Pulse-based Vegan Dog Food: Examination of Digestibility with or without Added Enzymes in Adult Dogs and Ileal Cannulated Pigs as a Model

by

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ABSTRACT

Demand for vegan dog foods is growing, yet little research has been conducted concerning the digestibility of these foods for dogs. The pulses and pulse protein concentrates that provide protein in these foods contain anti-nutritional factors (ANF), which may reduce nutrient digestibility. Addition of exogenous enzymes may help increase the digestibility of these foods, however, due to constraints on companion animal research, studies using dogs alone may not be able to accurately determine enzyme effectiveness.

For the present study two diets were formulated: a low protein diet (L) including mainly field pea and lentil flours, and a high protein diet (H) including mainly field pea and lentil protein concentrates. In chapter 2, ileal cannulated pigs were fed these diets either raw (R), extruded (E), extruded with enzyme added before extrusion (E+P), or enzymes added after extrusion (E+A). The enzyme blend included protease, phytase, cellulase and xylanase. Feces and digesta were collected to determine apparent total tract digestibility (ATTD) and standardized ileal digestibility (SID) of the diets. Extrusion alone increased ATTD of gross energy (GE) by 1% and SID total AA by 4% compared to R ($P < 0.05$). The addition of the enzyme blend after extrusion increased ATTD of crude protein (CP) and GE by 1%, ATTD of ash by 11-19% ($P < 0.05$), and increased SID of total AA by 2% ($P < 0.05$) compared to E. However, the addition of the enzyme blend before extrusion resulted in lower ATTD of organic matter (OM), CP, and GE, and lower SID of AA compared to E ($P < 0.05$), likely due to overheating during processing.

In chapter 3, dogs were fed the same formulations as controls which were extruded without enzymes (C), extruded with enzyme added before extrusion (P), or enzyme added after extrusion (A). Fecal samples were collected to measure ATTD, fecal metabolites and fecal

consistency. Unlike for pigs, ATTD of OM, CP, crude fat (EE), GE, or ash did not increase for dogs when enzymes were added after extrusion. However, the addition of enzymes after extrusion did increase concentrations of glucose, xylose, propionic and acetic acids in dog feces compared to C ($P < 0.05$), indicating the enzymes did hydrolyze some of the insoluble fiber in the diets leading to increased bacterial fermentation. The addition of the enzymes after extrusion also resulted in feces with higher moisture content than for C ($P < 0.05$), causing loose stools for the dogs. Addition of enzymes prior to extrusion resulted in ATTD of CP lower than C ($P < 0.05$). Although the ATTD values from pigs were greater than from the dogs, their results followed similar trends and comparison by regression analysis resulted in high R^2 values for GE, OM and CP ($R^2 > 0.7$).

In conclusion, extrusion and the addition of enzymes after extrusion increased the digestibility of pulse flour and protein concentrate based diets for growing pigs. The addition of enzymes after extrusion did not increase the digestibility of pulse flour and protein concentrate based diets for adult dogs, but did increase the concentrations of fecal metabolites indicating that the enzymes hydrolyzed some insoluble fiber. However, enzyme addition after extrusion also resulted in loose stools. Addition of enzymes before extrusion resulted in lower digestibility of the diets for both pigs and dogs. Overall, the addition of enzyme after extrusion can be used to increase the digestibility of pulse-based diets for growing pigs, and has some effect on dog intestinal microbiome and fecal quality. Comparison of digestibility values from the pigs and dogs resulted in high R^2 values, indicating that pigs can be used as a model for dog food digestibility studies.

PREFACE

This thesis was written in manuscript format with 4 chapters, including a literature review, manuscripts 1 and 2, and a general discussion. All chapters were written according to the guidelines for the Journal of Animal Science. Animal use in chapter 2 was reviewed and approved by the University of Alberta Animal Care and Use Committee for Livestock. Animal use in chapter 3 was reviewed and approved by the University of Guelph Animal Care and Use Committee for companion animals.

Manuscript 1 will be submitted to Journal of Animal Science as Whitney P. G. Van Straten, Li Fang Wang, A. Kate Shoveller, Eduardo Beltranena, Thava Vasanthan, and Ruurd T. Zijlstra, "Extrusion and enzyme addition enhanced ileal and total tract digestibility of pulse grain-based diets fed to growing pigs". Whitney P. G. Van Straten was responsible for investigation, sample processing, partial chemical analysis, and writing of the original draft. Li Fang Wang performed statistical analysis and validation, data curation, manuscript review and editing. A. Kate Shoveller was involved with funding acquisition, project conceptualization, methodology, and manuscript review and editing. Eduardo Beltranena was involved with resources acquisition, manuscript review and editing. Thava Vasanthan was responsible for funding acquisition, resource acquisition, conceptualization, methodology, and manuscript review and editing. Ruurd T. Zijlstra was responsible for funding acquisition, project administration, and manuscript review and editing.

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from a pig model". Whitney P. G. Van Straten was responsible for investigation, sample processing, chemical analysis, and writing of the original draft. A. Kate Shoveller was responsible for funding acquisition, project administration, conceptualization, methodology, and manuscript review and editing. Li Fang Wang performed statistical analysis and validation, data curation, manuscript review and editing. Eduardo Beltranena was involved with resources acquisition, manuscript review and editing. Thava Vasanthan was responsible for funding acquisition, resource acquisition, conceptualization, methodology, and manuscript review and editing. Ruurd T. Zijlstra was involved with funding acquisition and manuscript review and editing.

