kappeler et al (2023) evaluating digestibility of different yeast products by cats. The differences in dietary fiber composition did not seem to explain such discrepancy because it only existed in ATTD of crude protein but not that of dry matter, organic matter, crude fat and gross energy compared to both studies of Kilburn-Kappeler et al. (2022, 2023). Cats used in this study were all around 10-month old, the protein digestion capacity should have reached adult level as early as in 19-week old (Harper and Turner, 2000). Processing might have played a role in the relatively low protein digestibility in this study. Studies suggest that high specific mechanical energy (SME) levels generally improve digestibility by breaking down complex protein structures, making them more accessible for digestive enzymes (Holt and Aldrich, 2022; Wang et al., 2013). The SME in this study was notably lower than commonly seen in extruded pet food (Donadelli et al., 2021; Holt and Aldrich, 2022; Kilburn-Kappeler et al., 2023; Kim and Aldrich, 2023).

Colonic Fermentation

Though fecal pH did not differ among cats fed with different diets, the ammonia concentration in fresh feces increased linearly ($P < 0.05$) as MEP level increased in diets (Table 3.6). The concentrations of acetate, propionate, valerate, total SCFA, total BCFA and total fatty acids had a cubic relationship ($P < 0.05$) with MEP inclusion. The total SCFA concentration was lower ($P < 0.05$) in feces from cats fed with the 10MEP-C (384.9 μmol/g DM feces) than that from cats fed with the SBM-C (452.8 μmol/g DM feces). Cats fed with 10MEP-C (407.1 μmol/g

DM feces) also had the lowest ($P < 0.05$) total fatty acid concentrations in fresh feces compared to cats fed with SBM-C and 15MEP-C (480.1 and 483.6 μmol/g DM feces, respectively).

Short-chain fatty acids (SCFAs) refer to organic fatty acids with 1 to 6 carbon atoms (Brody, 1998). However, to differentiate fatty acids from carbohydrate fermentation and protein fermentation in this study, SCFAs specifically refer to acetate, propionate and butyrate while branched-chain fatty acids (BCFAs) refer to isobutyrate, valerate and isovalerate. Colonic fermentation in dogs and cats plays a crucial role in maintaining gastrointestinal health by breaking down undigested carbohydrates into SCFAs, which decrease fecal pH, provide metabolizable energy for colonic cells and promote overall gut health (Wong et al., 2006). Undigested proteins and peptides also can be substrates for colonic bacteria. The deamination and decarboxylation of amino acids produce a complex of protein fermentation products such as ammonia, branched-chain fatty acids (e.g. isobutyrate and isovalerate), indoles, phenols and volatile sulfur-containing compounds (Macfarlane et al., 1986). Many of these compounds are found to have adverse impacts on colonic health including causing colorectal cancer, promoting tumorigenesis, and exacerbating ulcerative colitis (Hussein et al., 1999). Generally speaking, increased SCFAs and decreased pH, ammonia and BCFAs are considered beneficial colonic fermentation parameters (Kim et al., 2023).

Carbohydrates in soybean meal are predominantly non-starch polysaccharides (NSP) and free sugars (e.g. sucrose, stachyose and raffinose) (Choct, 1997). The soybean NSPs can be further divided into insoluble cellulose, partially soluble non-cellulose polymers (e.g. mannans, galactans and fructans) and partially soluble pectic polysaccharides. The OSs in free sugars are indigestible but highly fermentable in monogastric animals (Bosch et al., 2008; Kim et al., 2023). However, cellulose in MEP has been deconstructed by manually added enzymes to release sugars for microbes to convert into proteins and exopolysaccharides (Gibbons and Brown, 2016). Major soybean OSs (stachyose and raffinose) are also barely detectable in the MEP (Sinn et al., 2017). Compared to regular soybean meal, the MEP is supposed to have less fermentable carbohydrates. However, chitin is the major carbohydrate in fungus and is usually insoluble (Zhang et al., 2001). The carbohydrates from fungal biomass in the MEP may have played a role in the overall dietary fiber composition of the product.

In this study, the fecal pH surprisingly was maintained similar regardless of significant differences in ammonia and SCFAs content in cats fed with each experimental diet. Such stable fecal pH and variable ammonia and total volatile fatty acids contents were also observed in the dog study of Beloshapka et al. (2016). This might be attributed to other fermentation compounds that also affect fecal pH such as lactic acid. Rochus et al. (2012) reported that viscous guar gum shifts dietary amino acids from metabolic use to fermentation substrate in domestic cats. Meanwhile, it has been reported that the microbe used in production of MEP can efficiently convert a broad range of difficult-to-metabolize oligosaccharides into cell mass and a microbial gum (Gibbons and Brown, 2016). The linear increase of fecal ammonia concentration as MEP inclusion increased might be explained by fungal gum shifting amino acid to colonic fermentation to some extent. The increase of isobutyrate/total fatty acids ratio and isovalerate/total fatty acids ratio with increase of MEP also supported this. As the ATTD of protein increased with MEP increase, it would be worthy to evaluate the in vivo apparent ileal digestibility of protein and amino acids of the MEP.

The cat fecal SCFA concentrations reported in this study were within the range reported by Santos et al. (2018) and Kilburn et al. (2023). The decreased fecal SCFA content in cats fed with 5MEP-C and 10MEP-C compared to cats fed with SBM-C was expected as MEP had lower fermentable carbohydrates than soybean meal. However, such effect seemed to be gone when soybean meal was completely replaced by MEP in cat diets. The possible reasons remain to be investigated but might be related to a combined influence of carbohydrates and proteins on colonic microbiota composition and fermentation patterns (Middelbos et al., 2007). It is also noted that fecal valerate concentration was comparable to or higher than isobutyrate or isovalerate in cats in this study and some other cat studies (Brosey et al., 2000; Kilburn-Kappeler et al., 2023; Santos et al., 2018) while it is usually the opposite in dogs (Beloshapka et al., 2016; Brosey et al., 2000; Hee S. Kim et al., 2023a; Maria et al., 2017). While fecal valerate mostly comes from protein fermentation in the colon, an *in vitro* study using cheetah feces showed that some carbohydrate can also generate a small but similar amount of valerate and isobutyrate but less isovalerate (Depauw et al., 2012). Such discrepancy in fecal BCFAs between cats and dogs reflected possible differences in colonic fermentation characteristics such as microbiota profile, preference for substrate and epithelial absorption for fermentation products in the two species.

Palatability

Cats showed preference ($P < 0.05$) for 5MEP-C to SBM-C on the first day according to first choice, but such preference did not last (Table 3.7). The intake ratio did not show any difference between SBM-C and 5MEP-C. When comparing SBM-C and 10MEP-C, cats showed preference ($P < 0.05$) for 10MEP-C indicated by two-day intake ratio but not first choice. For 15MEP-C, cats only showed preference ($P < 0.05$) for it over SBM-C on the second day indicated by intake ratio.

The results indicated that relatively low inclusion of MEP contributed to palatability more than high inclusion of MEP. Such observation was consistent with previous studies that reported palatability of diets with yeast fermented grain products (CFP) were greater than control diets only when inclusion level of CFP was lower (5% compared to 10 and 15%, 5% compared to 10 and 15%, 150 mg/kg BW compared to 300 mg/kg BW; Kilburn et al., 2021; Kilburn-Kappeler et al., 2022; Norton et al., 2023, respectively). The MEP is also a fungal fermented grain products that might have common characteristics with yeast products that yielded such distinctive palatability pattern in cats. According to the amino acid profile of MEP reported in this study and that of soybean meal from 10 facilities in the U.S. reported by Grieshop et al. (2003), the MEP had greater glutamic acids compared to soybean meal, which should have provided a stronger umami smell that is appealing to both dogs and cats (Ugawa and Kurihara, 1994). However, the reduced palatability at high level of MEP or yeast fermentation products remained unclear. In the review of Watson et al. (2023), many studies have shown that cats are more selective towards food compared to dogs and are more prone to display neophobia for foods. Norton et al. (2023) stated that higher inclusion level of *Saccharomyces cerevisiae* fermentation product may have resulted in overly strong and unfamiliar flavors and aromas that were questioned by cats. However, cats in our digestibility study willingly consumed all experimental diets and no refusals were observed.

The palatability of MEP differed between dogs and cats as dogs preferred 15% to lower MEP inclusion level while cats preferred 10% to either lower or higher MEP inclusion level in this study. This may also be explained that cats are more sensitive to unfamiliar flavors than dogs are.

Conclusion

Replacement of soybean meal with MEP at up to 15% increased kibble expansion without significant changes in extrusion settings. The nutrient digestibility in cats of MEP-containing diets were comparable to that of soybean meal control diet. A generally decreased colonic fermentation were observed when MEP was included at 5-10% but not at 15% in cat diets, but all colonic fermentation parameters fell in normal ranges. In combination with palatability test results, 10% seemed to be a favorable inclusion level of MEP in extruded cat diets. Further research on MEP carbohydrate composition and fermentability is needed to understand its influence on colonic fermentation.

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Table 3.1. Cat diet formulas with nutrient compositions of the experimental diets with increasing levels of MEP (SBM-C, 0%; 5MEP-C, 5%; 10MEP-C, 10%; and 15MEP-C, 15%).

Ingredient, %	SBM-C	5MEP-C	10MEP-C	15MEP-C
Soybean meal,	15.00	0.00	0.00	0.00
MEP	0.00	5.00	10.00	15.00
Corn	38.26	37.97	40.06	42.16
Chicken meal	29.50	28.44	18.27	8.10
Chicken meal, low ash	1.83	3.18	11.25	19.32
Fish meal	3.00	3.00	3.00	3.00
Beet pulp	4.00	4.00	4.00	4.00
Salt	0.25	0.25	0.25	0.25
Titanium dioxide	0.40	0.40	0.40	0.40
Potassium chloride	0.25	0.25	0.25	0.25
Choline chloride, 60% dry	0.20	0.20	0.20	0.20
Natural AOX, dry	0.04	0.04	0.04	0.04
Natural AOX, liquid	0.03	0.03	0.03	0.03
Vitamin premix[1]	0.15	0.15	0.15	0.15
Trace Mineral Premix[2]	0.10	0.10	0.10	0.10
Chicken fat (topical)	6.00	6.00	6.00	6.00
Digest-dry cat flavor (topical)	1.00	1.00	1.00	1.00
Analyzed nutrient composition				
Dry matter, %	95.72	94.60	94.06	93.73
	Dry matter basis			
Crude protein, %	35.59	37.65	37.93	36.79
Crude fat, %	13.24	12.95	13.68	13.26
Ash, %	9.70	9.44	8.33	6.81
Total dietary fiber, %	20.16	16.38	15.84	16.22
Soluble dietary fiber, %	3.13	4.44	3.30	3.31
Insoluble dietary fiber, %	17.03	11.95	12.55	12.91
Goss energy, kcal/kg	4686.4	4809.9	4998.9	4950.9

[1]Vitamin premix: 5.51% moisture, 4.02% crude protein, 34.5% ash, 13.4% calcium, 17,162,999 IU/kg Vitamin A, 920,000 IU/kg Vitamin D, 79,887 IU/kg Vitamin E, 14,252 mg/kg thiamine, 4,719 mg/kg riboflavin, 12,186 mg/kg pantothenic acid, 64,736 mg/kg Niacin, 5,537 mg/kg pyridoxine, 720 mg/kg Folic acid, 70 mg/kg biotin, 22 mg/kg vitamin B_{12}.

[2]Trace mineral premix: 0.66% moisture, 21.5% calcium, 0.02% sodium, 0.57% magnesium, 38,910 mg/kg iron, 11,234 mg/kg copper, 5,842 mg/kg manganese, 88,000 mg/kg zinc, 1,584 mg/kg iodine, 310 mg/kg selenium, 19% carbohydrate, and 1% crude fat.

Table 3.2. Content of essential amino acids (for dogs and cats) in MEP.

Amino acid	MEP, mg/g crude protein	NRC references, mg/g crude protein	AAS
Arginine	66.06	35.00	1.89
Histidine	26.40	18.75	1.41
Isoleucine	50.60	37.50	1.35
Leucine	79.70	67.50	1.18
Lysine	58.70	35.00	1.68
Phenylalanine	53.15	45.00	1.18
Phenylalanine + Tyrosine	83.46	73.75	1.13
Methionine	13.73	32.50	0.42
Methionine + Cystine	27.59	65.00	0.42
Threonine	38.60	42.50	0.91
Tryptophan	17.40	13.75	1.27
Valine	50.30	48.75	1.03
PDCAAS calculation			
Limiting amino acid	Methionine and SAA		
In-vitro protein digestibility, %	98.46		
PDCAAS	0.41		

AAS-amino acid score; SAA, sulfur amino acids (methionine and cystine); PDCAAS-protein digestibility corrected amino acid score.

Table 3.3. Extrusion processing data and post-extrusion kibble measurements for experimental cat diets.

	Treatment[1]					*P*-value			
Parameter	SBM-C	5MEP-C	10MEP-C	15MEP-C	MSE	SBM vs. T	L	Q	C
Sample size	5	5	7	4					
Feed rate, kg/h	597.80	595.00	597.57	596.75	2.44	0.3645	0.8194	0.1140	0.2991
PC steam, kg/h	29.60	29.20	29.57	30.00	0.47	0.8967	0.4276	0.9461	0.0240
Motor load, %	73.00	72.40	70.57	70.75	2.80	0.2713	0.1084	0.8680	0.6733
Motor power, kW	31.82	32.00	30.94	30.93	1.65	0.4163	0.2496	0.9136	0.5072
SME, kJ/kg	53.13	53.71	51.70	51.77	2.79	0.3762	0.2496	0.7956	0.4627
EX water, kg/h	17.20	17.20	17.29	17.50	0.49	0.9503	0.5508	0.6274	0.4358
Knife speed, rpm	1500 [a]	1500 [a]	1378 [b]	1406 [b]	22.37	<0.001	<0.001	0.1825	0.0033
OE Bulk density, kg/m^3	421.60	416.40	411.00	410.50	12.26	0.3762	0.1204	0.6083	0.7856
Sample size	50	50	70	40					
Diameter, mm	7.91 [c]	8.25 [b]	8.39 [a]	8.30 [ab]	0.27	<.0001	<.0001	0.0012	0.0708
Thickness, mm	4.71 [b]	4.57 [c]	4.88 [a]	4.86 [a]	0.27	<.0001	<.0001	0.0307	0.001
SEI	2.50 [c]	2.72 [b]	2.76 [ab]	2.82 [a]	0.17	<.0001	<.0001	0.0016	0.0798

[1]Control (SBM-C), no microbially enhanced protein added; and 5%, 10% and 15% (5MEP-C, 10MEP-C and 15MEP-C) microbially enhanced protein added to offset soybean meal in the diet, respectively.
[abc]Means within a row with different superscripts differ ($P < 0.05$).
PC-preconditioner; EX-extruder; OE-off the extruder.

Table 3.4. Least square means and contrasts (SBM vs. MEP5-15 [T]; linear [L]; quadratic [Q]; cubic [C]) for food intake, fecal output, fecal score, and defecation frequency of cats fed diets containing increasing levels of MEP.

	Treatment[1]					*P*-value			
Parameter	SBM-C	5MEP-C	10MEP-C	15MEP-C	SEM	SBM vs. T	L	Q	C
Intake (DM), g/d	79.13	77.32	76.13	77.37	3.875	0.1338	0.0585	0.7835	0.4936
Fecal output (As-is), g/d	58.10	52.14	53.25	54.23	4.543	0.5903	0.2737	0.3269	0.3292
Fecal output (DM), g/d	18.68	16.99	17.41	16.52	1.349	0.9843	0.2277	0.1053	0.9707
Fecal moisture, %	67.70[b]	67.04[b]	67.12[b]	69.78[a]	0.016	0.0241	0.5603	0.0139	<0.001
Fecal score[2]	3.86	3.84	3.86	3.82	0.083	0.7502	0.8413	0.4871	0.7836
Defecation time/day	1.10	1.05	1.00	1.08	0.091	0.3043	0.3632	0.7979	0.4933

[1] Control (SBM-C) no microbially enhanced protein added, and 5%, 10% and 15% (5MEP-C, 10MEP-C and 15MEP-C) microbially enhanced protein added to offset soybean meal in the diet, respectively.

[2] Subjective 1 to 5 scale with 1, runny; 2, soft; 3, firm and moist; 4, firm; 5, dry and hard.

[ab] Within a row, means without a common superscript differ ($P < 0.05$).

Table 3.5. Least square means and contrasts (SBM vs. MEP5–15 [T]; linear [L]; quadratic [Q]; cubic [C]) for nutrient ATTD calculated using titanium dioxide marker method by cats fed diets with increasing levels (0%, 5%, 10%, and 15%) of MEP.

	Treatment[1]					*P*-value			
Parameter	SBM-C	5MEP-C	10MEP-C	15MEP-C	SEM	SBM v.s. *T*	*L*	*Q*	*C*
Total fecal collection method									
Dry matter, %	78.23	79.66	78.73	80.57	0.009	0.4613	0.5432	0.0716	0.5745
Organic matter, %	84.65	85.60	84.54	84.97	0.010	0.4511	0.7269	0.2631	0.5203
Crude protein, %	75.71	78.21	78.07	80.11	0.017	0.9613	0.0802	0.0499	0.5049
Gross energy, %	82.91	84.20	85.53	83.83	0.007	0.0274	0.0239	0.7712	0.2474
Crude Fat, %	93.48[ab]	92.17[b]	94.56[a]	94.98[a]	0.007	0.0969	0.0131	0.3927	0.0031
TiO_2 marker method									
Dry matter, %	70.33[ab]	72.75[a]	68.33[b]	73.08[a]	0.010	0.0018	0.1913	0.0006	0.4850
Organic matter, %	78.83[ab]	80.67[a]	77.25[b]	79.33[ab]	0.015	0.0045	0.0502	0.0063	0.4239
Crude protein, %	67.33[b]	70.92[ab]	67.75[b]	72.50[a]	0.017	0.0632	0.5766	0.0008	0.3947
Gross energy, %	78.37	80.27	77.72	79.31	0.007	0.0471	0.3358	0.0139	0.4594
Crude Fat, %	91.33[ab]	89.67[b]	92.17[a]	93.08[a]	0.008	0.2347	0.0286	0.5194	0.0010

[1]Control (SBM-C) no microbially enhanced protein added, and 5%, 10% and 15% (5MEP-C, 10MEP-C and 15MEP-C) microbially enhanced protein added to offset soybean meal in the diet, respectively.
[abc] Within a row, means without a common superscript differ ($P < 0.05$).