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AA	Amino acid
ADF	Acid detergent fiber
ANF	Anti-nutritional factor
ATTD	Apparent total tract digestibility
AID	Apparent ileal digestibility
BW	Body weight
CP	Crude protein
DE	Digestible energy
DM	Dry matter
EE	Crude fat
GE	Gross energy
GET	Gastric emptying time
IDF	Insoluble dietary fiber
NDF	Neutral detergent fiber
NE	Net energy
OM	Organic matter
SDF	Soluble dietary fiber
SID	Standardized ileal digestibility
SITT	Small intestinal transit time

CHAPTER 1. LITERATURE REVIEW

1.1   Introduction

Pet food production is a large global industry, with North America alone producing 10.6 million metric tonnes of pet food in 2021 (Wall, 2022). In Canada approximately 50 percent of households own at least one dog or cat, and in 2017 Canada was the 6th largest pet food market in the world (Government of Alberta, 2019). One of the trends in this billion-dollar industry in the past few years, has been the increasing demand for specialty pet foods, including vegetarian, and vegan diets (Pet Food Industry, 2021). As such, research to understand the effects of plant-based diets has also increased.

The increased popularity of vegetarian and vegan pet foods has largely been due to vegetarian and vegan owners wanting to feed their pets in a way that coincides with their own lifestyle (Dodd et al., 2019), and due to the belief that plant-based diets are more environmentally friendly (Vanderhoydonck, 2022). Pet food producers have risen to the demand of consumers and started offering vegetarian and vegan pet food options, of which the majority are dry kibble foods. Although vegan pet food production is small compared to the entire pet food market, with an estimated global production of only 9.3 million USD in 2021, demand is increasing and the sector is predicted to grow to 15.6 million USD by 2028 (Pet Food Industry, 2021). These meatless diets rely heavily on the use of pulses, the dried seeds of non-oilseed legume plants, including lentils, beans, chickpeas, and field peas. Pulses contain more protein than cereal grains, which is especially beneficial for plant-based pet foods that contain little to no animal protein. Pulses also provide starch that is necessary to achieve the desired kibble quality from pet food extrusion.

Unfortunately, the use of pulses in pet food has garnered a bad reputation in the last few years, much of which can be attributed to the United States of America's Food and Drug Administration (FDA) investigation regarding a possible correlation between pulses in pet food and nutritionally mediated dilated cardiomyopathy (DCM) in dogs (U.S. Food and Drug Administration, 2019). Although a retrospective study did not detect a correlation between the rise in pulse inclusion in pet food and the number of DMC cases reported (Quest et al. 2022), the FDA investigation has nonetheless damaged veterinary professional and public opinions regarding the use of pulses in dog foods. A concern surrounding the use of pulses in pet food is their content of anti-nutritional factors. Pulses such as peas and chickpeas contain relatively high levels of the anti-nutritional factors (ANF) including phytate, tannins, and trypsin inhibitors that can inhibit the activity of digestive enzymes and reduce nutrient absorption if consumed in large quantities (Adamidou et al. 2011). In addition, pulses also contain large amounts of insoluble fiber, which consists mainly of cellulose and other cell wall structures that are not digestible for monogastric species, such as dogs, pigs, and humans (de-Oliveira et al., 2012). While insoluble fiber can help maintain bowel regularity and has benefits for glycemic control (Kimmel et al., 2000, McRorie and McKeown, 2017), insoluble fiber is also considered an ANF, with the potential to reduce the digestibility and absorption of protein, starch, and minerals, particularly in plant-based pet food (Wehrmaker et al., 2022). This has led many veterinary professionals and pet owners to question the safety and suitability of plant-based pet foods.

High temperatures during kibble extrusion help to reduce the effects of trypsin inhibitors and phytate (Adamidou et al., 2011). However, results regarding the effects of extrusion on insoluble fiber are mixed, with some studies showing IDF reduction with extrusion of pulses while others show no changes (Berrios, et al., 2010, Adamidou et al., 2011). Effects on IDF content may be

dependent on the conditions of extrusion, Therefore, a need exists for new technologies to reduce the effects of ANF, particularity insoluble fiber, in pulse-based pet food. One possible solution is the addition of exogenous enzymes.

Exogenous enzymes such as phytase, cellulase, xylanase, and protease have been researched for use in increasing the digestibility livestock feeds, with results showing increases in protein digestibility and animal growth (Zhao et al., 2020, Zouaoui, et al., 2018, Lee et al., 2020). However, few studies have applied this technology to pet food production, especially pulse-based pet foods. This review will discuss the nutritional value and digestibility of pulses and the role that ANF play in pulse protein digestibility. Additionally, available research concerning endogenous enzymes and their use in livestock feed and dog foods will be examined to determine if exogenous enzymes can be used to increase the nutritional quality of pulse-based pet foods. The research requirements to conduct studies with dogs or cats is more restrictive than for agriculture animals and often requires data to be generated in translation animal models. As such, a discussion on the use of animal models such as swine and roosters for studying pet food digestibility in place of dogs is also included.

1.2   Nutrition and digestibility

A wide variety of whole pulse grain and cereal grain ingredients are commonly used in commercial extruded pet food and these have varying chemical compositions (Table 1). Rice and corn are often used as sources of starch and micronutrients in extruded pet foods, however these ingredients provide a small amount of protein, making them of limited usefulness in plant-based extruded pet food. Hulled barley is also used as a starch source in traditional extruded pet food. While barley contains approximately 5% more crude protein than rice and corn, this cereal grain

still contains 37% less crude protein than chickpeas and 50% less protein than lentils. The high protein content of pulses compared to cereal grains, makes them a desirable protein source for plant-based vegetarian and vegan extruded dog food. The use of pulses and pulse protein concentrates can help achieve or exceed the minimum 18% protein regulatory requirement for adult dog diets (Association of American Feed Control Officials, 2014) without the use of animal protein ingredients. However, the high protein content in these ingredients is accompanied by higher amounts of fiber, with lentils having 28 and 47% more neutral detergent fiber (NDF) than barley and corn, respectively. This type of fiber includes insoluble structural plant fibers cellulose, hemicellulose, and lignin. Due to their insoluble nature, these fibers have limited fermentability and do not act as prebiotics.

Table 2 compares the essential amino acid profiles of these same ingredients. Due to their greater crude protein content, pulses can provide more essential amino acids compared to cereal grains. While the limiting amino acid in cereals is lysine, the sulfur-containing amino acids methionine and cysteine, and the aromatic amino acid tryptophan are the main limiting amino acids in pulses. This is reflected in the amino acid content of the ingredients, as the methionine content of the pulse ingredients is similar to the cereal grains despite the pulse's greater protein content. These shortcomings can be accounted for during pet food formulation through the addition of amino acid supplements or combining with other ingredients. Overall, the protein content of pulses makes them a desirable ingredient for plant-based dog foods, however this may be accompanied by some negative effects on digestibility.

The digestibility of pulses in dog foods has previously been examined. One such study compared the apparent total tract digestibility (ATTD) of extruded dog diets containing 49% or more of lentils, peas, brewer's rice or corn. The ATTD of crude protein was 3-10% lower for the

pea or lentil diets than the rice or corn diets (Carciofi et al., 2008). However, dietary inclusion of the test ingredient varied, with the pulse-based diets containing 66% peas or 70% lentils, while the cereal grain-based diets included only 49% brewer's rice or 54% corn. While this does indicate that dog diets high in pulses, such as is in vegan and vegetarian diets, may have reduced crude protein digestibility, it is also important to acknowledge the potential inaccuracies of only examining ATTD. For ATTD, dog feces are collected and analyzed to determine digestibility after the food has passed through the entire digestive tract. This method of determining food digestibility does not consider that fermentation that occurs in the animal's large intestine after digestion and absorption in the small intestine. Bacterial fermentation of nutrients and soluble fiber that are not absorbed by the dog can lead to incorrect estimates of nutrients digested and absorbed by the dog (Hendriks et al. 2013). Although the results of this comparison study may not be entirely accurate, the ANF content of pulses has been quantified and the mechanisms of ANF effects on food have been heavily studied.

1.3   Anti-nutritional factors common in pet food pulses

1.3.1   Trypsin inhibitor

As mentioned, several ANF exist in pulses; Table 3 compares the content of these ANF in some pulse ingredients commonly used in pet food. One of the main ANF of concern in pulses is trypsin inhibitors. Trypsin inhibitors are proteins that bind to the active site of the trypsin protease enzyme and prevent the enzyme from carrying out its function in protein digestion (Blow et al., 1974). As shown in table 3, chickpea, a relatively common ingredient in cereal grain free pet food, has the highest trypsin inhibitor activity (TIA) of the listed pulses, with more

than 5 times the TIA of field pea and lentil. Trypsin inhibitors are proteins, as such TIA is increased in pea protein concentrates that can contain double the TIA of field pea flour. Trypsin inhibitor content of pulse ingredients may reduce digestibility of protein in pet food, thereby reducing the bioavailability of amino acids for animals and potentially leading to negative health outcomes. This is especially a concern if the pulse ingredients are used at high dietary inclusion such as in vegan pet foods, which often contain less protein than traditional dog foods.

Heat processing, such as extrusion, can reduce TIA in pet food. While extrusion of peas at 140°C can reduce TIA by 84% (van der Poel et al., 1992), the temperatures required to reduce or eliminate TIA in pulses and soybean can also reduce chemically available lysine content by the formation of Maillard reaction products. The Maillard reaction occurs when proteins interact with reducing sugars during heat processing, and results in the formation of Maillard reaction products M*N*-ε-2-furoylmethyl-L-lysine and *N*-ε-(carboxymethyl) lysine (Hofmann et al., 2020). This crosslinking with lysine hinders hydrolysis of proteins by digestive enzymes, reducing protein digestibility. While studies in humans have found that up to 10% of Maillard reaction products may be absorbed and can be detected in serum, these bound lysine compounds are not bioavailable so cannot be used in metabolic processes. Instead, they are excreted in urine or deposited in body organs such as the liver and kidneys (van Rooijen et al., 2013). Depending on moisture content used, extrusion temperatures of 140°C or higher can reduce available lysine in peas by 16% (Hendriks et al. 1994). Additionally, heat processing can cause oxidation of heat sensitive vitamins and the sulfur containing amino acids methionine and cystine, further reducing nutrient bioavailability (Tran et al., 2008). This highlights the need for methods to increase nutrient digestibility and bioavailability without causing heat damage.