Table 3.6. Least square means and contrasts (SBM vs. MEP10–30 [*T*]; linear [*L*]; quadratic [*Q*]; cubic [*C*]) for short-chain fatty acid (SCFA), branched-chain fatty acid (BCFA), and total fatty acids (SCFA + BCFA) production from the fresh fecal sample collected from the cats fed diets with increasing levels (0%, 5%, 10%, and 15%) of MEP expressed in a mmol/g of feces in dry matter (DM) basis.

Parameter,	Treatment[1]				SEM	*P*-value			
	SBM-C	5MEP-C	10MEP-C	15MEP-C		SBM v.s.T	*L*	*Q*	*C*
Fecal pH	5.73	5.73	5.74	5.53	0.100	0.2358	0.4973	0.0608	0.0233
Ammonia, µmol/g DM feces	102.5[b]	122.0[ab]	127.0 [ab]	139.8 [a]	12.4	0.0498[2]	0.0434	0.1059	0.5142
SCFA, µmol/g DM feces	452.8[a]	392.1[ab]	384.9[b]	456.1[a]	34.81	0.0200	0.0793	0.7617	0.0020
Acetate	278.5[ab]	245.9[ab]	241.4[b]	283.6[a]	20.97	0.0290	0.1291	0.6517	0.0034
Propionate	125.3[ab]	104.5[b]	108.3[ab]	127.7[a]	11.34	0.0388[2]	0.2933	0.9015	0.0022
Butyrate	49.0	41.7	35.3	44.9	5.77	0.0505	0.0527	0.7879	0.2274
BCFA, µmol/g DM feces	27.35	22.36	22.22	27.53	4.710	0.1494	0.2766	0.9394	0.0331
Isobutyrate	5.98	6.05	6.10	6.94	1.42	0.5220	0.3571	0.1376	0.0666
Isovalerate	5.65	6.41	6.13	7.07	0.751	0.6154	0.2698	0.0517	0.4255
Valerate	15.72	9.91	9.98	13.52	3.049	0.1334	0.0879	0.5178	0.0402
Isobutyrate/total FA ratio[3]	1.23	1.44	1.44	1.48	0.002	0.2176	0.0347	0.2727	0.4665
Isovalerate/total FA ratio	1.16[b]	1.58[a]	1.53[ab]	1.49[ab]	0.001	0.0235[2]	0.0258	0.0575	0.1662
Total FA, µmol/g DM feces	480.1[ab]	414.4[bc]	407.1[c]	483.6[a]	38.86	0.0170	0.0712	0.7651	0.0015

[1]Control (SBM-C) no microbially enhanced protein added, and 5%, 10% and 15% (5MEP-C, 10MEP-C and 15MEP-C) microbially enhanced protein added to offset soybean meal in the diet, respectively.
[2]*P* value from Tuckey's post hoc pair comparison.
[3]FA, fatty acids.
[abc] Within a row, means without a common superscript differ ($P < 0.05$).

Table 3.7. Palatability assessment of diets containing MEP relative to the control (SBM) by cats.

Diet A vs. B	First choice, n[1]		Intake ratio of diet A[2]	
	Day 1	Day 2	Day 1	Day 2
SBM-C vs. 5MEP-C	24*	19	0.541	0.483
SBM-C vs. 10MEP-C	18	14	0.342*	0.296*
SBM-C vs. 15MEP-C	13	17	0.423	0.410*

[1]First choice number of first visits to bowl with diet A can be obtained by 30-n.
[2]Intake ratio of diet A = average of intake (g) of diet A/total intake (g) of diets A + B.
*P-value is < 0.05.

Chapter 4 - Evaluation of Inoculation Dosage of *Aspergillus oryzae* and Fermentation Duration on Protein Content in Fermented Soybean Meal and its Influence on Dog Food Extrusion Processing

Abstract

This study aimed to optimize the inoculation dosage and fermentation duration to enhance protein content and reduce soluble oligosaccharides of soybean meal using *Aspergillus oryzae*, and to assess the extrusion processing and nutritional changes of the resulting product in a dry dog food product. In experiment 1, a 3×5 factorial arrangement of treatments was used to determine a the optimal combination of dosage and fermentation time durations. In experiment 2, soybean meal was bulk fermented under the optimal conditions determined in experiment 1to further evaluate nutritional changes through fermentation. The fermented samples were analyzed for macronutrients, amino acids, sugars, and trypsin inhibitors. In experiment 3, four dog diets were formulated with different soybean products (SBM with 30% soybean meal; AMF with 30% soybean meal plus 1% dried *A. oryzae* biomass product; FSBM with 30% fermented soybean meal; and SPI with 18% soybean protein isolate) and produced through a single-screw extruder with processing data and samples collected every 30 minutes. Kibbles were analyzed for physical characteristics. Raw mixes of all diets were analyzed for particle size distribution and viscosity. Results were analyzed using a generalized linear mixed model with least-square means compared by Tukey's test (α=0.05). The optimal combination was inoculation at 1×10^4 spore/g substrate and a 36-hour fermentation duration. Fermentation decreased (P<0.05) organic matter, insoluble fiber, and gross energy in soybean meal while increasing (P<0.05) crude protein and soluble dietary fiber. Methionine content and cysteine content increased from 0.72 and 0.76% DM to 0.81 and 0.82% DM, respectively. Fermentation eliminated sucrose and greatly reduced raffinose, stachyose, and verbascose concentrations. Trypsin inhibitors (TI) were inactivated by heat during retort, making it impossible to evaluate the effect of fermentation on TI inactivation. The required specific mechanical energy (SME) was highest (P<0.05) for SPI and lowest (P<0.05) for SBM, with FSBM intermediate; the FSBM required less (P<0.05) in-barrel moisture than all other diets. Kibble sectional expansion index of FSBM (1.80 mm^2/mm^2) was

greater (P<0.05) than SBM (1.30 mm^2/mm^2) but less (P<0.05) than SPI (2.55 mm^2/mm^2). FSBM exhibited intermediate particle size distribution and the least raw mix viscosity compared to SBM and SPI. These results suggest that *Aspergillus oryzae*-fermented soybean meal offered an enhanced nutrient profile, increased SME and kibble expansion compared to unfermented soybean meal.

Introduction

The US produced 4.2 billion bushels of soybeans in 2023, contributing to around 29% of worldwide soybean supply (USDA FAS, 2024b). Soybean meal is the by-product of the soybean oil separation through solvent and/or mechanical extraction (Grieshop et al., 2003). At least 70% of soybean meal in the US is used as feed for poultry, swine and cattle while less than 5% is used in pet food (Ruiz et al., 2020; Stein et al., 2008). A portion of the pet food industry is looking for more sustainable ingredients with health benefits for animals. Soybeans are considered a good plant protein source for monogastric animals with less impacts on the environment than animal proteins. However, utilization of soybean products in pet foods are limited for reasons such as the incomplete amino acid profile, presence of anti-nutritional factors including trypsin inhibitors and oligosaccharide, and an overall negative consumer impression to name a few.

Fermentation is a natural process during which microorganisms produce bioactive enzymes to metabolize the substrates and converts or modifies them into products with desirable features. The fungus, *Aspergillus oryzae* is very commonly used in soybean meal fermentation to produce human food such as saké, shoyu (soy sauce), and miso (Gomi, 2014). Many studies have proved that fermentation with *A. oryzae* can effectively decrease protein molecular weight, increase protein content, modify amino acid composition, and eliminate antinutritional factors in soybean meal (Frias et al., 2008; Hong et al., 2004; Teng et al., 2012; Yang et al., 2020). Fermented soybean products also bring benefits when fed to animals such as improved growth performance, nutrient digestibility, intestinal enzyme activities in both ruminant and monogastric animals

(Beloshapka et al., 2016; Cervantes-Pahm and Stein, 2010; Feng et al., 2007b; Kim et al., 2012; Sumi et al., 1990; Teng et al., 2012; Yuan et al., 2017). However, previous studies conducted with various fungus inoculation dosages, fermentation durations, and fermentation conditions such as substrate moisture content, environment humidity, solid versus liquid fermentation phase, have made it complicated to determine the optimal protocol to modify soybean meal with desired nutrient modifications through fermentation with *A. oryzae*. Additionally, previous animal studies have provided scant information on processing methods for the ingredient and diets of interest. If fermented soybean meal is to be utilized in pet food, its extrusion impact is an inevitable topic to investigate in order to better understand how the derived ingredient will affect the production of kibbles (the most popular form of dog/cat food). Therefore, the objectives of this study were to 1) determine the proper inoculation dosage and fermentation time to improve protein content in soybean meal, 2) investigate changes in macronutrients, amino acid, sugars and trypsin inhibitors in soybean meal fermented with a certain protocol, and 3) evaluate extrusion performance of the fermented soybean meal incorporated in a practical complete and balanced dog food formula. We hypothesized that both inoculation dosage and fermentation duration would affect the nutrient content of soybean meals. Fermentation was expected to significantly alter the nutrients and anti-nutritional factors profile of soybean meal without introducing technical challenges in extrusion process.

Materials and Methods

Spore Suspension Preparation

Freeze dried *Aspergillus Oryzae* (lyophilized, ATCC 42149) was obtained from the American Type Culture Collection (Manassas, Virginia, USA) and recovered on a Czapek-Dox agar plate and harvested in 50% glycerol solutions. A hemacytometer chamber (Hausser

Scientific, Horsham, Pennsylvania, USA) was used for spore counting under a microscope. Solutions with estimated spore concentrations were used for inoculation.

Fermentation of Soybean Meal

Commercial soybean meal (SBM) was purchased from a local distributor (MKC, Manhattan, KS). Distilled water was added to soybean meal to reach a 50% initial moisture content before retorting for 2 hours under 121.1 °C. Retorted soybean meal was evenly divided into 3 groups and inoculated with *Aspergillus oryzae* spore suspensions at dosage of $1×10^4$ (dose 1), $5×10^5$ (dose 2), and $1×10^6$ (dose 3) spore/g SBM (DM basis). Fermentation was carried out in a ventilation chamber (GEN2000, Conviron, Pembina, ND) under 37°C，80±5% RH and light cycle of a 2-hour darkness/day. Substrate was sprayed on surface with distilled water and then flipped with ethanol sanitized spatulas every 12 hours. Samples were collected from each group at 0, 12, 24, 36 and 48 hours of fermentation and immediately dried in oven at 60°C for 36 hours to achieve moisture content less than10% and stored at room temperature for further analysis. The above fermentation process was repeated 3 times blocked by days and tray location in the chamber (left, middle or right).

Chemical analysis

Dry matter (DM), organic matter (OM), and ash were determined according to AOAC methods 934.01 and 942.05. Crude protein (CP) was determined by Dumas combustion method (AOAC 990.03) using a nitrogen analyzer (FP928, LECO Corporation, Saint Joseph, MI). The conversion factor from nitrogen to crude protein was 6.25. Crude fat was determined by acid hydrolysis followed by hexane extraction (ISO11085:2008) using semi-automated equipment (Hydrotec 8000 and ST 255 Soxtec, Foss, Denmark). Gross energy was measured as total heat of sample combustion by calorimetry (Parr 6200 Calorimeter, Parr Instrument Company, Moline, IL). The soluble dietary fiber (SDF), insoluble dietary fiber (IDF) and total dietary fiber (TDF) content of diet samples was measured (AOAC 991.43) by an ANKOM Dietary Fiber Analyzer (ANKOM Technology, Macedon, NY, USA). Aflatoxin (B_1, B_2, G_1 and G_2) analysis was conducted with test strips (Reveal Q+, Neogen Ltd., Lansing, MI) and read with an AccuScan Gold (Neogen Ltd., Lansing, MI).

Sugars (sucrose, stachyose, raffinose and verbascose) and amino acids were determined at a cooperating University (University of Missouri Agricultural Experiment Station Chemical

Laboratories; Columbia, MO). Sucrose, stachyose, raffinose and verbascose contents were determined by gas chromatography (Bhatti et al., 1970). All amino acids, except methionine, cysteine, and tryptophan, were digested with 6 N HCl for 24 h at 110°C. The amino acids were then separated by ion-exchange chromatography and the concentration was determined with a Beckman 6300 amino acid analyzer (Beckman, Palo Alto, CA). Methionine and cysteine were first oxidized by performic acid to methionine sulfone and cysteic acid, respectively, prior to acid hydrolysis. Tryptophan was hydrolyzed in 3 M mercaptoethanesulfonic acid before analysis. Available lysine was determined (AOAC 975.44) and lysine availability (%) was calculated as the ratio of available lysine to total lysine.

The Kunitz trypsin inhibitors (KTI) and Bowman-Birk trypsin inhibitors (BBI) were determined in triplicate by a simplified spectrophotometer method (Kim and Krishnan, 2023) and expressed a trypsin inhibitor unit (TIU)/g of sample. For KTI analysis, 20 mg ground sample and 1 mL of 10 mM NaOH were added into a 2 mL tube and vigorously agitated on a vortex mixer at room temperature for 10 minutes followed by a 10-minute centrifugation at 16,000 g. Protein concentration in the supernatant was measured by Bio-Rad protein assay (Bradford, 1976). Add 50 μL of protein concentration and 150 assay buffer (50mM Tris-HCl, pH8.2 and 20 mM $CaCl_2$) to a 1.5 mL tube followed by addition of 500 μL of BAPNA solution (0.4mg/mL) and 200 μL of trypsin (20 μg/mL) and vortex of the solution. Reaction was run for 10 minutes at 37 ℃ in a water bath and terminated by adding 100 μL of 30% (v/v) acetic acid. Absorbance was measured at 410 nm. The positive control, substrate blank and enzyme blank were used in parallel. For BBI analysis, the same procedure was used but BAPNA and trypsin were replaced with AAPF (N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide) and α-chymotrypsin. In the analysis, one TIU was defined as a decrease in 0.1 absorbance units at 410 nm.

In-vitro **Protein Digestibility**

The *in-vitro* pepsin-pancreatin protein digestibility was determined in duplicates according to Godoy et al (2009) with modifications. Two additional blanks were created with 1 mL of deionized water. For each replicate, 1 g of sample was weighed into 50 mL centrifuge tubes, followed by addition of 15 mL of 0.1N HCL-pepsin (porcine; Merck Millipore 516360-2.5GM) solution and placed in a shaking water bath for 6 hours at 37°C. The pepsin hydrolysis was stopped by adding 7.5 mL of 0.5 N NaOH to neutralize the solution. Next, a mixture of 4 mg porcine pancreatin (Sigma-Aldrich P1750-100G), 7.5 mL of phosphate buffer (pH 8) and 1 mL of sodium azide (for microbial control) was added into each test tube. Tubes were then placed in the shaking water bath for 18 hours at 37°C. After the 18-hour incubation, 1 mL of 10% trichloroacetic acid was added to each tube to end the reaction and help with protein precipitation. Tubes were then centrifuged at 3,700 g for 20 minutes. The supernatant was discarded after filtration with Whatman 541 filter paper. Then the sediments were washed with distilled water, centrifuged and filtered again for 3 times (the supernatant became clear). Final sediments on the filter paper were oven-dried overnight at 105°C and then analyzed for crude protein (CP) by nitrogen (AOAC 990.03) using a nitrogen analyzer (FP928 LECO Corporation, Saint Joseph, MI). *In-vitro* protein digestibility was calculated using the following equations:

Weight of sample CP = Sample weight (g) × sample CP (%)

Weight of residue CP = residue weight (g) × residue CP (%)

$$\text{CP digestibility (\%)} = \frac{\text{Weight of sample CP} - \text{Weight of residue CP}}{weight\ of\ sample\ CP} \times 100$$

Diet Formulation

Soybean meal fermented with *A. oryzae,* following the protocol established in experiment 1, was used as an ingredient in formulation of a dog diet. To produce adequate dog food for feeding trial, a total of 19 fermentation sessions were conducted to produce over 200 lbs of fermented soybean meal. Four diets, nutritionally adequate for maintenance of adult dogs (AAFCO, 2020), were formulated with different soybean meal products for comparison: SBM with 30% soybean meal; AMF with 30% soybean meal plus 1% dried *A. oryzae* wheat bran fermentation product (Amaferm®, BioZyme, Inc., St. Joseph, MO) where the latter served to differentiate any effects from *A. oryzae* biomass on processing conditions; FSBM with 30% *A. oryzae* fermented soybean meal; and SPI with 18% soybean protein isolate (Table 4.3). Note that the soybean meal and fermented soybean meal had closer crude protein content (51-55% DM basis) while the soybean protein isolate had 92% crude protein on DM basis. We aimed to include similar amount of soybean protein in each diet, which resulted in the 18% instead of 30% of soybean protein isolate in SPI diet. Brewers' rice was used only in the SPI diet to complete the formula. All ingredients but Amaferm® and fermented soybean meal were purchased and mixed from a commercial mill (Wilbur-Ellis Holdings, Inc., St. Joseph, MO). Diets were formulated to have similar nutritional composition and included titanium dioxide (TiO2; 0.4%) as an indigestible marker for determination of apparent total tract digestibility (ATTD) of dietary nutrients. As predicted by the formulation, the concentrations of minerals (calcium, phosphorous, potassium, magnesium, sodium, sulfur, manganese, copper iron, and zinc) were similar among diets and met AAFCO (2020) nutrient profile recommendations for adult dogs at maintenance.