1.3.2 Phenolic compounds

Phenolic compounds are another ANF that is common in pulse grain ingredients. Over 8,000 different phenolic compounds have been identified with varying chemical structures, but all containing an aromatic ring and one or more hydroxyl groups (Soto-Vaca et al., 2012). This group of compounds includes benzoic acid derivatives, cinnamic acid, flavonoids, and tannins. Phenolic compounds have many potential health benefits including the prevention of certain cancers and bacterial infections, much of which is associated with their antioxidant capabilities (Soto-Vaca et al., 2012). Some phenolic compounds in peas have strong antioxidant activity (Troszyńska et al., 2002), however phenols have poor absorption from the intestines (Soto-Vaca et al., 2012) and may reduce nutrient digestibility.

Phenolic compounds, particularly plant polyphenols, can interact with digestive enzymes produced by animals and reduce their activity (Rohn, Rawel and Kroll, 2002). Binding of phenolic compounds to the free amino and thiol groups of digestive enzymes such as amylase, trypsin, pepsin, and lipase, can reduce digestibility of saccharides, lipids, and proteins (Rohn et al., 2002; He et al., 2007). Although reduced digestibility is sometimes seen as a positive for overweight humans, these effects could impact the health of companion animals.

In Table 3, lentils contain the most total phenolics, which could be a concern for the pet food industry due to the wide use of lentils in grain-free and plant-based extruded dog food. Unlike trypsin inhibitors, the content of phenolic compounds does not increase during pulse protein concentrate production from pulse flours, likely due to the location of phenolic compounds in the seed. In pulses such as peas, phenolic compounds are mainly concentrated in the hull/seed coat, which is removed during the production of pea flour and protein concentrate (Dueñas et al.,

2004, Troszyńska et al., 2002). The phenolic compounds located in the cotyledon of the seed end up in the course, starch fraction after air classification production of protein concentrates (Gómez-Caravaca et al., 2015). As with trypsin inhibitors, phenolic compounds are damaged by heat during extrusion (Zeng et al., 2016), which reduces their activity and may help prevent their anti-nutritional effects.

1.3.3 Phytate

Phytate is the main storage form of phosphorus in plant material, making up 60-80% of phosphorus in seeds (Cowieson et al., 2017). As such, phytate's main effect is on mineral availability; however, phytate (phytic acid) can also reduce the digestion of protein. Monogastric mammals do not produce the phytase enzyme needed to break down phytate (Moita et al., 2022). Due to its insoluble nature, phytate has poor intestinal absorption, causing reduced bioavailability of phosphorus for animals (Torre et al., 1991). Phytate also commonly forms stable insoluble chelates with iron, zinc, copper, magnesium, and calcium, thereby reducing the bioavailability of these minerals.

Phytate can also form complexes with proteins. At low pH, basic amino acids arginine, histidine and lysine become positively charged and form binary complexes with negatively charged phytate (Selle at al., 2012). The low pH of the monogastric stomach exacerbates the formation of these complexes that prevent digestion by pepsin. In the small intestine, where pH rises, these binary complexes dissociate. However, pH above the isoelectric point of proteins is ideal for the formation of ternary phytate-protein complexes (Selle at al., 2012). These complexes involve Ca+ as a bridge to link negatively charged amino acids and phytate, causing

further inhibition of protein hydrolysis. The isoelectric point of pulse proteins is at a relatively low pH of 4 to 5 (Shevkani et al., 2019) compared to corn zein protein that has an isoelectric point at pH of 6.2 (Elzoghby et al., 2015). The average pH of the canine small intestine is 7.3 (Lui et al., 1986), as such, the low isoelectric point of pulse proteins may cause a propensity for the formation of ternary phytate - protein complexes in the dog intestinal tract. However, little research exists regarding the effects of these specific complexes on pulse protein digestibility.

The comparison of the 3 whole pulse ingredients in table 3 indicates that chickpeas contain the most phytate, and therefore would have the greatest potential decrease in protein digestibility and amino acid bioavailability caused by phytate. Lentils likely have the least phytate effect due to their comparatively low phytate content. Phytate becomes concentrated through the production of pulse protein concentrates, as pea protein concentrate contains over twice the amount as the native pea flour. This concentration of phytic acid in pulse protein concentrates can further reduce nutrient digestibility compared to pulse flours (Carnovale et al., 1988)

Although phytic acid is heat labile, degradation may only occur at temperature above 150°C (Daneluti and Matos. 2013). Normal extrusion temperatures used in pet food processing are 80-200°C, with higher temperatures during processing resulting in greater Maillard reactions and reduced vitamin and amino acids availability, as previously discussed. Again, this highlights the need for alternative methods to increase digestibility of pulse ingredients.

1.3.4 Fiber

As previously discussed, pulses contain more fiber than cereal grains, with lentils having 28 times more insoluble fiber than rice. Dogs and cats do not naturally produce the enzymes

necessary to digest fibrous plant materials, such as cellulose, lignin, pectin and gums. While

passing through the digestive tract, soluble fibers including psyllium, inulin and pectin, attract

water, increasing digesta viscosity, reducing transit time and decreases enzyme access to

nutrients, thereby reducing nutrient digestibility (Silvio et al., 2000). However, in the large

intestine, soluble fibers act as a prebiotic, providing an energy source for bacterial fermentation

(Biagi et al., 2010) and producing short chain fatty acids which can have benefits for canine

intestinal health (Pilla and Suchodolski, 2020). Certain soluble fibers, including pectin, improve

the intestinal health of dogs by increasing the population size of beneficial *lactobacilli* and

bifidobacteria, and reducing the population of harmful *clostridium perfringens* (Biagi et al.,

2010). While these effects make soluble fiber very beneficial for gut health, other types of fiber

do not have the same benefits.

Unlike soluble fiber, insoluble cell wall fibers such as cellulose, hemicellulose and lignin, do

not provide an energy source for fermentation by intestinal bacteria and therefore do not have the

same benefits for intestinal health. Coarse insoluble fiber can aid with regularity by irritating the

intestinal mucosa to stimulate water and mucus secretion, however, insoluble fiber also has

potential to decrease nutrient digestibility (McRorie and McKeown, 2017). As per table 3,

approximately 75% of dehulled chickpea dietary fiber, and 81% of lentil dietary fiber, is

insoluble. The intact cell walls of pulse grains are largely impermeable to digestive enzymes

(Dhital et al., 2016). Therefore, encapsulation of starch and protein within pulse cotyledon cells

is the main barrier to pulse grain macronutrient digestion (Brummer et al., 2015). The anti-

nutritional effects of insoluble fiber encapsulation can be reduced by milling and cooking to

break apart cell wall structures (Dhital et al., 2016), however this does not reduce the insoluble

fiber content of the ingredient. The presence of insoluble fiber even after milling can still have negative effects on digestibility.

The low glycemic index of pulses caused by lower digestibility due to fiber and resistant starch could benefit animals with diabetes. Studies comparing glycemic response in dogs found that a lentil-based diet had a glycemic index of 85.5 compared to 95.7 for a rice-based diet (Quilliam et al., 2021). Although this can benefit overweight and diabetic dogs, reduced digestibility of nutrients due to high insoluble fiber content may be detrimental in plant-based diets that may already have relatively low protein content compared to traditional dog food.

1.4   Enzymes for Increasing Digestibility

Although ANF could reduce pet food nutrient digestibility, exogenous enzymes may be used to ameliorate these effects. Exogenous enzymes are enzymes produced by bacterial or fungal synthesis, that can be added to animal diets to aid with the hydrolysis of nutrients and reduce the effects of ANF (Facchini et al., 2011). Fiber degrading enzymes such as cellulase and xylanase, along with phytase and protease, may be beneficial for use in pulse-based extruded dog foods.

1.4.1   Fiber Hydrolyzing enzymes

Fiber hydrolyzing enzymes are categorized as carbohydrase enzymes and include a variety of different cellulase, xylanase, and β -glucanase enzymes. These enzymes break down the β, 1-4 bonds of insoluble and indigestible plant fibers, converting them into soluble polysaccharides

that may have fewer negative effects on nutrient digestibility. Cellulase includes 3 different types of enzymes, endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase and β-glucosidase that carry out different reactions to hydrolyze cellulose (Ejaz et al., 2021). These enzymes consist of a single peptide chain with two domains, one that links to the cellulose strand while the other catalyzes the reaction. Endo-β-1,4- D glucanase, also known as endocellulase, binds and hydrolyses the cellulose β, 1-4 bonds at random intervals, resulting in oligomers of various sizes. Exo-1,4-β-D-glucanase, also known as exocellulase, binds to the non-reducing end of the cellulose and hydrolyses it into cellobiose disaccharides and glucose (Ejaz et al., 2021). Finally, β -glucanase further hydrolysis cellobiose into glucose from the reducing end. Together these cellulase enzymes may degrade cellulose completely into glucose, thereby preventing some of the antinutritional effects of the insoluble fiber.

Xylanase is another enzyme available for use as an exogenous fiber degrading enzyme. Endo-1,4-β-xylanases, also known as endoxylanases, are a group of enzymes that hydrolyses the β-1,4-glycosidic linkages in the xylan backbone of hemicellulose (Ustinov et al., 2008). Like endocellulase, endoxylanase acts randomly, hydrolyzing bonds and releasing oligosaccharides of various sizes (Puls, 1997). Endoxylanases are divided into 2 families, F and G, with F endoxylanase producing shorter oligosaccharides than G endoxylanase. Different plant species have differences in hemicellulose structure that can affect the activity of xylanase enzymes and the products of hydrolysis (Puls, 1997). While endoxylanases cannot reduce hemicellulose completely into simple sugars, the xylooligosaccharides and xylose produced by these enzymes are soluble and may have less negative impact on digestibility of nutrients in vegan dog food. Soluble xylooligosaccharides and xylose derived from endoxylanase are also fermentable by gut

bacteria, including beneficial lactobacillus species, as such they can act as a prebiotic and may have positive effects on gut health (Singh et al., 2021).