Rapid Viscosity Analysis

Rapid Visco Analysis (RVA) is used to measure the viscosity of a material as heat and shear are applied to it. The raw mixes were analyzed using AACC method 76-21.02 on a RVA 4500 (Perten Instruments, Waltham, MA, USA). Each sample was weighed 3.5 grams and mixed with 25 mL deionized water in the test cup. The testing cycle kept the sample at 50°C for 1 minute, and then heated up to 95°C at a rate of 12.2°C/minute; it is then held at 95°C for 2.5 minutes. The sample was then cooled to 50°C at 11.8°C per minute before being held for 2 minutes.

Particle Size Distribution

An alpine jet sieve (E200 LS, Hosokawa Micron, Augsberg, Germany) was used to measure the particle size distributions (PSD) of raw mix of four experimental diets. The ASTM standard sieve sizes used for understanding the granulation ranges from 125 µm to 1000 µm. The analysis was carried out in duplicates by taking 100 g of raw mix for each run. The PSD was measured based on the amount of raw mix (g) passing through the sieves. Additionally, by measuring the weights of materials retained over each sieve, the geometric mean diameter (d_{gw}) and geometric standard deviation of the particle diameter (Sgw) was calculated using the equations stated in the study of Patwa et al. (2014).

Extrusion Processing

Prior to extrusion, feed rate calibration was done with raw mixes of SBM diet and SPI diet to understand influences of feeder screw speed and the material height in the feed hopper on actual feeding rate. Measurements were taken at two different hopper fill levels (High, and Low) and two different screw speeds (11 and 14 RPMs). On the day of production, raw mixes were fed into the preconditioner (PC) via a volumetric screw feeder on a live bottom feed hopper. The materials were then hydrated with steam and water in the PC (Model 2 Differential Conditioner, Wenger Manufacturing, Sabetha, KS, USA) before entering a 3.25-inch diameter barrel single screw pilot-scale extruder (X-20, Wenger Manufacturing, Sabetha, KS, USA). The extruder had six-barrel heads which were divided into three heating zones. The feed intake used single flight full pitch screws and then transitioned into double flighted half pitch cone screws at the discharge end followed by a die with a single 7.07 mm opening. Four steam locks were used (two small, one medium and one large) inter-screw (Figure 4.2).

Die temperature and pressure was collected during processing using two separate probes inserted in the material stream at 5 cm behind the die exit. Products were cut with 6 hard knives upon leaving the extruder. Samples were then transported by a negative pressure air conveying system to a pilot scale dryer at 220°F for 12 minutes, and 6 minutes in the cooler (Wenger Manufacturing, Sabetha, KS, USA) to reach moisture content below 10%. Processing parameters were kept constant within treatments, although some natural variability occurred. Kibble samples out of extruder and process data were collected at 0, 30, and 60 minutes of the production of each diet. After drying, the chicken fat and digest (dry dog flavor) were applied to the kibbles in a double-ribbon blender (Wenger Manufacturing, Sabetha, KS, USA). Samples before and after coating were collected for further analysis.

Throughput of the extruder was measured twice within the last ten minutes of each diet production by weighing extrudates collected from extruder discharge portal in 1 minute. Raw mix feed rate (as-is basis, kg/h) was calculated by mass balance with the equation:

$$\text{Feed rate (kg/h)} = \frac{\text{Throughput}\times\text{Moisture content of extruder kibbles}}{\textit{Moisture content of raw mix}}$$

In-barrel moisture (IBM) was calculated using the following equation:

$$\text{IBM (\%)} = \frac{m_f * X_{fw} + m_{ps} + m_{pw} + m_{es} + m_{ew}}{m_f + m_{ps} + m_{pw} + m_{es} + m_{ew}} \times 100$$

Where m_f is feed rate (as-is), X_{fw} is moisture content of feed material, m_{ps} and m_{pw} are steam and water injection rate in preconditioner respectively. The m_{es} and m_{ew} are steam and water injection rate in extruder barrel respectively.

SME was calculated using the following equation:

$$\text{SME (kJ/kg)} = \frac{\frac{(\tau - \tau_0)}{100} \times \frac{N}{Nr} \times Pr}{m}$$

where, τ is the extruder motor load, τ_0 is the extruder no load % torque, N is the extruder screw speed (rpm), Nr is the rated extruder screw speed, Pr is the rated extruder motor power, and m is dry matter feed rate (kg/s).

Kibble Physical Characteristics

Sectional expansion index (SEI) was determined by comparing the squared diameter of the dried extruded kibbles by the squared die diameter of the extruder:

$$\text{SEI} = \frac{D^2}{d^2}$$

where D is the extrudate diameter and d is the extruder die diameter;

Specific length in cm/g was determined by equation:

$$\text{specific length} = \frac{l}{m}$$

where l is the extrudate length and m is extrudate mass;

Piece volume (V, cm^3) was determined by equation:

$$\text{Piece volume (V)} = \frac{\pi D^2 l}{4}$$

Piece density (g/cm^3) was determined by equation:

$$\text{Piece density} = \frac{m}{V}$$

where m is the piece mass.

Wet bulk density was measured off the extruder in three replicates manually during each treatment processing using a 1 L cup and leveling the kibbles with a metal ruler and weighing on a digital scale with 0.1 g sensitivity. Dry bulk density was measured off the dryer in three replicates using the same method.

Statistical Analysis

A 2-way ANOVA was performed to analyze the effects of inoculation dosage, fermentation duration and their interaction on crude protein content and *in-vitro* protein digestibility using a generalized linear mixed model in a statistical software (GLIMMIX, SAS version 9.4, SAS Institute, Inc., Cary, NC) with position and day as random effects. Tukey's post hoc test was applied for the least-square means separation, with significance considered at $P<0.05$. A paired t-test was used to analyze the nutrient content changes through fermentation. Extrusion and kibble characteristics data were analyzed with a generalized linear mixed model in a statistical software (GLIMMIX, SAS version 9.4, SAS Institute, Inc., Cary, NC). Tukey's post hoc test was applied for the leas-square means separation, with significance considered at $P<0.05$. For each diet production, sampling was conducted at evenly spaced intervals (30 minutes) which were considered replicates.

Results

Experiment 1- Optimal Inoculation Dosage and Fermentation Duration

Content of organic matter (OM), crud protein (CP), total dietary fiber (TDF) and insoluble dietary fiber (IDF) on dry matter basis were influenced only by fermentation duration ($P<0.05$) but not by inoculation dosage or the interaction of inoculation dosage and fermentation duration (Figure 4.1). The crude protein content was greater (55.77%, $P<0.05$) in soybean meal fermented with *A. oryzae* for 48 hours compared to those fermented for 24 hours or less (at an average of 52.60%). The *in-vitro* protein digestibility of *A. oryzae* fermented soybean meal was not affected by any factors. The OM and IDF in *A. oryzae* fermented soybean meal decreased ($P<0.05$) after 24-hour fermentation (93.01%) compared to unfermented soybean meal (93.33%), but no changes after 24 hours. The SDF in the *A. oryzae* fermented soybean meal showed a trend (P=0.097) of increase from 36-hour to 48-hour fermentation duration; but was not influenced by inoculation dosage, or the interaction between inoculation dosage and fermentation duration. To achieve our goal of increasing protein content and limiting SDF in fermented soybean meal, an inoculation dosage of 1×10^4 spore/g SBM (DM basis) and a fermentation duration of 36 hours was determined to be optimal.

Experiment 2- Impact of Fermentation on Nutrient Profiles and Antinutritional Factors

Over 200 lbs of soybean meal fermented with *A. oryzae* from 19 sessions using optimal fermentation condition identified from Experiment 1 were used produce adequate ingredient for experimental diet production and further animal feeding trial. However, 11 sessions of unfermented soybean meal samples were molded due to accidental exposure to tap water. Unfermented soybean meal and the corresponding fermented soybean meal samples from the rest 8 sessions were used for analysis of macronutrients. One composite sample of unfermented soybean meal and another composite sample of fermented soybean meal from these 8 sessions were used for analysis of amino acid composition, sugars (sucrose, stachyose, raffinose and verbascose) and Aflatoxins. The trypsin inhibitor (Kunitz trypsin inhibitor and Bowman-Birk trypsin inhibitor) analysis data came from corresponding unfermented soybean meal, retorted but not fermented soybean meal and fermented soybean meal from 5 sessions. Fermentation of soybean meal with *A. oryzae* decreased ($P < 0.05$) OM, IDF and gross energy (GE) in soybean meal while it increased ($P < 0.05$) CP and SDF content (Table 4.1). The crude fat and TDF content were not influenced by fermentation. For amino acid composition, methionine content numerically increased from 15.12 to 15.51 mg/g CP and cysteine content numerically decreased from 16.02 to 15.71 mg/g CP (Table 4.2). The total essential amino acid content numerically decreased from 487.82 to 467.86 mg/g CP. Sucrose was eliminated while major oligosaccharides significantly decreased in fermented soybean meal (Table 4.2). Both KTI and BBI in unfermented soybean meal (3.17 and 2.29 TIU/g) were completely inactivated by retort processing. The Aflatoxin content of fermented soybean meal was 2.6 ppm which is much below the U.S. Food and Drug Administration (FDA) guideline limit in pet food ingredient (20 ppb).

Experiment 3-Extrusion Performance of *A. oryzae* Fermented Soybean Meal

Rapid viscosity analysis. A peak viscosity for each experimental treatment appeared in rapid viscosity analysis (RVA, Figure 4.3). The results showed a clear difference among soybean products with SPI exhibiting the highest viscosity (1200 cp), followed by SBM and AMF (277 and 272 cp, respectively), and FSBM showing the lowest viscosity (110 cp). The inclusion of 1% Amaferm® in AMF did not appear to affect viscosity, as their viscosity curves overlapped. The SPI also took less time for hydration and swelling. The FSBM showed less changes in viscosity compared to the other three diets during the entire cycle, but it started to build up viscosity and reached the peak viscosity faster than SBM and AMF.

Particle size distribution. Both the geometric particle size (D_{gw}) and geometric standard deviation of the particle diameter (S_{dw}) decreased in an order of SBM, AMF, FSBM and SPI (Table 4.4). When using the selected series of 8 sieves (diameter from 125 to 1000 μm), raw mixes of all four diets showed a unimodal particle size distribution (Figure 4.4). They all had a mode in the center of the size class between 212 and 500 μm, but the height of the peaks varied among them. The raw mix of SPI had the lowest peak while FSBM had lower peak than both SBM and AMF.

Extrusion processing. The goal of extrusion process was to compare the performance of fermented soybean meal with that of regular soybean meal and soybean protein isolate in terms of extrusion conditions and outcomes. During extrusion, some input variables were kept the same across diets, including feeder screw speed (13 rpm), PC screw speed (400 rpm), PC steam flow (13.1 ± 0.09 kg/h), barrel set temperatures (50°C, 70°C, and 90°C for each head zone), and knife speed (1842 rpm). The extruder shaft speed was 636 rpm for SBM, AMF and FSBM but had to decrease to 342 rpm for SPI due to surging (Table 4.5).

The PC discharge temperature was similar among treatments while PC water injection rate was greater ($P < 0.05$) for SBM and AMF (18.50 and 19.30 kg/h, respectively) than for FSBM and SPI (11.27 and 13.07 kg/h, respectively). The motor power and die temperature were both greater ($P < 0.05$) for FSBM and SPI (10.57 kW,117 ℃ and 10.03 kW, 117.33 ℃, respectively) than for SBM and AMF (5.90 kW, 100.33 ℃ and 7.57 kW, 104.67 ℃, respectively). The in-barrel moisture (IBM) content was the lowest ($P < 0.05$) for FSBM (28.21%), while SPI (33.45%) had lower ($P < 0.05$) IBM than SBM and AMF (34.46% and 34.69%, respectively). The SPI (305.3 kJ/kg) generated the greatest ($P < 0.05$) SME with the SBM (83.7 kJ/kg) generating the least ($P < 0.05$) SME.

Kibble characteristics. The kibbles from SBM and AMF diets had poor expansion, leading to inefficient drying with a moisture content of about 15% after the initial session. These kibbles were manually re-fed into the dryer at a rate similar to the extrusion throughput for a second drying session on the following day. All reported dry kibble data are from those that underwent two drying sessions. Wet kibbles and dry kibbles followed similar ranking of sectional expansion index (SEI) with the kibbles from SPI most ($P < 0.05$) expanded, followed by kibbles from FSBM, and kibbles of the rest two diets least ($P < 0.05$) expanded (Table 4.5). The differences in bulk density among diets were more distinguishable in dry kibbles than in wet kibbles. For the former ones, bulk density decreased ($P < 0.05$) in an order of SBM, AMF, FSBM and SPI (520.3, 481.0, 385.0 and 324.7 g/L, respectively).

Experimental diets. All experimental diets reached a moisture content below 10% with FSBM slightly dryer that other three diets (Table 4.6). Crude protein and crude fat contents were similar among diets at an average of 33.4% and 11.8% DM basis, respectively. For dietary fiber composition, the SPI was lower in TDF (14.0% DM basis) and IDF (9.4% DM basis) than the other three diets (at an average of 18.4% TDF and 13.9% IDF on DM basis). The gross energy content was similar among all experimental diets at an average of 4925 kJ/kg DM basis.

Discussion

Experiment 1 - Optimal Inoculation Dosage and Fermentation Duration

Many studies investigating factors affecting enzyme production of *A. oryzae* used single material or a combination of wheat, wheat bran, rice hull, rice bran and other grains that are common in the commercial enzyme industry and a focus on enzyme harvest instead of nutrient changes in the substrate through fermentation(Chutmanop et al., 2008; Das et al., 2023; Eugster et al., 2015; Ferreira et al., 2011; Hoa and Hung, 2013; Samarntarn et al., 1999; Shankar and Mulimani, 2007; Vishwanatha et al., 2010; Zhao et al., 2019). The industrial soybean meal fermentation with *A. oryzae* to produce traditional human foods has mostly utilized enzyme assisted fermentation or co-fermentation with other microorganisms in liquid state rather than solid state fermentation (Frias et al., 2008; Gao et al., 2013; Hong et al., 2004; Suprayogi et al., 2022; Teng et al., 2012; Yang et al., 2020). To our knowledge, this is the first study to evaluate the effects of inoculation dosage and fermentation duration on nutritional properties in soybean meal through pilot-scale solid state fermentation with *A. oryzae* alone. In this study, we evaluated three inoculation dosages (1×10^4， 5×10^5 and 1×10^6 spore/g SBM dry matter) and fermentation durations up to 48 hours.

A. oryzae is a major source of commercial protease production in the food industry with an additional advantage that this species lacks the gene to produce aflatoxins (Gomi, 2014). This fungus species produces multiple types of proteases including acid, neutral, alkaline proteases, and prolyl endopeptidases (Eugster et al., 2015; Samarntarn et al., 1999; Sandhya et al., 2005;

Vishwanatha et al., 2010). These enzymes play a crucial role in modifying soybean proteins during fermentation, making them essential to the production of fermented soy-based products. Battaglino et al. (1991) found that initial pH of 7.0 and moisture content of 35-40% were favorable for protease production while Chutmanop et al. (2018) found that 50% is the ideal substrate moisture content for *A. oryzae* to produce protease. The latter also reported that initial pH of 7.5 yielded about 30% greater protease activity than initial pH of 5.5. However, Battaglino et al. (1991) measured protease activity with a buffer pH at 7.0 while Chutmanop et al. (2018) used a buffer pH at 10.0. This is a critical factor to consider when interpreting results from different studies. The initial pH of substrate was not investigated in this study, which may be a limitation of the results. Other affecting factors including temperature, initial moisture content, relative humidity and solid versus liquid/submerged state were all set at optimal levels for *A. oryzae* growth according to previous studies (Chutmanop et al., 2008; Hoa and Hung, 2013; Park et al., 2019; Zhao et al., 2019).

Crude protein. Surprisingly, inoculation dosage did not affect crude protein (CP) content in the fermented soybean in this study though Battaglino et al. (1991) reported a positive relation between protease activity and inoculation dosage (from 10^4 to 10^6 spore/g substrate). This indicates that protease production/activity may not always be a proper predictor of crude protein content in the final fermentation product. The CP content remained unchanged until hour 24, after which it began to increase. This can be attributed to the fact that protease activity was low at early stage of fermentation and kept increasing over time as observed by Chutmanop et al. (2008) and Battaglino et al. (1991). At early state of rice bran fermentation with *A. oryzae*, the amylase activity reached a peak at between 10 to 20 hours and started to decrease while protease activity increased mildly at the beginning and started to increase fast from hour 24 until hour 60

(Chutmanop et al., 2008). Meanwhile, Sardjono et al. (1998) found that protease activity reached the plateau at hour 48 during solid state fermentation of cracked whole soybean with *A. oryzae*. Soybean meal is a much less starchy, but protein-rich substrate compared to rice bran, but such nutrient composition did not seem to accelerate the increase in protease activity under our fermentation conditions, as indicated by the trend of CP content change. Neutral proteases are the most important type of enzymes to break down soybean proteins during *A. oryzae* fermentation, though acidic proteases may dominate in the early stages of solid-state fermentation (Vishwanatha et al., 2010). Instead of substrate nutrient composition, the pH may play a more significant role in regulating protease activity, warranting further investigation.