Studies using livestock have observed that cellulase and xylanase enzymes may increase feed digestibility. A blend of xylanase, cellulase and β-glucanase added to growing swine diets containing 25% maize bran, sugar beet pulp or soybean hulls, increased apparent total tract digestibility (ATTD) of gross energy by 2%, and apparent ileal digestibility (AID) of crude protein by 1-3% (Zhao et al., 2020). Studies adding fiber-degrading enzyme blends to wheat and soybean meal-based broiler chicken diets resulted in similar increases (Cozannet et al., 2017). As well as increased production of short chain fatty acids (SCFAs) and reduced populations of pathogenic bacteria for broilers fed xylanase or cellulase with diets containing palm kernel meal (Sharmila et al. 2014). Higher SCFA concentrations including increased propionate, acetate and butyrate indicate increased bacterial fermentation in the large intestine, which consequently can improve gut health and reduce pathogenic bacteria loads (Sharmila et al. 2014).

Although several studies have been successful at using fiber degrading enzymes to increase digestibility of livestock feed, few involve diets containing pulses. A summary of livestock studies examining enzyme use in feeds containing pulses is presented in table 4. One study examined the effects of a blend of cellulase, xylanase and amylase added to broiler chickens fed diets containing 50% pea meal from one of five cultivars (Cowieson, et al., 2003). However, only one of the five pea cultivars showed a significant increase in ATTD of DM, with a 9% increase for the enzyme supplemented diet compared to the control. Similarly, another study involving broilers fed a 30% pea diet found no increase in AID of starch or protein after addition of a multi enzyme blend of fibrolytic enzymes (Meng and Slominski, 2005). In studies involving growing pigs there were no increases for ATTD of DM, CP, or GE after an enzyme blend

containing cellulase, xylanase, galactosidase and amylase was added to diets containing 36%

hulled or dehulled peas (Thacker and Racz, 2001). While all these studies point towards fiber

degrading enzymes being ineffective when used in diets containing pulses, it is important to note

that these studies only examined apparent digestibility. Apparent digestibility does not consider

endogenous losses, the loss of amino acids from the animal due to digestive enzymes and

sloughed epithelial cells. As previously discussed, total tract digestibility does not consider

fermentation in the large intestine. If endogenous losses are not considered, it may prevent

increases in nutrient digestibility from being observed, as endogenous losses and use of nutrient

by bacteria cannot be differentiated and therefore may cause over or underestimation of nutrient

digestibility.

1.4.2   *Phytase enzymes*

Phytase enzymes have been known for over 100 years, with the first mention of these

enzyme in 1907 (Lei et al., 2013). These enzymes are naturally occurring in many

microorganisms including fungi; however, the most common source of phytase for use in animal

feed is produced through fermentation by a variety of different bacterial species (Thorsen et al.,

2021). Phytase is one of the most common enzymes added to monogastric livestock feeds to

increase phosphorus digestibility.

Several different classes of phytase enzyme are used in animal feeds. Class 1 phytase

includes histidine acid phytases (HAP) (Singh et al., 2018). This class of phytase enzyme is most

effective in acidic environments, binding to positively charged amino acid groups to produce

myo-inositol monophosphate by hydrolyzing five of the six phosphate groups from phytate

(Singh et al., 2018). Class 1 phytase has broad specificity and can hydrolyze phytate molecules with varying numbers of phosphorus groups, however HAP are not thermostable and are destroyed at temperatures above 60°C. Because this class of phytase is heat labile and works best at acidic pH, HAPs works well when added as a supplement to animal feeds.

Class 2 phytase enzymes includes alkaline phytase enzymes (Singh et al., 2018). Different from HAP, these phytase enzymes work best at alkaline pH and have more specific action. Alkaline phytases bind to negatively charged amino acid groups and are unable to hydrolyze phytate molecules with three or less phosphorus groups (Singh et al., 2018). These types of phytase enzymes are more heat stable and able to withstand temperatures up to 95°c. Because of their greater heat resistance and better action at alkaline pH, these enzymes have the potential to be added to animal feed during pelleting or other processes prior to feeding.

Both classes of phytase enzyme work to increase feed digestibility by hydrolyzing phytate and freeing phosphorus for better availability for monogastric animals. This hydrolysis of phytate can also help increase protein digestibility. As phosphate groups are hydrolyzed from phytate, it loses its ability to bind proteins (Yu et al., 2012). Phytase molecules with 6 phosphorus groups (IP6) have the greatest affinity for protein binding, binding capacity decreases when IP6 is hydrolyzed into IP5, and protein binding ability is lost completely with further hydrolysis into IP1-4. In this way, phytase enzymes can reduce protein binding by phytate and increase protein digestibility.

These positive effects of phytase are well researched, with livestock studies consistently observing benefits of phytase supplementation. A meta-analysis of 34 papers relating to phytase addition to swine diets, found a strong positive association (R^2=0.85-0.99) between increased

AID of amino acids and phytase supplementation (Zouaoui, et al., 2018). Similar results have been achieved with broiler chickens. One such study involved broilers fed diets of corn and soybean meal with 20% rapeseed meal or sunflower meal, with or without a phytase supplement (Siegert et al., 2019). The AID of crude protein and all essential amino acids was 6% higher for phytase-supplemented diets than controls.