***In-vitro* protein digestibility.** Unexpectedly, the *in-vitro* protein digestibility in fermented soybean meal was not affected by inoculation dosage, fermentation or the interaction of these two factors. Fermentation with *A. oryzae* has been shown to be able to decrease the overall protein molecular weights in soybean meal (Frias et al., 2008; Hong et al., 2004; Yang et al., 2020). Dias et al. (2010) reported increased *in-vitro* pepsin and trypsin digestibility of proteins in common bean (*Phaseolus vulgaris L.*) flour treated with protease from *Bacillus* species. Teng et al. (2012) reported improved *in-vitro* protein digestibility by pepsin but not by pepsin and trypsin in *A. oryzae* fermented soybean meal. Amadou et al. (2010) reported that fermentation with *Lactobacillus plantarum* increased *in-vitro* trypsin digestibility of soybean meal protein. In this study where *in-vitro* protein digestibility was measured using porcine pepsin and pancreatin, protein digestibility of fermented soybean meal was between 80 to 90% regardless of inoculation dosage and fermentation duration. It is important to note that we used a prolonged retort process (2 hours) to sterilize fermentation substrate, which very likely have decreased protein digestibility due to the formation of indigestible protein aggregates or complexes with other dietary components (e.g., protein, fiber and starch). This decreased protein digestibility due to harsh retort process has been reported in other studies (Li et al., 2017; Wei et al., 2021). The *in-vitro* protein digestion is a rather simple process compared to *in-vivo* digestion; but protein structure (including amino acid composition and sequence), protein solubility, formation of disulfide bonds and cross-linking, molecular size/peptide length and presence of antinutritional factors still significantly influence protein digestibility. An additional observation during trypsin inhibitor analysis in this study was that NaOH soluble protein markedly decreased after fermentation, which agreed to the study of Frias et al. (2008). This might have compromised protease accessibility of the soybean protein despite smaller molecular weight. The maintained *in-vitro* protein digestibility of *A. oryzae* fermented soybean meal may be related to amino acid profile change, decreased protein solubility (as observed during trypsin inhibitor activity analysis) and possible negative influences from fungal biomass that requires further investigation.

Fiber composition. The soluble dietary fiber (SDF) in soybean meal consists of pectic polysaccharides, hemicelluloses (e.g. β-glucans, mannans, xyloglucan and arabinoxylans), and other non-starch polysaccharides (NSP) and oligosaccharides (Choct, 1997; Choct et al., 2010). They all serve as substrates for colonic fermentation, leading to complex impacts on the host. The effects of dietary NSP and oligosaccharide (OS) on nutrient digestibility depends on the inclusion level, sources and composition. Soy NSPs seem to decrease nutrient digestibility to a greater extent than other common dietary fiber sources such as wheat bran and sugar beet pulp (Freire et al., 2000). The addition of soy OS in diets also decreased nutrient digestibility in swine (Smiricky et al., 2002; Smiricky-Tjardes et al., 2003). This study focused on improving soybean protein content and digestibility through fermentation with *A. oryzae*. Therefore, avoidance of increased SDF was desired. Note that the SDF analysis method used in this study did not take account of soybean OS. Our results showed that neither total dietary fiber (TDF) or insoluble dietary fiber (IDF) content in the soybean meal changed from hour 24 to 48 regardless of inoculation dosage. However, the SDF content maintained until late stage of fermentation, showing a trend of increasing from hour 36 to hour 48 (P=0.0969, from 5.85% to 7.8% of DM). Fermentation for 36 hours seemed to be a conservative and time-efficient choice to balance the changes in CP and SDF in soybean meal during the solid-state fermentation with *A. oryzae*.

The trend of increased SDF was very likely multifactor. Gomi's (2014) review summarized the genes in *A. oryzae* related to extracellular enzymes, among which cellulase, polygalacturonase, pectin lyase and xylanase contribute to solubilization of carbohydrates. The *A. oryzae* extract was found to stimulate *in-vitro* degradation of cellulose and hemicellulose after 12 hours of fermentation (Varel et al., 1993). Mohammadi et al. (2024) reported 100% solubilization of arabinoxylan fiber with xylanase treatment. Souza et al. (2021) reported a linear increase of dry matter and neutral detergent fiber *in-vitro* digestibility as *A. oryzae* dosage increased. However, it is insensible to compare the dosage impacts on fiber modification observed in their study and ours due to completely different inoculum preparation method and fermentation types (anerobic liquid state versus aerobic solid state). The *A. oryzae* is also famous for reducing OS which is also a type of SDF in soybean meal. Note that The synthesis of α-galactosidase from *A. oryzae* is able to remove/decrease OS content in soybean meal by breaking down α-1,6 glycosidic bonds while β-galactosidase is able to breakdown the chain at the β-1,6 glycosidic bonds (de Fátima Viana et al., 2005; Shankar and Mulimani, 2007). A study comparing multiple factors on α-galactosidase production of *A. oryzae* found that the enzyme synthesis stopped increasing after inoculation dosage reached 1×10^5 spore/g substrate in a liquid state fermentation (Shankar and Mulimani, 2007). Though the substrate for α-galactosidase (e.g. the OS) was not measured in this experiment, the lack of dosage effects at higher level on general SDF hydrolase was consistent with our results as the second-high dosage (5×10^5 spore/g substrate) used in our study was already greater than the “adequate” dosage found by Shankar and Muliani (2007). Additionally, the fungal biomass of *A. oryzae* formed during fermentation also contained dietary fiber in the cell wall and hyphae, mostly β-glucans and chitin (Garcia-Rubio et al., 2020). In filamentous fungus species such as *A. oryzae*, the soluble β-glucans and insoluble chitin comprise around 50–60% and 10-20% of dry cell wall weight, respectively. Therefore, the accumulation of biomass also contributed to increase in SDF in the fermented soybean meal. The maintained SDF content during early to middle stage of fermentation in this study could be a balance of degradation of soybean SDF, solubilization of soybean IDF and formation of fungal SDF.

Based on the results of this experiment regarding inoculation dosage and fermentation duration, we decided to scale up the soybean meal fermentation with *A. oryzae* using an inoculation dosage of 1×10^4 spore/g and a fermentation duration of 36 hours. This approach was chosen to increase CP content while avoiding an increase in SDF.

Experiment 2 - Impact of Fermentation on Nutrient Profiles and Antinutritional Factors

Changes of macronutrient contents. In this experiment, all soybean meal was fermented with *A. oryzae* at 1×10^4 spore/g DM for 36 hours while other conditions remained the same as in experiment 1. Samples were analyzed for organic matter, CP, CF, TDF, IDF, SDF and GE in soybean meal before and after fermentation with *A. oryzae* for 36 hours (Table 4.1). The reduction in organic matter and CP, along with the retention of TDF and decrease in IDF after fermentation, were consistent with the findings in experiment 1 (Figure 4.1). The SDF increased ($P < 0.001$) here unlike experiment 1 was probably due to a greater number of replicates (from 3 to 8 replicates) which may have improved the power of the statistical model. The decrease in GE after fermentation was likely due to sampling and analytical variation because ash, CP and CF content all increased; which should have increased GE content from a chemical perspective. Hong et al. (2004) reported an average 5% increase in soybean meal CP content after solid state fermentation with *A. oryzae*. This was similar to the 4.65% increase observed in this study. However, they also observed an increase of CF after fermentation, this was probably due to longer fermentation duration (48 hours) that increased the biomass proportion leading to higher CF concentration when compared to soybean meal (Pedersen et al., 1999). Teng et al. (2012) reported an average 2.9% increase in CP in soybean meal fermented by *A. oryzae* over 72 hours. Because the unfermented soybean meal had a lower CP concentration (34.46%) compared to the unfermented soybean meal used in this study (50.05%) and similarly the study of Hong et al. (50.32%, 2004), it may indicate the percentage increase in CP depends on the initial concentration in the substrate. Nevertheless, the extent of increased CP calculated by [(fermented value- unfermented value) /unfermented value] remained similar among the three studies (9.21 ± 0.76 %). Other studies have also supported that soybean meal fermented by *A. oryzae* alone or in combination with other microorganisms or enzymes under various conditions significantly increased CP content (Chen et al., 2013; Gao et al., 2013; Suprayogi et al., 2022; Yang et al., 2020).

Amino acid composition. Compared to CP content, amino acid composition provides a better reflection of the quality of a protein ingredient when used alone. The limiting amino acid in soybean for cats and dogs is methionine and/or sulfur-containing amino acids (SAA) (Berry et al., 1962; Zarkadas et al., 2007). Frias et al. (2008) reported that solid-state fermentation with *A. oryzae* increased methionine content per unit of protein in cracked soybean seeds. However, our study found that solid-state fermentation with *A. oryzae* did not increase methionine content per unit of protein in soybean meal. This is consistent with findings from previous studies using similar procedures (Chen et al., 2013; Gao et al., 2013; Hong et al., 2004; Teng et al., 2012; Yang et al., 2020). Das et al. (2023) reported that methionine synthase was one of the five most abundant proteins in *A. oryzae* during solid state fermentation of pea protein isolate, of which the limiting amino acid is also methionine; and the abundance of this enzyme peaked at 36-h fermentation though much lower than abundance of α-amylase A. They postulated that the failure of *A. oryzae* in efficiently increasing methionine or SAA content in pea protein eliminated the possible increased activity in the methionine degradation pathway. Methionine plays a significant role in the biosynthesis of aflatoxins in *Aspergillus* species, primarily through its conversion into S-adenosylmethionine (SAM), which acts as a methyl donor in various methylation reactions essential for aflatoxin production (Yu et al., 1993). The *A. oryzae* is known to be incapable of aflatoxin synthesis (Gomi, 2014) which may have decreased demands for methionine biosynthesis. Betaine and choline supplementation were reported to repress methionine synthase transcription by decreasing the demand for methyl group while homocysteine supplementation enhanced the transcription (Kacprzak et al., 2003). The amino acid composition analysis of a commercial *A. oryzae* biomass product (Amaferm®, BioZyme, Inc., St. Joseph, MO) showed that it had higher cysteine content but similar methionine content compared to soybean meal (data shown in table 5.1, Chapter 5). This indicated that the *A. oryzae* might be able to accumulate cysteine but barely convert it to methionine. In summary, multiple factors may have limited the capacity of *A. oryzae* in accumulating methionine within fungal cells, such as low cysteine content in substrate (true for the soybean meal used in this study), limited inorganic sulfur resources from substrate and the environment, low demands for methyl from methionine due to existence of alternative methyl resources of the fungus, etc. Meanwhile, the majority of proteins in the final product of solid-state fermentation originate from the initial substrate, rather than the fungal biomass, accounting for 93.2% and 6.8%, respectively, after 36 hours of fermentation, as determined in the study by Das et al. (2023). Therefore, even if the fungus itself is able to efficiently accumulate a certain amino acid, the accumulation in the biomass would have minor impacts on the overall amino acid content in the fermentation end product.

Oligosaccharides. Research have shown that the galacto-oligosaccharides (GOS, raffinose, stachyose and verbascose) are fermented more rapidly in the colon of monogastric animals compared to other types of soluble dietary fibers (SDF) found in soybean meal (Hee Seong Kim et al., 2023; Smiricky-Tjardes et al., 2003). This has led to complaints of soybean OS causing flatulence and abdominal discomfort in monogastric animals. The removal or reduction of sucrose and GOS through fermentation in this study was not surprising as *A. oryzae* is able to produce α-galactosidase (Gomi, 2014). It was extraordinarily effective in reducing stachyose compared to raffinose and verbascose (relative reduction of 95.2, 87.4 and 41.8%, respectively) in the soybean meal according to our results. Opazo et al. (2012) reported 88% and 76% relative reduction in stachyose and raffinose respectively after solid-state fermentation of soybean meal with *A. oryzae* for 10 days. Our reduction percentages were higher than those. Chen et al. (2013) observed that sucrose in soybean meal was fully digested after 20 hours of fermentation while the extent for GOS needs at least 28 hours. Shankar and Muliani (2007) found that increasing solid substrate spatial concentration (from 100 to 400 g/tray) during solid-state fermentation, while keeping the inoculation dosage constant (1×10^5 spores/g substrate), decreased enzyme production likely due to poor ventilation from the increased substrate thickness on the tray. This highlights the importance of proper ventilation during fermentation to minimize OSs as much as possible. Our practice of breaking, flipping and spraying surface of caked solid substrate every 12 hours in this study may have been crucial for GOS degradation.

Trypsin inhibitor. Previous studies have confirmed that trypsin-inhibitors (TI) in soybean meal can be greatly reduced or inactivated through fermentation due in part to the potent proteases from *A. oryzae* (Chen et al., 2013; Gao et al., 2013; Hong et al., 2004; Teng et al., 2012). There are two major types of TI in soybeans that these enzymes target on trypsin or both trypsin and chymotrypsin: Kunitz TI (KTI) and the Bowman-Birk TI (BBI), respectively (Birk, 1961; Kunitz, 1945). The BBI is more resistant to physical and chemical inactivation such as heat or acids for proteolysis by pepsin compared to KTI owing to its different compositions and conformations (Huang et al., 2008). Both trypsin and chymotrypsin are important serine proteases with similar catalytic mechanisms but different substrate specificities: KTI prefers basic amino acids like lysine and arginine, while BBI prefers aromatic amino acids like phenylalanine, tryptophan, and tyrosine (Vajda and Szabó, 1976). The standard methods for soybean TI activity analysis are American Oil Chemists' Society (AOCS) and the American Association of Cereal Chemists International (AACCI) approved methods (Kim and Krishnan, 2023). These methods measure total inhibition on trypsin by KTI and BBI. The residual 10-20% TI activity in soybean meal after standard inactivation treatments usually comes from the heat-insensitive BBI (Birk, 1961; Murphy, 2008). In order to evaluate impacts of solid-state fermentation with *A. oryzae* on BBI in soybean meal, chymotrypsin inhibition was also measured in addition to trypsin inhibition, which would be able to reflect the level of BBI alone. However, our study found that retort at 121℃ for 2 hours eliminated total TI activity in the unfermented soybean meal. Other studies either did not measure TI activities in retorted soybean meal or retorted at relatively low temperature or/and much shorter duration (Gao et al., 2013; Hong et al., 2004; Teng et al., 2012). The reason for longer retort duration in our study was to control field contamination during fermentation as our batch size was much greater than that of previous studies (at least 14 kg per batch vs. less than 100 grams in the flask). Chen et al. (2013) reported 2.1 TIU/mg autoclaved (121℃, 15 minutes) soybean meal, which was completely eliminated after 36-hour solid state fermentation with *A. oryzae*. Our study did not detect any TI activity in retorted soybean meal probably due to longer retort time and the use of different measuring method. Moreover, our study used a relative novel method of trypsin inhibitor activity analysis which seemed to be less sensitive than traditional methods (Kim and Krishnan, 2023).

Experiment 3-Extrusion Performance of *A. oryzae* Fermented Soybean Meal

Raw mix characteristics and extrusion process. Considering the experimental diet formulations, the distinct particle size distribution of the SPI raw mix is likely attributed to the fine particles in soybean protein isolate and possibly the brewer's rice as well. It was reported that soybean protein isolate is more viscous than corn starch under similar shear rate (Zhang et al., 2022). This could explain the extraordinarily high viscosity of the SPI raw mix in our study. The fermented soybean meal ingredient was ground through 1.00 mm sieve before mixing with the rest of the formula, which explained that the raw mix of FSBM had less sample retained on the 1000 μm sieve compared to that of the SBM and AMF.

Under the same feeder screw speed, the raw mix of FSBM tended to have higher feed rate which indicated this raw mix was more flowable than raw mix of the other three diets. The FSBM had relatively higher preconditioner (PC) discharge temperature in spite of its lower PC water injection rate compared to SBM and AMF. The moisture content of raw mix for FSBM was approximately 1.5% lower than that of SBM and AMF (data not shown). This indicated that the raw mxi of FSBM required lower moisture to generate heat for cooking in the PC. Considering the low feed rate of the SPI, the extruder shaft speed was necessarily decreased to avoid surging due to inadequate material supply to maintain the die pressure. The greater motor power, die temperature and specific mechanical energy (SME) for FSBM compared to SBM and AMF were expected as FSBM feed rate was relatively high. The in-barrel moisture (IBM) content for FSBM was the lowest among all experimental diets. The extruder water injection rate was automatically adjusted by the extruder system to maintain the fixed barrel temperature. This suggested that FSBM material might have required much less moisture than the other three diet materials to build up viscosity to generate SME. But this did not seem to be the case based on the RVA analysis results in this study. Instead, the increased SME for FSBM may also be an effect of particle size as smaller particle size usually creates more mechanical energy due to increased contact surface per unit volume (Al-Rabadi et al., 2011; Carvalho et al., 2010). The particle size distribution analysis showed that the FSBM had smaller geometric mean diameter compared to both SBM and AMF. Particle size distribution analysis results also explained the greatest SME in the SPI when it had slightly lower IBM than both SBM and AMF. The greater IBM in the SPI compared to the FSBM may explained the similar diet temperature regardless of greater SME in the former: water acts as a heat sink that absorbs thermal energy which results in cooler die temperatures (Bhattacharya and Hanna, 1987). Bhattacharya et al. (1987) also reported that lower IBM increased die pressure due to more viscous material, which is opposite to observation in our study between the FSBM and the SPI. This may be explained by the RVA results showing that the SPI raw mix had the greatest viscosity while the FSBM raw mix barely built-up viscosity. Feed rate and extruder shaft speed influence die pressure in an opposite way. Another possible reason for the higher die pressure for SPI than FSBM could be that the decreased extruder shaft speed increased barrel fill, despite the slightly decreased feed rate.