Unlike the fiber degrading enzymes, studies involving pulse-based diets for livestock also show positive effects of phytase supplementation (Table 4). For growing pig diets containing low phytate or normal phytate field pea as the only protein source, the addition of a phytase supplement increased standardized ileal digestibility (SID) of arginine, leucine, isoleucine, phenylalanine and valine compared to controls (Kahindi et al., 2015). While few studies examining protein digestibility have been conducted using only phytase added to pulse-based livestock diets, a growth performance study involving broiler turkeys fed diets containing 24% raw chickpea meal did observe increased body weight gain and feed efficiency when phytase was added to the diets (Ciurescu at al., 2020). Increases in AID of dry matter and phosphorus have also been achieved in broiler chickens when diets containing 12-35% black eyed peas were supplemented with phytase enzymes (Iyayi ae al., 2013). Together these studies indicate that phytase enzymes can be effective when used in diets containing pulses.

1.4.3   Protease enzyme

As their name suggests, protease enzymes are designed to hydrolyze proteins. Although the goal of these enzymes is not to reduce ANF, protease enzymes are often included in enzyme blends for livestock feed to aid protein digestion. While there are several different types of

protease enzymes, including acidic proteases derived from fungi, and neutral proteases derived from plants (Razzaq et al., 2019), protease enzymes used in animal feed are typically alkaline proteases from bacterial origins. Bacterial produced protease enzymes include chymosin, pepsin aminopeptidase, chymotrypsin, and several other types of protease enzymes (Velázquez-De Lucio et al., 2021). These enzymes work by hydrolyzing peptide bonds, degrading proteins into smaller peptides and free amino acids. These exogenous protease enzymes may increase protein digestibility by hydrolyzing proteins into smaller peptides that are more quickly digested in the animal.

Several studies in livestock have shown the effectiveness of protease enzymes at increasing digestibility of feeds containing a variety of ingredients. For corn and soybean-based grower-finisher pig diets, the addition of protease enzymes increased average daily gain by 0.3-0.5 kg/day and ATTD of crude protein by 7-9% compared to controls (Lee et al., 2020). Broilers fed corn and soybean-based diets with protease enzyme addition have also demonstrated the enzyme's effectiveness. For diets supplemented with 10,000 phytase units per kg there was a 4% increase in AID of dry matter, and 5% increase in AID of protein (Borda-Molina et al., 2019). Meta-analysis of 25 studies involving swine and poultry feed diets supplemented with protease concluded that protease supplementation has an overall positive effect on AID of protein, with a mean 4.5% increase in apparent ileal digestibility of lysine, cystine, methionine and threonine (Cowieson and Roos, 2013). However, the effectiveness of the protease was dependent on the inherent amino acid digestibility of the test diets used, with the greatest increase in AID being when control diet AID of amino acids was less than 70%.

When examining studies using diets containing pulses (Table 4), protease addition has shown inconsistent results. In one study involving broilers fed diets containing 10, 20 or 30% of protein

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provided by peas, addition of protease enzyme did not result in any increase in AID of amino acids (Boroojeni et al., 2017). However, other studies have achieved promising results with increased SID of essential amino acids when protease enzyme was added to broiler chicken diets containing 71-81% field pea (Szczurek and Świątkiewicz, 2020). Increased AID of several amino acids was also achieved in a study with broiler chickens fed diets containing 30% field pea, faba bean or lupin (Hejdysz et al., 2020). However, in this study the effects of the enzyme were dependent on the type of pulse in the diet. While the protease supplementation of the field pea and faba bean diets resulted in increased AID of some essential amino acids, the enzyme supplementation had less effect on the lupin diet. These differences among studies could be explained by differences in methods. In the study by Boroojeni et al. (2017), only the peas in the diets were treated with the enzyme by mixing pea flour with water and the enzyme blend then incubating the mixture at 30 for 24 hours, which is different from the other two studies that included the protease enzyme dry mixed into the entire diet. Mixing the enzyme into the entire diet means that the results may be due in part to the enzyme's effects on the other ingredients in the diet; however, these studies do still indicate that the addition of an enzyme supplement can increase protein digestibility of livestock diets containing pulses.

1.5   Available research using enzymes in dog foods

Despite these enzymes being commercially available for use in livestock feed, little information is available regarding their use in pet foods. Studies that are available using enzymes in dog food have given mixed results and none of these studies include pulses. However, by

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looking at these studies, determining whether enzyme addition could be beneficial in pulse-based dog foods might be possible.

One such study looked at the addition of an enzyme blend to dog food containing different levels of non-starch polysaccharides (Twomey et al., 2003; Table 5). This study used mixed breed dogs fed extruded barley and wheat-based diets containing 11, 16, or 20 grams of non-starch polysaccharides (NSP)/ kg with or without a solution fiber and starch degrading enzymes sprayed on the food at feeding. The ATTD of CP for the food with 20 g/kg NSP was 7% lower than the food containing 11g/kg NSP. The addition of the enzyme blend increased ATTD of CP of the 20g/kg NSP diet by 6%. This increase in digestibility was attributed to the enzymes ability to hydrolyze the added NSP into smaller molecules that have less water holding capacity, and therefore would not cause the increased digesta viscosity that is associated with a decrease in nutrient digestibility (Choct and Annison, 1992).