Kibble characteristics. Smaller particle size has been found to promote expansion in extruded high-protein pet food and high-fiber snacks (Alam et al., 2014; Mathew et al., 1999). The kibbles from SBM and AMF were poorly expanded, hence the high dry kibble bulk density. The cause might be the relatively large particle size of the raw mix of these two diets (Table 4.5), mostly from soybean meal. Large particle size decreased contact surface per volume in the barrel and decreased mechanical energy from friction between materials, barrel wall and the screw. The low viscosity of FSBM raw mix, as indicated by RVA, appeared to contradict the significant kibble expansion observed this diet. It is important to note that the RVA results may not accurately represent the actual material viscosity within the extruder barrel, where significantly higher pressure, moisture content, heat and shear forces are present compared to the conditions in the RVA test chamber. Studies showed that high pressure increased soybean protein viscosity (Torrezan et al., 2007; Zhang et al., 2005). Additionally, increased moisture content may decrease overall protein mixture viscosity because water acts as a plasticizer that reduce the viscosity of protein mixtures and moderating the effect of pressure (Guy, 2001). The FSBM had low IBM compared to the other three diets, this may decrease their differences in regard to viscosity. The small particle size, extra starch content from brewer's rice, high viscosity of raw mix, great SME, high die temperature and pressure were well reflected on the greatest kibble expansion of SPI diet. Additionally, insoluble dietary fiber (IDF) decreases kibble expansion (Robin et al., 2012). The low IDF content in SPI should also have contributed to kibble expansion. The specific length of FSBM kibbles was greater than that of SBM and AMF (wet) or

SBM and SPI (dry kibbles), indicating greater longitudinal expansion of FSBM kibbles. Compared to SPI, the greater longitudinal expansion of FSBM kibbles was probably at the expense of sectional expansion as the two are usually negatively related (Kumar et al., 2007; Robin et al., 2012). However, the FSBM kibbles had both greater sectional and longitudinal expansion than SBM and AMF kibbles. This may assist in the assumption of low actual viscosity of FSBM material in the barrel as lower viscosity usually leads to less resistance to flow and more pronounced longitudinal expansion. We also observed shrinkage (decreased SEI) of kibbles after drying for the SBM, AMF and FSBM diets but the opposite for SPI diet. The extended drying time may have caused significant shrinkage after drying as more moisture was removed. Mild fluctuation in kibble expansion during extrusion and sampling timing for wet and dry kibbles may also have contributed to the observed changes in SEI after drying.

Experimental diets. The SBM and AMF formulas only differed by less than 2% of corn. In the latter, corn was replaced by Amaferm® to produce this experimental diet. As anticipated the macronutrient compositions of these two diets was very similar except for the SDF concentration, which is more likely due to analytical variation. The slightly higher IDF and SDF in FSBM than in SBM and AMF reflects the differences in fiber contents between their corresponding ingredients (soybean meal and fermented soybean meal). The SPI had markedly lower IDF and TDF content compared to all other diets. The primary variation among experimental diet formulas was the type of soybean product used, with 30% inclusion. Due to soybean protein isolate's high crude protein content (over 90%), matching the protein levels of the other diets without major ingredient adjustments was not feasible. Instead, we maintained similar ratios of soybean protein to overall diet protein, resulting in 18% soybean protein isolate and 10.68% brewers' rice to balance the composition. Both soybean protein isolate and brewers' rice contain significantly lower levels of TDF and IDF compared to the soybean meal and fermented soybean meal, leading to a decrease in IDF and TDF in the SPI diet. However, the SDF levels in the SPI diet remained comparable to the other diets, likely due to effective compensatory increases in brewers' rice, corn, and/or reductions in poultry by-product meal.

Conclusion

This study found that the inoculation dosage higher than 1×10^4 spore/g substrate did not impact protein quality and dietary fiber content in soybean meal fermented by *A. oryzae*. To increase crude protein content while avoiding an increase in soluble dietary fiber in soybean meal, a fermentation duration of 36 hours was required. However, substrate pH was also identified as a critical factor for protease activity of the *A. oryzae* during fermentation, suggesting that optimization in future studies is needed. Fermentation under conditions determined in this study barely changed methionine or sulfur-containing amino acid contents in the soybean meal but eliminated sucrose and decreased undesirable OS. Trypsin inhibitors were completely inactivated by extended heat treatment. If fermentation is to be done in a space with strict microorganism control, the potential of *A. oryzae* to remove TI would still be valuable. The pet food containing fermented soybean meal product from this study had well expanded kibbles regardless of the very low viscosity raw material compared to similar formulas that contained unfermented soybean meal. This indicated that fermentation may have altered some physicochemical properties of the soybean meal, warranting further investigation. In conclusion, solid-state fermentation with *A. oryzae* improved protein content and decreased certain ANFs in soybean meal. The fermentation product can be successfully incorporated into adult dog maintenance food formula at a 30% inclusion rate and produce well-expanded dry kibble.

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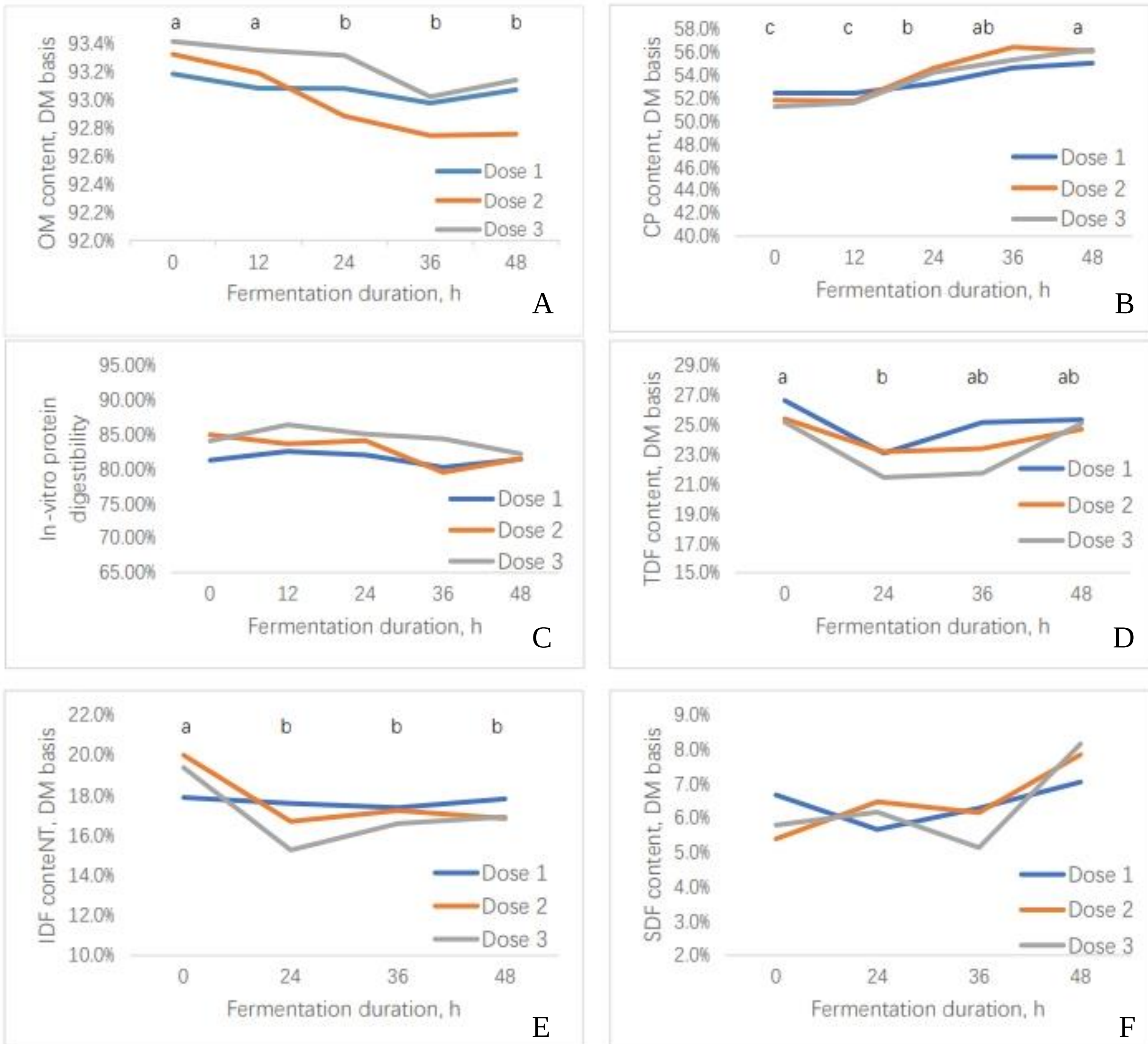

Figure 4.1. Nutrient contents of soybean meal fermented with *Aspergillus oryzae* with different inoculation dosage and fermentation duration. (A) Organic matter, (B) Crude protein (CP) content, (C) *in-vitro* protein digestibility and (D) total dietary fiber, (E) insoluble dietary fiber, (F) soluble dietary fiber.

[abc] Means fermentation durations without a common superscript letter differ ($P < 0.05$).

Table 4.1. Paired t-test analysis results of nutrient changes from 8 batches of unfermented soybean meal (U-SBM) and *Aspergillus oryzae* fermented soybean meal (F-SBM).

Nutrient	U-SBM	F-SBM	Difference[1]	SEM of difference	*P*-value
Organic matter, %DM	92.52	91.51	-1.01	0.001	<.001
Crude protein, %DM	50.05	54.69	4.63	0.004	<.001
Crude fat, %DM	2.21	2.40	0.19	0.002	0.2874
TDF, %DM	25.06	23.88	-1.18	0.009	0.2104
IDF, %DM	19.32	15.29	-4.03	0.009	0.0026
SDF, %DM	5.74	8.59	2.85	0.005	<.001
GE, kcal/kg DM	4720.2	4555.6	-164.6	13.78	<.001

[1]Calculated by (F-SBM) - (U-SBM).
TDF-total dietary fiber; IDF-insoluble dietary fiber; SDF-soluble dietary fiber; GE-gross energy.

Table 4.2. Amino acid composition, common soybean sugar contents and trypsin inhibitor units (TIU) in unfermented soybean meal (U-SBM) and *Aspergillus oryzae* fermented soybean meal (F-SBM).

Chemicals	U-SBM		F-SBM
Amino acids, % DM			
Arginine	3.64		3.46
Histidine	1.34		1.36
Isoleucine	2.46		2.72
Leucine	3.99		4.43
Lysine	3.21		2.96
Methionine	0.72		0.81
Phenylalanine	2.64		2.92
Threonine	1.96		2.17
Tryptophan	0.63		0.63
Valine	2.55		2.84
Alanine	2.23		2.52
Aspartic acid	5.69		6.21
Cysteine	0.76		0.82
Glutamic acid	9.34		10.11
Glycine	2.14		2.35
Serine	2.15		2.33
Proline	2.56		2.85
Tyrosine	1.90		2.11
Hydroxylysine	0.02		0.01
Hydroxyproline	0.07		0.08
Taurine	0.09		0.06
Lanthionine	0.00		0.07
Ornithine	0.03		0.08
Sugars, % DM			
Sucrose	6.58		0.00
Raffinose	0.95		0.12
Stachyose	2.93		0.14
Verbascose	0.55		0.32
Trypsin inhibitor, TIU/g DM	U-SBM	R-SBM	F-SBM
Kunitz TI	3.17	Not detected	Not detected
Bowman-Birk TI	2.29	Not detected	Not detected

DM-dry matter; R-SBM-retorted but not fermented soybean meal.

Table 4.3. Experimental diet formulas (SBM, 30% soybean meal; AMF, 30% SBM and 1% Amaferm®; FSBM, 30% fermented soybean meal; SPI, 18% soybean protein isolate).

Ingredient, %	SBM	AMF	FSBM	SPI
Soybean meal	30.00	30.00	0.00	0.00
Amaferm®	0.00	1.00	0.00	0.00
Fermented soybean meal	0.00	0.00	30.00	0.00
Soybean protein isolate	0.00	0.00	0.00	18.00
Brewer's rice	0.00	0.00	0.00	10.68
Corn	37.23	35.87	39.11	40.00
Beet pulp	3.79	3.79	3.79	3.79
Poultry by-produce meal	19.31	19.25	16.00	16.06
Chicken fat	5.82	6.06	6.07	6.00
Dog dry digest	1.00	1.00	1.00	1.00
Fish oil	1.00	1.00	1.00	1.00
Calcium carbonate	0.47	0.51	0.60	0.68
Dicalcium phosphate	0.37	0.31	0.82	0.84
Potassium chloride	0.00	0.20	0.60	0.95
Salt	0.25	0.25	0.25	0.25
Choline chloride	0.10	0.10	0.10	0.10
Titanium dioxide	0.40	0.40	0.40	0.40
Vitamin Premix[1]	0.15	0.15	0.15	0.15
Trace mineral premix[2]	0.10	0.10	0.10	0.10

[1]Vitamin premix: 5.51% moisture, 4.02% crude protein, 34.5% ash, 13.4% calcium, 17,162,999 IU/kg Vitamin A, 920,000 IU/kg Vitamin D, 79,887 IU/kg Vitamin E, 14,252 mg/kgthiamine, 4,719 mg/kg riboflavin, 12,186 mg/kg pantothenic acid, 64,736 mg/kg Niacin, 5,537 mg/kg pyridoxine, 720 mg/kg Folic acid, 70 mg/kg biotin, 22 mg/kg vitamin B12.
[2]Trace mineral premix: 0.66% moisture, 21.5% calcium, 0.02% sodium, 0.57% magnesium, 38,910 mg/kg iron, 11,234 mg/kg copper, 5,842 mg/kg manganese, 88,000 mg/kg zinc, 1,584 mg/kg iodine, 310 mg/kg selenium, 19% carbohydrate, and 1% crude fat.
DM, dry matter.

Figure 4.2. Schematic of extruder screw profile. Inlet starting on the left to discharge ending on the right.

Screw element 1: inlet screw, single flight full pitch;

Screw element 2: single flight, full pitch screw;

Screw element 3: small steam lock;

Screw element 4: single flight full pitch screw;

Screw element 5: small steam lock;

Screw element 6: Single flight, full pitch screw;

Screw element 7: medium steam lock;

Screw element 8: double flight, ½ pitch screw;

Screw element 9: Large steam lock;

Screw element 10: double flight, ½ pitch, cone screw.

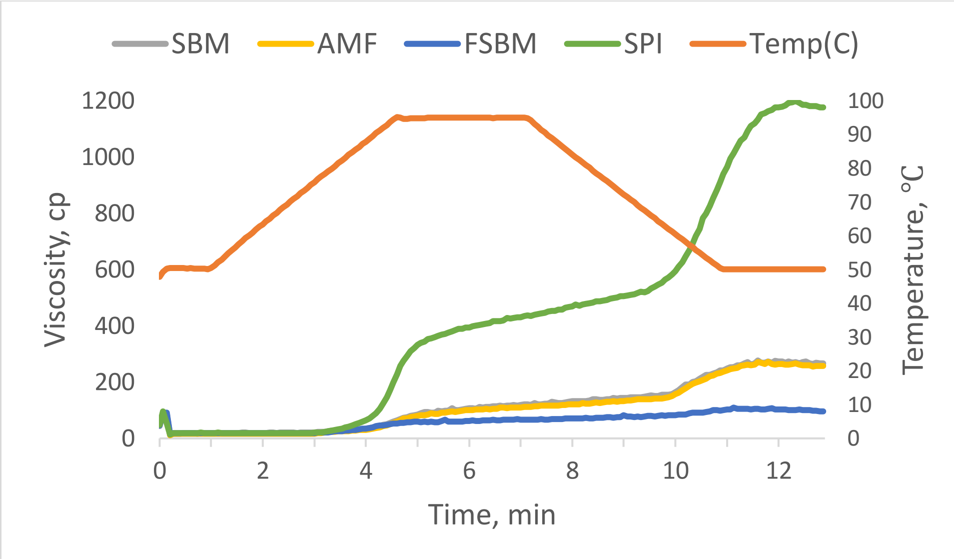

Figure 4.3. Rapid viscosity analysis results of raw mixes of four experimental diets.

Table 4.4. Geometric mean diameter (D_{gw}) of raw mixes and geometric standard deviation of the particle diameter (S_{dw}) of each raw mix for four experimental diets.

	SBM	AMF	FSBM	SPI
D_{gw}	382.8	347.1	286.8	223.1
S_{gw}	300.63	284.28	240.80	206.93

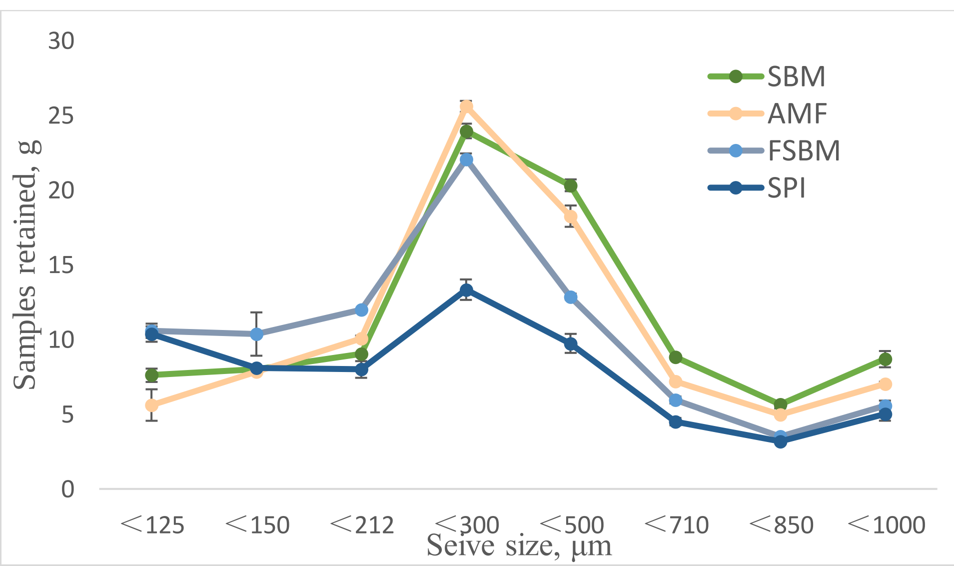

Figure 4.4. Particle size distribution of raw mixed material of four experimental diets.

Table 4.5. Extrusion processing data and out-of-extruder kibble measurements of treatment diets (SBM, 30% soybean meal; AMF, 30% SBM and 1% Amaferm®; FSBM, 30% fermented soybean meal; SPI, 18% soybean protein isolate).

Parameter	SBM	AMF	FSBM	SPI	SEM	*P*-Value
Feed rate (as-is), kg/h	113.05	117.00	124.20	98.85	4.583	0.0681
PC water injection, kg/h	18.50^{a}	19.30^{a}	11.27^{b}	13.07^{b}	0.604	<.001
PC discharge	93.9	92.7	95.0	94.3	0.71	0.1723
EX Shaft speed, rpm	636^{ab}	637^{a}	636^{b}	342^{c}	0.2	<.001
EX Water injection,	12.60	13.23	11.60	11.90	0.533	0.2109
Motor power, kW	5.90^{b}	7.57^{b}	10.57^{a}	10.03^{a}	0.512	<.001
Die temperature, ℃	100.33^{b}	104.67^{b}	117.00^{a}	117.33^{a}	1.581	<.001
Die pressure, psig	200^{b}	250^{a}	210^{b}	250^{a}	5.0	<.001
In-barrel moisture, %	34.46^{a}	34.69^{a}	28.21^{c}	33.45^{b}	0.002	<.001
SME, kJ/kg	83.7^{c}	115.9^{bc}	198.7^{b}	305.3^{a}	14.57	0.0015
Wet kibble characteristics						
SEI, mm^2 /mm^2	1.44^{c}	1.50^{c}	1.87^{b}	2.45^{a}	0.030	<.001
Piece density, g/cm^3	0.788^{a}	0.759^{a}	0.575^{b}	0.449^{c}	0.022	<.001
Specific length, mm/g	22.95^{b}	22.69^{b}	25.75^{a}	23.68^{ab}	0.651	0.0049
Bulk density, g/L	520.7^{a}	484.0^{ab}	419.0^{bc}	348.3^{c}	17.31	<.001
Dry kibble characteristics						
SEI, mm^2 /mm^2	1.30^{c}	1.31^{c}	1.80^{b}	2.55^{a}	0.024	<.001
Piece density, g/cm^3	0.74^{a}	0.68^{b}	0.49^{c}	0.41^{d}	0.010	<.001
Specific length, mm/g	26.63^{b}	28.60^{a}	29.19^{a}	24.26^{c}	0.314	<.001
Bulk density, g/L	520.3^{a}	481.0^{b}	385.0^{c}	324.7^{d}	5.73	<.001

[abc] Means within a row lacking a common superscript letter are different ($P < 0.05$).
PC-preconditioner; EX-extruder; SEM-specific mechanical energy; SEI-sectional expansion index.

Table 4.6. Nutrient composition of the coated experimental diets with different soybean products (SBM, 30% soybean meal; AMF, 30% soybean meal plus 15 Amaferm®; FSBM, 30% A. oryzae fermented soybean meal; SPI, 18% soybean protein isolate).

Nutrient	SBM	AMF	FSBM	SPI
DM, %	93.6	93.9	96.00	93.9
Crude protein, % DM	33.2	33.8	31.5	35.0
Crude fat, % DM	12.4	12.3	11.5	11.0
Ash, % DM	7.78	7.28	8.61	7.18
Total dietary fiber, % DM	17.9	17.6	19.8	14.0
Insoluble dietary fiber, % DM	13.3	13.7	14.7	9.4
Soluble dietary fiber, % DM	4.6	3.9	5.1	4.6
Gross energy, kcal/kg DM	4955.7	4975.6	4829.5	4938.1

DM-dry matter.

Chapter 5 - Digestibility and Palatability of Extruded Dog Food with *Aspergillus oryzae* Fermented Soybean Meal and Its Influence on Colonic Fermentation

Abstract

Fermentation has been demonstrated to improve the nutritional value of soybean meal for companion dogs. This study aimed to compare *Aspergillus oryzae* fermented soybean meal with traditional soybean products in regard to nutrient digestibility, colonic fermentation and palatability when fed to adult dogs. Four diets were formulated with similar macronutrient contents but using different soybean products (30% soybean meal, SBM; 30% soybean meal plus 1% dried *A. oryzae* biomass product, AMF; 30% fermented soybean meal, FSBM; and 18% soybean protein isolate, SPI). Diets were produced on a single-screw extruder and fed to 12 adult dogs in a replicated 4 x 4 Latin square design experiment. Dogs were adapted to diets for 9 days followed by a 5-d total fecal collection. Fresh fecal samples were collected for hind-gut fermentation analysis. Apparent total tract digestibility was calculated by total fecal collection (TFC) and indirect marker (titanium dioxide) methods. Data were analyzed using the generalized linear mixed model by statistical software (GLIMMIX; SAS version 9.4, SAS Institute, Inc., Cary, NC) with treatment as a fixed effect and dog and period as random effects. Least square means were separated with Tukey's post hoc test with significance level of $\alpha = 0.05$. Dogs on SPI had lower ($P < 0.001$) fecal output and defecation frequency compared to dogs on other three diets. Dogs fed with FSBM had similar ATTDs of organic matter, crude protein and gross energy to dogs fed with SBM, but the ATTDs were lower ($P < 0.05$) than those of dogs fed with the SPI regardless of estimation method. Notably, the ATTD of dry matter, as calculated by the marker method, was higher ($P < 0.001$) in dogs on the FSBM diet compared to those on the SBM diet. Fecal pH was higher ($P < 0.05$) in dogs on the SPI diet compared to those on SBM and AMF, with dogs on the FSBM intermediate. While fecal ammonia content was similar across all dogs, total short-chain fatty acid (SCFA) content was lowest ($P < 0.05$) in dogs on the SPI and highest ($P < 0.05$) in those on the AMF. Acetate, propionate, and total fatty acid contents mirrored the ranking of total SCFA. Fecal isovalerate content was higher ($P < 0.05$) in dogs on the SPI diet than those on the FSBM diet. Percentage in total fatty acid content of propionate and total SCFA were lower ($P < 0.05$), whereas those for butyrate, isovalerate, and total branched-chain fatty acids were higher ($P < 0.05$) in dogs on the SPI diet compared dogs on other diets. Dogs showed a preference ($P < 0.05$) for SBM over FSBM, but differences between SBM, AMF, and SPI were significant. In conclusion, the *A. oryzae* fermented soybean meal induced similar stool quality, nutrient digestibility and colonic fermentation pattern in dogs compared to regular soybean meal, but was still distinctive from soybean protein isolate. Palatability was less favorable for the fermented soybean meal.

Introduction

The inclusion of soybean meal in dog food has occurred for decades due to its affordability and distinctive amino acid composition compared to cereal grains (Berry et al., 1962; Zarkadas et al., 2007). However, traditional soybean meal contains anti-nutritional factors like trypsin inhibitors and oligosaccharides (OS) that can impair protein digestibility and reduce the overall nutritional value of dog foods (Blow et al., 1974; Patten et al., 1971; Silvio et al., 2000; Yamka et al., 2003). Further processing of soybean meal into soybean protein concentrates or isolates can remove some of these negative influences and improve nutrient digestibility, fecal quality and reduce flatulence but with a marked increase in cost (Clapper et al., 2001). This is where fermented soybean meal has emerged as a potential alternative for pet food industry.

Soybean fermentation has a long and rich history, particularly in East Asian countries such as China, Japan, and Korea, where it plays a crucial role in the production of traditional foods like miso, soy sauce, and tempeh. The key microorganism used in soybean fermentation is *Aspergillus oryzae*, a filamentous fungus commonly referred to as koji mold in Japan and China (Gomi, 2014). This fungus has the capacity to degrade large protein molecules into smaller, more digestible peptides while simultaneously reducing or removing some anti-nutritional factors like phytic acid and trypsin inhibitors (Chen et al., 2013; Frias et al., 2008; Gao et al., 2013; Hong et al., 2004; Suprayogi et al., 2022; Teng et al., 2012; Yang et al., 2020). The inability to produce aflatoxin greatly contributed to the utilization of *A. oryzae* in the human food industry for centuries (Kusumoto et al., 1990). These modifications are not just beneficial for humans but also for monogastric livestock (Feng et al., 2007b, 2007a; Kim et al., 2012; Mathivanan et al., 2008; Yang et al., 2007; Zamora and Veum, 1988).

For companion animals, there are studies on functionalities of enzyme processed and enzyme-assisted fermented soybean meal as a protein source or a prebiotic in extruded dog food (Beloshapka et al., 2016; Félix et al., 2012; Yamka et al., 2006; Zuo et al., 1996). However, there are rarely studies exploring the potentials of soybean meal fermented with single microorganism as a pet food protein source. The objectives of this study were to evaluate effects of *A. oryzae* fermented soybean meal on nutrient digestibility, diet palatability and colonic fermentation when included in extruded dog food. We hypothesized that the diet with such fermented soybean meal would have greater overall nutrient digestibility, palatability and less colonic fermentation in dogs compared to a diet with traditional soybean meal.

Materials and Methods

Experimental Diets

The fermented soybean meal ingredient was produced with the protocol described in Chapter 4 in a laboratory in the Department of Biological and Agricultural Engineering, Kansas State University, KS. This ingredient was ground through a 1.00 mm sieve and then mixed with the rest of the formula before extrusion. The *A. oryzae* biomass product which derived from wheat and bran fermentation was purchased from BioZyme Inc. (St. Joseph, MO). Other raw materials for diet extrusion were purchased and blended by a commercial mill (Wilbur-Ellis Holdings, Inc., St. Joseph, MO). The analyzed nutrient composition of soybean products and *A. oryzae* biomass product used in this study was presented in Table 5.1.

Four diets, nutritionally adequate for maintenance of adult dogs (AAFCO, 2016), were formulated with different soybean meal products: SBM with 30% soybean meal; AMF with 30% soybean meal plus 1% Amaferm® where the latter served to differentiate any effects from *A. oryzae* biomass on processing and animals; FSBM with 30% *A. oryzae* fermented soybean meal; and SPI with 18% soybean protein isolate (Table 5.2). Note that the soybean protein isolate had 92% crude protein on dry matter basis, much higher than that of the soybean meal and fermented soybean meal used in this study. We aimed to include similar amounts of soybean protein in each diet, which resulted in the 18% instead of 30% of soybean protein isolate in the SPI diet. Brewers' rice was used only in the SPI diet to complete the formula. Diets were formulated to have similar nutritional composition and included titanium dioxide (TiO_2; 0.4%) as an indigestible marker for determination of apparent total tract digestibility (ATTD) of dietary nutrients. As predicted by the formulation, the concentrations of minerals (calcium, phosphorous, potassium, magnesium, sodium, sulfur, manganese, copper iron, and zinc) were similar among diets and met AAFCO (2020) nutrient profile recommendations for adult dogs at maintenance. The experimental diets were produced using a single screw extruder (EX; model X115, Wenger Manufacturing, Sabetha, KS, USA) at the extrusion facility of the Department of Grain Science and Industry, Kansas State University, KS. Processing conditions were set as described in Chapter 4. After drying, the chicken fat (Pekingese, International Dehydrated Foods, Inc., Monett, MO) and dry digest (AFB International, St. Charles, MO) were applied to the kibbles in a double-ribbon blender (Wenger Manufacturing, Sabetha, KS, USA). Coated diets were kept in laminated bags and stored at room temperature until fed.

Feeding Trials

The dog digestibility trial was conducted at the Kansas State University Large Animal Research Center (LARC) under the Institutional Animal Care and Use Committee (IACUC) protocol #4348. Dogs were housed in USDA approved kennels (Large Animal Research Center at Kansas State University; Manhattan, KS, USA). Twelve healthy adult beagles (6 neutered male and 6 spayed female) of similar age (3.0 $\pm$ 0.2 year) and similar body weights (9.8 $\pm$ 1.6 kg) were individually housed in metabolic pens (1.83m × 1.20m) equipped with an acrylic-mesh floor to allow for the separation of urine and feces. The dogs were housed on a 12 h light cycle with lights off from 1900 to 0700.The dogs were randomly assigned and fed a specific diet. Initial dietary intake on day 0 was determined by calculating daily metabolizable energy (ME) requirements for laboratory kennel dogs [130 $\times$ $BW_{kg}^{0.75}$; NRC (2006)]. The ME of experimental diets was calculated using the prediction equation in pet food [(8.5 $\times$ CF) +(3.5 $\times$ CP) + (3.5 $\times$ NFE)]. The daily food allowance was calculated using the daily ME requirement divided by predicted dietary ME. Dogs were fed twice daily (8:00 AM and 5:00 PM) in equal rations to meet daily food allowance. The body weights of dogs were measured at the beginning, middle, and end of each period. If necessary, food allowances were adjusted by 5 or 10% for the following week to maintain dog BW. Water was provided for *ad libitum* consumption.

The feeding trial used a replicated 4×4 Latin square design, where each animal served as its own control. Each of the four periods composed a 9-day adaptation followed by a 5-day collection. During collection period, all feces and orts were collected daily. Every fecal sample was weighed and scored on a 5-point scale where 1 = liquid diarrhea, 2 = very soft consistency, unformed stool; 3 = soft stool that retains shape; 4 = well-formed firm stool that does not leave residue when picked up; and 5 = very hard, dry stool. The weighted mean fecal score was calculated for each dog in each period. Fecal samples were stored at -20℃ until the end of trial and then thawed at room temperature, pooled by dog, weighed, and freeze-dried (Harvest Right LLC., Salt Lake City, UT) with a protocol revised from Molnar et al. (2021) until the moisture level reached below 10%. The partially dried fecal samples were also weighed, and the values were used when calculating the fecal dry matter (DM) content. Diet samples and partially dried fecal samples were ground through a 1.00-mm screen by a laboratory fixed blade impact mill (Retsch, type ZM200, Haan, Germany) and stored in Mason jars at room temperature for further chemical analysis.

In addition, the pH of one fresh fecal sample per dog per period (within 15 min of defecation) was recorded in triplicate with a calibrated glass-electrode pH probe (FC240B, Hanna Instruments, Smithfield, RI) immediately after collection. Fresh fecal samples were stored at -80℃ until further analysis.

Chemical Analysis

Dry matter (DM), organic matter (OM), and ash were determined according to AOAC methods 934.01 and 942.05. Crude protein (CP) was determined by Dumas combustion method

(AOAC 990.03) using a nitrogen analyzer (FP928, LECO Corporation, Saint Joseph, MI) with nitrogen to CP conversion factor 6.25. Crude fat was determined by acid hydrolysis followed by hexane extraction (ISO 11085:2008) using a semi-automated equipment (Hydrotec 8000 and ST 255 Soxtec, Foss, Denmark). Gross energy (GE) was measured as total heat of sample combustion by calorimetry (Parr 6200 Calorimeter, Parr Instrument Company, Moline, IL). The titanium dioxide content in diet and fecal samples was analyzed with a colorimetric method (Myers et al., 2004). The soluble dietary fiber (SDF), insoluble dietary fiber (IDF) and total dietary fiber (TDF) content of diet samples was measured (AOAC 991.43) by an ANKOM Dietary Fiber Analyzer (ANKOM Technology, Macedon, NY, USA).

Analysis for sugars (sucrose, stachyose, raffinose and verbascose) and amino acid composition in soybean products and *A. oryzae* biomass product (Amaferm®) was conducted at a cooperating university (University of Missouri Agricultural Experiment Station Chemical Laboratories; Columbia, MO). Sucrose, stachyose, raffinose and verbascose contents were determined by gas chromatography (Bhatti et al., 1970). All amino acids, except methionine, cysteine, and tryptophan, were digested with 6 N HCl for 24 h at 110°C. The amino acids were then separated by ion-exchange chromatography and the concentration was determined with a Beckman 6300 amino acid analyzer (Beckman, Palo Alto, CA). Methionine and cysteine were first oxidized by performic acid to methionine sulfone and cysteic acid, respectively, prior to acid hydrolysis. Tryptophan was hydrolyzed in 3 M mercaptoethanesulfonic acid before analysis. Available lysine was determined (AOAC 975.44) and lysine availability (%) was calculated as the ratio of available lysine to total lysine.

Ammonia concentration in the fresh fecal samples was analyzed by colorimetric method described by Chaney and Marbach (1962) in duplicates. The fresh fecal samples were thawed and diluted with deionized water and 0.1N HCl and homogenized. The homogenized samples were centrifuged at 3,000 g for 20 min to separate the suspended solids. The 1 mL of the supernatant of the centrifuged samples was collected and kept frozen at −20 °C for at least 24 h to complete deproteinization. The acidified samples were thawed, centrifuged at 20,000 g for 15min, and plated.

Fecal SCFA including BCFA contents were analyzed on a gas chromatography (GC; Agilent 7890, Agilent Technologies, Santa Clara, CA). The fresh fecal samples were thawed and diluted with deionized water and homogenized, followed by centrifugation at 3,000 g for 20 min to separate the suspended solids. The 1mL of the supernatant of the centrifuged samples was collected acidified with 0.25mL of 25% m-phosphoric acid was before deproteinization at −20 °C for at least 24 h. The GC is equipped with flame ionization detector (FID) and a capillary column (DB-WAX, Agilent 127-7012, 10m × 0.1mm × 0.1μm, Agilent Technologies, Santa Clara, CA). Hydrogen was used as a carrier gas with a flow rate of 35 mL/min, and the split ratio was 100:1 with injection volume of 0.2 μL. Nitrogen was used as the makeup gas with a flow rate of 25 mL/min. The detector and injector temperatures were set at 300℃, and the initial oven temperature was set to 40℃ with a ramp rate of 20℃/min to 180℃ for a total run time of 8 min. The peak area of chromatograms was determined using an integrative software (OpenLab CDS, Agilent Technologies, Santa Clara, CA). The concentrations of SCFA (acetate, propionate, and butyrate) and BCFA (isobutyrate, isovalerate, and valerate) in samples were quantified by comparing the sample peak area to three standards with known concentrations of each volatile free acid and corrected for the fecal dry matter content.

Digestibility Calculation

Two methods were utilized to estimate nutrient apparent total tract digestibility (ATTD). The total fecal collection (TFC) method requires the collection of all feces excreted by the experimental animals. The marker method uses TiO_2 as an indigestible dietary marker. In current study, the ATTD of DM, OM, CP, CF and GE were calculated according to the TFC and marker methods by following equations:

3. TFC method:

$$Nutrient\ digestibility\ (\%) = \frac{(nutrient\ consumed\ (g) - nutrient\ excreted\ (g))}{nutrient\ consumed\ (g)} \times 100\%$$

4. Marker method:

$$Nutrient\ digestibility\ (\%) = [1 - \frac{(nutrient\ in\ feces\ \% \times TiO2\ in\ food\ \%)}{(nutrient\ in\ food\ \% \times TiO2\ in\ feces\ \%)}] \times 100\%$$

Palatability Trial

Palatability trials were conducted at a commercial research kennel (Summit Ridge Farms; Susquehanna, PA, USA) with a two-bowl test with the AMF, FSBM and SPI diet each compared to the SBM diet. Twenty male and female Beagles identified by ear tattoo and cage number were presented the test diets on an individual basis. Two stainless steel bowls, each containing approximately 400 grams of diet, were offered once daily for 2 days. Bowl placement was reversed daily and both bowls were presented for 30 minutes. Food consumption and first choice (FC) preference were recorded for each dog. Food intake ratios (IR) of each dog were calculated by dividing the intake of each diet into the total intake for both test diets. Preference was determined based on animals' FC and IR.

Statistical Analysis

The nutrient ATTD, food intake, fecal characteristics, SCFA, BCFA, and ammonia contents from the fresh fecal samples were analyzed using the generalized linear mixed model in a statistical software (GLIMMIX, SAS version 9.4, SAS Institute, Inc., Cary, NC) with diet as the fixed effect and period and dog as random effects. Least square means were separated with Tukey's post hoc test, with significance considered at $P < 0.05$. In palatability tests, the intake ratio was analyzed using a t-test in a 2-way ANOVA and the first-choice preference was analyzed using a Chi^2 test.

Results

Feed Types and Nutrient Composition

The soybean meal and fermented soybean meal had comparable CP contents of 51.44% and 54.69% DM, respectively, with Amaferm® and soybean protein isolate representing the lower (15.00% DM) and higher extremes (92.14% DM) respectively (Table 5.1). Fermentation improved the methionine and cysteine contents of soybean meal from 0.75% to 0.81% DM and 0.75% to 0.82% DM, respectively, though they remained lower than those in soybean protein isolate (1.2 and 1.14% DM, respectively). Despite its much lower CP, Amaferm® matched the soybean meal in taurine content (0.11% and 0.13% DM, respectively). Sugar contents decreased from soybean meal to fermented soybean meal, and then to soybean protein isolate, except for verbascose, with Amaferm® having the highest raffinose content (1.40% DM). No trypsin inhibitors were detected in the diets (Table 5.3) though they were present in the soybean meal ingredient. All diets had similar contents of DM, CP, ash and GE but the SPI had much lower IDF (9.4% DM) than the other three diets (averagely 13.9% DM). The inclusion of 1% Amaferm® in the AMF diet notably increased stachyose and raffinose levels (1.12% and 0.44%

DM, respectively) compared to the SBM diet (0.53% and 0.37% DM, respectively). The content of galacto-oligosaccharide (GOS) in the FSBM (0.22% DM) was lower than that of the SBM (6.80% DM) but still greater than that of the SPI (0.09% DM).

Food Intake and Fecal Characteristics

Generally, the food was well tolerated and consumed by dogs in this study as food intake were not different among dogs regardless of diets (Table 5.4). However, one dog was removed from the panel due to stress-induced bloody stool confirmed by veterinary staff at the LARC at Kansas State University (Manhattan, KS, USA) since the second period. Dogs fed with the SPI had the lowest ($P < 0.001$) wet fecal output, dry fecal output, fecal moisture content and defecation frequency (96.5 g, 157.5 g, 67.5% and 1.8 times/day, respectively) compared to dogs fed with the other three diets (averagely 171.6 g, 233.4g, 72.7% and 2.2 times/day, respectively). Fecal score tended (P=0.0558) to increase in dogs fed with SPI compared to that of dogs on other three diets. The average fecal score of 3.73±0.09 from all dogs was within the ideal range.

Apparent Total Tract Digestibility

The food amounts offered to each dog during the feeding trial were adjusted weekly to maintain body weight of dogs, which proved successful with an average bodyweight of 9.81±1.63 kg at day 0 and 9.75±1.80 kg at day 56.

Overall, the ATTD of macronutrients calculated by TFC method were greater than those calculated by marker method. Regardless of methods, the ATTD of DM, OM, CP and GE were the greatest ($P < 0.05$) in dogs fed the SPI. The ATTD of CF was similar among dogs fed with experimental diets. The rankings of ATTD of OM and GE in dogs fed with the SBM, AMF and FSBM calculated by both methods were similar. The ATTD of DM and CP were similar among dogs fed with SBM, AMF and FSBM if calculated by TFC method; but the ATTD of DM in dogs fed with FSBM (74.05%) was greater ($P < 0.05$) than that in dogs fed with SBM (72.67%) when calculated by marker method.

Colonic Fermentation

The fecal pH was higher ($P < 0.05$) in dogs fed with SPI (5.93) compared to that in dogs fed with SBM and AMF, with dogs on FSBM intermediate (5.60, 5.48 and 5.64, respectively). The fecal ammonia content was similar among dogs fed with experimental diets. The content of total fatty acids, SCFA, acetate and propionate in fresh feces were greater ($P < 0.05$) in dogs fed with AMF (583.9, 571.8, 305.9 and 201.8 μmol/g DM feces, respectively) than those in dogs fed with SPI (417.1, 403.2, 222.2 and 112.0 μmol/g DM feces, respectively), with dogs on SBM and FSBM intermediate. Though isovalerate was the only BCFA that showed greater ($P < 0.05$) concentration in fresh feces from dogs fed with SPI compared to that of dogs fed with FSBM (5.13 and 2.27 μmol/g DM feces, respectively), the total BCFA content and isobutyrate content in fresh feces seemed to be numerically greater in dogs fed with SPI.

In regard to the proportion of individual short chain fatty acids relative to total SCFA, the proportion of acetate was similar among fresh feces from dogs fed with experimental diets (averagely 53.8%). The relative proportion of propionate to total SCFA was less ($P < 0.05$) in fresh feces from dogs on the SPI (27.2 and 96.72%, respectively) than those from dogs on the other three diets (averagely 33.7 and 97.74%, respectively). For butyrate, isovalerate and total BCFA, the proportions were greater ($P < 0.05$) in fresh feces from dogs on the SPI (15.5, 1.182 and 3.28%, respectively) than those from dogs on the other three diets (averagely 10.32, 0.429 and 2.26%, respectively). Amaferm and FSBM did not move the SCFA proportions in a manner that would merit note.

Palatability

Dogs did not show a preference between the SBM and AMF (Table 5.8). The SBM was preferred ($P < 0.05$) to FSBM by the dogs reflected by IR of two days and FC of the second day. In regard to choice between experimental diets SBM and SPI, dogs only showed a preference ($P < 0.05$) for SPI on the first day but not the second day based on FC and IR.

Discussion

Nutrient Composition of Ingredients and Diets

To facilitate the experiments to evaluate comparisons among fermentation and diet production with fermented treatments in this study, the soybean meals used were derived from different suppliers. This was necessitated by the time frame between experiments and research chronological path. The former had greater protein content, which was close to the fermented soybean meal used in this study. Additionally, the soybean meal used for fermentation was high in verbascose (0.51% DM) compared to the data of soybean meal from 10 domestic plants (from 0.16-0.24% DM, Grieshop et al., 2003) while that of the soybean meal (0.23% DM) used for diet production fell in the range that Grieshop et al. (2003) reported. The content of CP, CF, sulfur-containing amino acids, sugars and TI fell in an expected rankings among three soybean protein products in this study. The Amaferm®, which is a commercial source of *A. oryzae* biomass, was high in taurine content per unit of DM compared to the fermented soybean meal. Considering the low CP content in Amaferm®, its taurine content per unit of protein was way greater than the soybean meal, fermented soybean meal and soybean protein isolate used in this study.

The stachyose contents in the SBM (0.57% DM) and AMF (1.19% DM) diets differed substantially, which was contrary to our anticipation for the formulas based on ingredient sugar analysis results: when added at 1%, the low-stachyose Amaferm® (0.03% DM) should not have influenced total stachyose content of the AMF compared to SBM. The studies of Zuo at al. (1996) and Yamka et al. (2006) also included traditional soybean meal in extruded dog diets at levels from 18.55 to 37%, the consequent dietary stachyose contents were all above 1.0% DM. The SBM in this study had 30% soybean meal but the stachyose content was 0.57% DM. On contrary, the AMF had 1.19% stachyose (DM basis) that seemed to be reasonable. In summary, the unexpected low stachyose content in SBM diet was very likely an analysis error. Meanwhile, Berrios et al. (2010) reported that extrusion at 160°C decreased stachyose in formulated chickpea flour and lentil flour by 37% and 28%, respectively. Borejszo and Khan (1992) also found stachyose content decreased 60% in a pinto bean starch product after high temperature extrusion. The SBM in our study has more complicated nutrient composition than materials in the two studies mentioned above, which may have led to a different stachyose interaction during extrusion. But there is still possibility that extrusion partially removed stachyose in the SBM but not in the AMF, indicating a potential protective function from the *A. oryzae* biomass on stachyose. Trypsin inhibitors (TI) were present in the soybean meal ingredient but not in the SBM diet, agreeing with Kim and Aldrich (2023) that extrusion and drying process effectively denatured TI in soybeans. Further discussion on macronutrient contents of experimental diets were available in Chapter 4.

Food Intake and Fecal Characteristics

Dogs maintained body weights throughout the whole trial even though the GE and DM intake were both numerically lower in FSBM. The actual metabolizable energy in the FSBM may be greater than the other diets. Also, the palatability test results showed that the FSBM was the least palatable diet in this study. It may have played a role in the reduced food intake as well. The resulting minor decease in calorie intake was compensated by dogs to result in a maintained bodyweight. Generally, the food intake and fecal dry matter output relationships and rankings among treatments were consistent with nutrient ATTD results in this study.

When evaluating fecal quality parameters, dogs fed SBM, AMF and FSBM had similar scores. The minor difference in fecal moisture between dogs fed AMF and FSBM were not of practical significance. On the other hand, dogs fed SPI showed distinctive fecal characteristics that were most probably related to the level and composition of dietary fiber in this diet. The SPI diet had considerably lower IDF and moderate amounts of SDF among four diets (Table 5.3) due to replacement of soybean meal with soybean protein isolate and brewer's rice. Our results suggest that elevated IDF increased fecal bulk and moisture content. In the review of McRorie and McKeown (2017), they pointed out that "laxative effect of IDF was due to mechanical irritation of the mucosa, causing secretion of water and mucous, leading to bulky/soft/easy-to-pass stools." Silvio et al. (2000) observed a linear increase of fecal DM content and fecal DM output as SDF (pectin) gradually replaced by IDF (cellulose). Similar relationship between fiber composition and dog fecal characteristics were also found in studies of Beloshapka et al. (2016) and Kim et al. (2023), Kilburn-Kappeler and Aldrich (2023) and Kilburn-Kappeler et al. (2023).

Apparent Total Tract Digestibility

Based on the *in-vitro* protein digestibility analysis results for traditional and *A. oryzae* fermented soybean meal ingredients in Chapter 4, it was not unexpected that the ATTD of DM, OM, CP, CF and GE were not affected by dietary treatments SBM, AMF or FSBM (Table 5.5). It seems neither formulation or processing augmented protein digestibility among the two soybean meal treatments. The studies of Yamka et al. (2006) and Zuo et al. (1996) both corroborate our findings in that when a diet contained less than 2.7% stachyose and less than 0.4% raffinose, replacing traditional soybean meal in it with low-oligosaccharide low-phytate soybean meal did not improve nutrients ATTD in dogs. The SBM, and AMF diets were in this range. The SDF usually absorbs water and increases viscosity of digesta. This allows more time for digestion and absorption but also creates physical barrier for the contact of nutrient and digestive enzymes. For certain types of SDF, like the OS in soybean meal, there may be a comprehensive negative effect on nutrient digestibility (Ruiz et al., 2020; Yamka et al., 2003). The *A. oryzae* is a filamentous fungi whose cell wall has a major meaningful level of β-1,3-glucan and chitin (Garcia-Rubio et al., 2020). The β-glucans together with other soluble polysaccharide complexes from the biomass in the fermented soybean meal may have offset the effects of reduced soybean OS on total fermentable carbohydrate contents in FSBM (Table 5.3), as well as the benefits form reduction of OS, hence the similar nutrient ATTD in this study. From another research group (Rochus et al., 2012), it was reported that viscous guar gum shifts dietary amino acids from metabolic use to fermentable substrate in domestic cats. This indicates that diets with high SDF may have decreased protein digestibility in the small intestine, but the extra peptides/amino acids that enter the colon can still be efficiently utilized by microorganisms as the SDF is degraded, resulting in the same total tract digestibility compared to diets with lower SDF. This may also partially explain the similar crude fat digestibility among all experimental diets in spite of dietary fiber composition difference: lipids are incompatible with water and are hardly affected by water soluble dietary fiber. Meanwhile, the ATTD of DM, OM, CP, CF and GE were considerably greater in dogs on SPI. This should be the combined result of decreased IDF (partially replaced with starch), possible change of SDF profile due to addition of brewer's rice, smaller particle size of raw material (as shown in Chapter 4) and potentially greater removal of other antinutritional factors through production of soybean protein isolate compared to soybean meal.

Colonic Fermentation

Overall, less colonic fermentation in dogs on SPI was reflected by lower total fatty acid concentration that resulted in higher fecal pH. The numerical increase in fecal ammonia of dogs on SPI may not only reflect a trend for increased protein fermentation, but also a consequence of conversion of ionic ammonium to gaseous ammonia due to increased pH.

Interestingly, the greatest difference in fecal short chain fatty acid concentrations were between dogs on AMF and dogs on SPI although those concentrations were similar between dogs fed SBM and dogs fed FSBM. The low concentration in SCFA, acetate, propionate and valerate in dogs on SPI can be easily explained by the lower total fermentable carbohydrate content in this diet. Though the high protein digestibility in SPI allowed less protein to enter the colon compared to the other diets, the numerically increased isobutyrate and isovalerate concentrations from fresh dog feces from those animals fed SPI might indicate a promotion of protein fermentation due to decreased fermentable carbohydrates. The increased percentage of isobutyrate, isovalerate and BCFA in Table 5.7 also supported this. The results from dogs on AMF, however, showed greater impact from *A. oryzae* biomass product (Amaferm) on regulating dog colonic fermentation even after extrusion. Published research has shown that Amaferm efficiently increased fiber digestibility of certain feedstuffs by rumen fluid simply via stimulation of rumen bacterial activities (Beharka and Nagaraja, 1991, 1993). The unaltered fecal short chain fatty acid percentage in dogs on AMF compared to dogs in SBM and on FSBM (Table 5.7) suggest that Amaferm only increased fermentation quantity but did not seriously affect fermentation pattern.

If considered as the fermentation product solely from carbohydrates, the butyrate concentration and percentage looked a bit contradictory to the fermentation level and pattern stated above. Interestingly, dietary protein has been found to increase the abundance of butyrate-producing bacteria in dogs but may not directly increase butyrate concentration as noted in one study (Xu et al., 2017). Addition of prebiotics (fructo-oligosaccharides) has been found to increase fecal butyrate concentration in dog fed with diet with a low protein digestibility and high dietary protein (Pinna et al., 2016). Some *in-vitro* fermentation studies with dog feces also suggested that butyrate concentration and percentage are affected by substrate protein content (Calabrò et al., 2013; Cutrignelli et al., 2009). In that sense, the similarly high fecal butyrate concentration in dogs on SBM and on SPI may be explained by prebiotic function of Amaferm and increased protein fermentation in dogs on SPI.

The valerate was in a similar situation to butyrate: both the concentration and the percentage of valerate seemed to be affected by both dietary protein and fermentable carbohydrate contents. According to previous research with dogs, valerate seemed to be a common colonic fermentation product from both carbohydrate and protein (Nery et al., 2012; Peixoto et al., 2018): the absolute concentration (μmol/g substrate) was more affected by protein content but not TDF or starch content in the fermentation substrate (Cutrignelli et al., 2009; Hee Seong Kim et al., 2023), while the ratio of valerate to total short chain fatty acids (%) were less affected to protein content in fermentation substrate (Calabrò et al., 2013). Based on the previous fecal quality and nutrient digestibility results, it is not surprising that dogs fed the SPI diet attracted attention when considering short chain volatile fatty acid content expressed as a percentage to total fatty acid content.

The lack of difference in colonic fermentation parameters between dogs on SBM and dogs on FSBM is possibly due to the additional dietary fiber from fungal cell wall inherent in the fermented soybean meal ingredient. Fungal cell wall contains a remarkable amount of fermentable carbohydrates, especially in filamentous fungi like *Aspergillus spp.* (Garcia-Rubio et al., 2020). However, the biomass content in the fermented soybean meal ingredient, and fermentable carbohydrate contents in the *A. oryzae* biomass remain to be investigated to verify our assumption.

Palatability

Fermentation of beans can reduce the “green,” “floral,” “earthy” flavors that are not appreciated by dogs who are oriented towards a more carnivorous nature, while generated esters commonly appeal to this species (Senanayake et al., 2023). The flavor change during fermentation is greatly influenced by the variety of microorganisms, substrate, and fermentation conditions. We were surprised to find the compromised palatability of FSBM compared to SBM for dogs. The FC was not different between FSBM and SBM on the first test day but greater for SBM on the second day. This indicates that the flavor of FSBM could be novel yet not distasteful for dogs; some dogs gave it a try out of curiosity. But IR were greater for SBM instead of FSBM for both test days, indicating a better comprehensive palatability (texture, flavor, taste, etc.) of the SBM. Felix et al. (2012) suggested that low molecular weight sugars in soybean may contribute to its palatability for dogs, which may partially explain what we observed here based on sugar composition of experimental diets (Table 5.3). Dogs were found to prefer high-fat and low-fiber diets (Hewson-Hughes et al., 2013; Koppel et al., 2015), which is the opposite for FSBM compared to SBM. But such disadvantage of the FSBM is not directly related to the fermented soybean meal ingredient and can be easily optimized. Another critical factor for dry dog food palatability is processing and kibble characteristics (Koppel et al., 2015). The FSBM was dryer (4% moisture content) than the other three diets (6-7% moisture content) in this study which might have made the kibbles too hard (Zhang et al., 2023). Based on the results of Chapter

2, high inclusion level (over 15%) of bioprocessed soybean meal seems to have compromised palatability compared to low inclusion levels. Beloshapka et al. (2016) also reported that a diet with 12% bioprocessed soybean protein (SP) was more palatable than a diet with 48% same SP for dogs. The influence of fermented soybean products on pet food palatability may require further research to understand the reasons.

The SBM and AMF unsurprisingly had similar palatability as the formulas were very similar. As for the SBM and SPI, dogs preferred SPI on the first day based on both FC and IR but did not show preference for either diet on the second day. This indicates that the SPI was tasteful for dogs at the beginning, but dogs may have become “fatigued” or “bored” with the flavor sooner. The four diets were extruded with the same extruder and die configuration; but the sectional expansion ratio (SEI) varied markedly among kibbles from different treatment as shown in Chapter 4, which may have influenced kibble texture. According to the palatability test between SBM and SPI, the two experimental diets with the greatest difference in SEI, texture seems to have been a minor factor in this study.

Conclusion

To our knowledge, this is the first study investigating *A. oryzae* fermented soybean meal as an ingredient in extruded dog food with traditional soybean meal and soybean protein isolate as comparison. Neither the nutrient digestibility, stool quality and colonic fermentation was affected by fermentation of soybean meal compared to traditional soybean meal, while the diet using soybean protein isolate showed great advantages. The aroma of the diet with fermented soybean meal did not appear to be disliked by dogs, but the general palatability did seem to be compromised, which requires further investigation and resolution if this protein modification is to be used in commercial dog foods.

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Table 5.1. Analyzed nutrient composition of soybean products and *A. oryzae* biomass ingredients used in this study.

Nutrient	Soybean meal	Amaferm®	Fermented soybean meal	Soybean protein isolate
Dry matter, %	89.67	92.57	94.40	96.24
Dry matter basis				
Crude protein, %	51.44	15.00	54.69	92.14
Crude fat, %	2.44	5.22	2.47	-
Sugars, %				
Sucrose	6.87	2.11	0.00	0.00
Stachyose	5.30	0.03	0.14	0.00
Raffinose	1.27	1.40	0.12	0.00
Verbascose	0.23	0.00	0.32	0.00
Trypsin inhibitor, TIU/mg				
Kunitz TI	3.17	-	ND	-
Bowman-Birk TI	2.29	-	ND	-
Amino acids, %				
Arginine	3.95	1.04	3.46	7.53
Histidine	1.45	0.43	1.36	2.42
Isoleucine	2.55	0.51	2.72	4.64
Leucine	4.19	0.94	4.43	7.51
Lysine	3.53	0.58	2.96	5.79
Methionine	0.75	0.22	0.81	1.20
Phenylalanine	2.82	0.62	2.92	5.28
Threonine	2.15	0.49	2.17	3.35
Tryptophan	0.68	0.21	0.63	1.16
Valine	2.68	0.73	2.84	4.67
Alanine	2.37	0.72	2.52	3.75
Aspartic acid	6.14	1.04	6.21	11.26
Cysteine	0.75	0.36	0.82	1.14
Glutamic acid	10.08	2.92	10.11	19.83
Glycine	2.31	0.84	2.35	3.91
Serine	2.45	0.56	2.33	4.21
Proline	2.71	0.93	2.85	5.10
Tyrosine	2.03	0.40	2.11	3.54
Taurine	0.13	0.11	0.06	0.00

Table 5.2. Diet formula with calculated nutrient compositions of the experimental diets (SBM, 30% soybean meal; AMF, 30% SBM and 1% Amaferm®; FSBM, 30% fermented soybean meal; SPI, 18% soybean protein isolate).

Ingredient, %	SBM	AMF	FSBM	SPI
Soybean meal	30.00	30.00	0.00	0.00
Amaferm®	0.00	1.00	0.00	0.00
Fermented soybean meal	0.00	0.00	30.00	0.00
Soybean protein isolate	0.00	0.00	0.00	18.00
Brewer's rice	0.00	0.00	0.00	10.68
Corn	37.23	35.87	39.11	40.00
Beet pulp	3.79	3.79	3.79	3.79
Poultry by-produce meal	19.31	19.25	16.00	16.06
Chicken fat	5.82	6.06	6.07	6.00
Dog dry digest	1.00	1.00	1.00	1.00
Fish oil	1.00	1.00	1.00	1.00
Calcium carbonate	0.47	0.51	0.60	0.68
Dicalcium phosphate	0.37	0.31	0.82	0.84
Potassium chloride	0.00	0.20	0.60	0.95
Salt	0.25	0.25	0.25	0.25
Choline chloride	0.10	0.10	0.10	0.10
Titanium dioxide	0.40	0.40	0.40	0.40
Vitamin Premix[1]	0.15	0.15	0.15	0.15
Trace mineral premix[2]	0.10	0.10	0.10	0.10

[1]Vitamin premix: 5.51% moisture, 4.02% crude protein, 34.5% ash, 13.4% calcium, 17,162,999 IU/kg Vitamin A, 920,000 IU/kg Vitamin D, 79,887 IU/kg Vitamin E, 14,252 mg/kgthiamine, 4,719 mg/kg riboflavin, 12,186 mg/kg pantothenic acid, 64,736 mg/kg Niacin, 5,537 mg/kg pyridoxine, 720 mg/kg Folic acid, 70 mg/kg biotin, 22 mg/kg vitamin B12.
[2]Trace mineral premix: 0.66% moisture, 21.5% calcium, 0.02% sodium, 0.57% magnesium, 38,910 mg/kg iron, 11,234 mg/kg copper, 5,842 mg/kg manganese, 88,000 mg/kg zinc, 1,584 mg/kg iodine, 310 mg/kg selenium, 19% carbohydrate, and 1% crude fat.
DM, dry matter.

Table 5.3. Nutrient composition of the coated experimental diets with different soybean products (SBM, 30% soybean meal; AMF, 30% soybean meal plus 15 Amaferm®; FSBM, 30% A. oryzae fermented soybean meal; SPI, 18% soybean protein isolate).

Nutrients	SBM	AMF	FSBM	SPI
Dry matter, %	93.6	93.9	96.00	93.9
	Dry matter basis			
Crude protein, %	33.2	33.8	31.5	35.0
Crude fat, %	12.4	12.3	11.5	11.0
Ash, %	7.78	7.28	8.61	7.18
Total dietary fiber, %	17.9	17.6	19.8	14.0
Insoluble dietary fiber, %	13.3	13.7	14.7	9.4
Soluble dietary fiber, %	4.6	3.9	5.1	4.6
Gross energy, kcal/kg	4955.7	4975.6	4829.5	4938.1
Sugars, %				
Sucrose	2.97	3.01	0.97	1.05
Stachyose	0.57	1.19	0.13	0.01
Raffinose	0.40	0.47	0.10	0.09
Verbascose	0.00	0.00	0.00	0.00
Total fermentable carbohydrate[1], %	5.57	5.56	5.33	4.60
Trypsin inhibitors[2], TIU/g	ND	ND	ND	ND

[1]The sum of soluble dietary fiber, stachyose and raffinose.

[2]Include Kunitz trypsin inhibitor and Bowman-Birk trypsin inhibitor.

ND-not detected.

Table 5.4. Food intake and stool quality parameters of dogs fed diets with different experiment diets (SBM, 30% soybean meal; AMF, 30% SBM and 1% Amaferm®; FSBM, 30% fermented soybean meal; SPI, 18% soybean protein isolate).

	SBM (n=12)	AMF (n=11)	FSBM (n=11)	SPI (n=11)	MSE	*P*-Value
Food intake (as-is), g/d	212.6	212.0	202.6	204.1	26.94	0.0779
Wet fecal output, g/day	171.4^{a}	179.4^{a}	164.1^{a}	96.5^{b}	38.21	<0.001
Dry fecal output, g/day	45.8^{a}	47.3^{a}	47.0^{a}	31.5^{b}	7.36	<0.001
Fecal moisture, %	72.85^{ab}	73.80^{a}	71.33^{b}	67.50^{c}	0.024	<0.001
Defecations per day	2.2^{a}	2.3^{a}	2.1^{a}	1.8^{b}	0.40	<0.001
Fecal score	3.66	3.67	3.72	3.85	0.326	0.0558

Table 5.5. The ATTD of dogs fed diets with different soybean protein sources (SBM, 30% soybean meal; AMF, 30% SBM and 1% Amaferm®; FSBM, 30% fermented soybean meal; SPI, 18% soybean protein isolate) estimated by TFC and TiO2 as dietary marker methods.

Nutrient	SBM (n=12)	AMF (n=11)	FSBM (n=11)	SPI (n=11)	MSE	*P*-Value
			TFC method			
Dry matter, %	77.02^{b}	77.62^{b}	76.57^{b}	83.47^{a}	0.050	0.001
Organic matter, %	80.17^{b}	79.54^{b}	80.48^{b}	86.96^{a}	0.044	<0.001
Crude protein, %	78.69^{b}	80.05^{b}	78.21^{b}	86.77^{a}	0.055	<0.001
Crude fat, %	95.81	95.19	95.75	95.69	0.014	0.582
Gross energy, %	81.15^{b}	80.55^{b}	81.10^{b}	87.03^{a}	0.042	<0.001
			Marker method			
Dry matter, %	72.67^{c}	73.78^{c}	74.05^{b}	81.65^{a}	0.023	<0.001
Organic matter, %	77.22^{b}	77.61^{b}	77.55^{b}	85.96^{a}	0.019	<0.001
Crude protein, %	75.59^{c}	78.15^{b}	75.01^{c}	85.77^{a}	0.031	<0.001
Crude fat, %	95.21	94.58	95.24	95.39	0.012	0.597
Gross energy, %	78.37^{b}	78.71^{b}	78.22^{b}	86.05^{a}	0.016	<0.001

Table 5.6. Concentrations of short-chain fatty acid (SCFA), branched-chain fatty acid (BCFA), and total fatty acids (SCFA + BCFA) production from the fresh fecal sample collected from the dogs fed diets with different soybean protein products expressed in a µmol/g of feces in dry matter basis.

Parameter,	SBM (n=12)	AMF (n=11)	FSBM (n=11)	SPI (n=11)	MSE	*P*-value
Fecal pH	5.60^{b}	5.48^{b}	5.64^{ab}	5.93^{a}	0.372	0.0058
Ammonia, µmol/g DM feces	100.4	114.7	115.1	132.6	48.40	0.4435
SCFA, µmol/g DM feces	463.5^{ab}	571.8^{a}	464.0^{ab}	403.2^{b}	162.37	0.0397
Acetate	254.8^{ab}	305.9^{a}	256.5^{ab}	222.2^{b}	86.45	0.0420[1]
Propionate	155.3^{ab}	201.8^{a}	163.8^{ab}	112.0^{b}	56.65	0.0017
Butyrate	52.73	63.69	42.56	69.03	34.509	0.1097
BCFA, µmol/g DM feces	11.10	12.38	10.56	13.92	5.378	0.3880
Isobutyrate	5.30	5.50	4.83	6.35	1.591	0.1315
Isovalerate	2.71^{ab}	2.70^{ab}	2.27^{b}	5.13^{a}	2.255	0.0171
Valerate	3.08	4.18	3.46	2.44	2.065	0.1177
Total, µmol/g DM feces	474.6^{ab}	583.9^{a}	474.4^{ab}	417.1^{b}	166.20	0.0486

[1]*P* value of AMF and SPI from Tukey-Kramer post hoc analysis.

Table 5.7. Least square means for short-chain fatty acid (SCFA) and branched-chain fatty acid (BCFA) production from the fresh fecal sample collected from the dogs fed diets different soybean protein products expressed as a percentage of total fatty acids.

Parameter	SBM (n=12)	AMF (n=11)	FSBM (n=11)	SPI (n=11)	MSE	*P* value
Acetate	54.67	52.31	54.25	54.05	0.045	0.6001
Propionate	31.87[a]	35.14[a]	34.17[a]	27.20[b]	0.449	＜0.0001
Butyrate	11.16[b]	10.44[b]	9.37[b]	15.50[a]	0.052	0.0034
Isobutyrate	1.26[ab]	0.98[b]	1.06[b]	1.52[a]	0.004	0.0046
Isovalerate	0.442[b]	0.415[b]	0.429[b]	1.182[a]	0.0037	＜0.0001
Valerate	0.705	0.728	0.736	0.586	0.0032	0.5461
SCFA[1]	97.58[a]	97.87[a]	97.77[a]	96.72[b]	0.007	0.0019
BCFA[2]	2.42[b]	2.13[b]	2.23[b]	3.28[a]	0.007	0.0019

[1]SCFA, short-chain fatty acids; sum of acetic acid, propionic acid, and butyric acid.
[2]BCFA, branched-chain fatty acids; sum of isobutyric acid, isovaleric acid, valeric acid.
[ab] Within a row, means without a common superscript differ ($P < 0.05$).

Table 5.8. Palatability assessment of diets containing different soybean protein products by dogs.

Diet A vs. B	First choice, n[1]		Intake ratio of diet A[2]	
	Day 1	Day 2	Day 1	Day 2
SBM vs. AMF	9	9	0.364	0.417
SBM vs. FSBM	11	18*	0.766*	0.803*
SBM vs. SPI	5*	13	0.372*	0.639

[1]First choice number of first visits to bowl with diet B can be obtained by 20-n.
[2]Intake ratio of diet A = average of intake (g) of diet A/total intake (g) of diets A + B.
*P-value was < 0.05.

Chapter 6 - Conclusions and Future Steps

This thesis evaluated two types of bioprocessed soybean meal products as a protein ingredient in extruded pet food: the enzyme-assisted fermented soybean meal (MEP) and the solid-state A. oryzae fermented soybean meal (F-SBM). Chapter 2 and 4 verified that both MEP and F-SBM had increased nutritional value in regard to CP content and methionine content per unit and decreased antinutritional factors per unit of dry matter. However, the nutrient digestibility in dogs and cats did not seem to be much affected when replacing traditional soybean meal with either bioprocessed soybean meal at up to 30% of the formulas. Such results may be attributed to the compensatory protein fermentation in the colon. It is worth investigating the apparent ileal digestibility instead of the ATTD of protein. Additionally, the dietary fiber content in the bioprocessed soybean meal should be carefully interpretated as the fungal biomass that formed during fermentation can play an important role on nutrient digestibility and colonic fermentation as seen in Chapter 3 and 5.

Processing can affect diet palatability to dogs and cats by altering kibble characteristics and nutrient interactions. Both the MEP and F-SBM promoted kibble expansion compared to traditional soybean meal. The optimal inclusion level of MEP in dog diet was 15% as supported by nutrient digestibility and palatability tests while that in cat food was 10% as supported by colonic fermentation and palatability tests. In context with palatability results in Chapter 5, the diet palatability seemed to have a non-linear relation with bioprocessed soybean meal inclusion level and relatively low level would be beneficial for palatability.

For further research, there is a need to keep looking for an efficient way of increasing methionine and other sulfur-containing amino acids per unit of soybean protein to promote utilization of soybean protein as a complete plant protein source. The potential switch of protein digestion between the small and large intestine due to inclusion of soybean meal or bioprocessed soybean meal needs further investigation to verify. If true, it may be significant for prescription diet application for those who need great protein supply but are not able to properly digest protein in the small intestine. Other potential benefits of biosynthesized compounds during soybean meal fermentation, such as antioxidants, and anti-inflammatories, are also worth exploring to add value to the fermented products. This thesis showed that palatability may be limiting factor for utilization of bioprocessed soybean meal. The causes and possible solutions also remain to be addressed.

The additional values of fermented soybean meal include improving consumer perception of low quality for soybean proteins and supporting the sustainability of the feed industry. In conclusion, the bioprocessed soybean meal can be included in extruded pet food as a complementary protein source without compromising nutrient digestibility, stool quality and palatability at moderate levels (10-15%) to support the well-being of companion animals.