Although the study by Twomey et al. indicated a possible benefit of enzyme additions, other studies examining enzyme inclusion in dog food have not found the same positive results. Another study using enzymes in dog food examined extruded kibble diets that contained 25% wheat bran resulting in 14.7% total dietary fiber (Sa et al., 2013). Adult beagle dogs were fed these diets that had a blend of enzymes (β -glucanase, cellulase, xylanase, phytase and glucoamylase) added before extrusion, or as a coating after extrusion. The results of this study showed no increase in digestibility of nutrients for either of the enzyme treatments; however, the amount of enzyme used in this study was much lower than in the previous study by Twomey et al (2003). While the study by Twomey et al. used 340 units/kg xylanase and 30 U/kg of cellulase, the study by Sa et al., (2013) only used 16 U/kg xylanase, 1.5 U/kg cellulase and 4.5 U/kg β-glucanase. The lower dose of insoluble fiber degrading enzymes used by Sa et al. (2003)

may not have been enough to create an increase in digestibility. The addition of enzymes prior to extrusion is also unlikely to increase digestibility because heat during extrusion would inactivate the enzymes before they are able to act on the chemical matrices of the ingredients.

Two studies have been conducted using enzymes in extruded dog kibble diets containing dried distillers' grains with solubles (DDGS). In the first, beagles were fed diets with 0, 60, 120 or 180 g/kg of DDGS with or without a coating of 80,000 U/kg xylanase added after extrusion (Silva et al.,2016). The addition of DDGS without enzymes decreased ATTD of DM, OM, CP and GE due to the higher fiber content of the DDGS. However, the addition of the xylanase enzyme increased ATTD of DM, OM, CP, and GE compared to diets without the enzyme, especially for the diets with the highest inclusion of DDGS (120 and 180g/kg). Despite these promising results with xylanase and DDGS by Silva et al. (2016), the second study by Risolia et al. (2019) did not find these same positive results. Risolia et al. (2019) fed beagles kibble diets with 200 g/kg DDGS with xylanase (32,000 U/kg) and or protease (425 U/kg) mixed with soybean oil sprayed onto the kibble after extrusion. The addition of DDGS reduced ATTD of DM, OM, CP, and GE, however, the addition of the enzymes did not affect nutrient digestibility.

Finally, one study has examined the use of enzymes with kibble containing mostly corn and soybean meal (Machado et al., 2021). For this study beagles were fed the diet with or without a coating of phytase, amylase, β-glucanase and mannanase after extrusion. While enzyme addition did not increase ATTD of nutrients, the diet with added enzymes did result in increased fecal SCFA concentrations. The increase in fecal SCFA content indicates successful hydrolysis of non-starch polysaccharides by the exogenous enzymes added to the diet. As discussed earlier, increased microbial fermentation in the large intestine may prevent differences in ATTD from being detected.

Together these results indicate that enzymes may increase digestibility of nutrients in kibble containing high fiber ingredients. However, the effectiveness of the enzymes may depend on the diet ingredients, concentration of enzymes used, and the application method. There are no studies currently available that have applied enzymes to vegan or grain free diets that contain high quantities of pulses. As such there is a knowledge gap for which research is needed. However, since the research requirements to conduct studies with dogs are more restrictive than for agriculture animals, these future studies may need to use translation animal models.

1.6   Comparing animal models for canine digestibility studies

Although canine nutrition research using laboratory dogs is valuable for improving our knowledge of canine digestion and nutrient utilization, the use of dogs in laboratory settings can be controversial. As companion animals, the welfare of research dogs can be a concern for the public who often view dogs as family members (Clement, 2011). Due to the public's proximity to dogs in everyday life they develop ideas of what they think good dog husbandry looks like. While regulations are in place for the use of dogs in research to ensure their basic needs are being met (National Research council, 2010), the need for reduced variability in research can lead to controlled environments that may look different from what the public may consider to be suitable husbandry. Public perceptions of laboratory dogs can also be influenced through social media campaigns by animal rights organizations who oppose the use of animals for research (Ormandy and Schuppli, 2014). As concerns over laboratory companion animal welfare grow, the use of dogs in research may become difficult. As such, using livestock species as models for

canine nutrition research may become necessary, however the effectiveness of these models first needs to be assessed.

1.6.1   Cecectomized roosters

Cecectomized roosters are male chickens that are subjected to surgery to remove the cecum. The ceca in birds is where the majority of bacterial fermentation occurs (Mansano et al., 2019). Removal of the ceca allows for collection of digesta directly from the bird's small intestine, decreasing the interference caused by bacterial fermentation. This allows the determination of AID and SID. The AID does not consider the endogenous losses by the animal, so is not as accurate of a measurement of the animal's digestion and absorption of nutrients compared to SID. The SID is typically calculated from AID by feeding the animal a diet devoid of nitrogen to measure basal endogenous nitrogen losses from the animal so that basal endogenous protein losses can be accounted for. Ileal cannulated dogs often have postoperative complications such as abscess, and skin ulceration due to leakage of caustic digesta from the cannula site (Hill et al., 1996). Instead, cecectomized roosters can be used with little to no postoperative complications (Mansano et al., 2019). Their small size, lack of postoperative complications and potential for long term use makes cecectomized roosters a desirable model that is already used for canine nutrition research.

Cecectomized roosters are used with a precision feeding protocol for canine nutrition research, in which a tube is used to deposit a specific amount of food into the crop of the roosters (Johnson et al., 1998). The bird's excreta are then collected for 48 hours and assessed for nutrient content to enable calculation of AID and SID. This method is thought to give the most accurate

results and has been compared to ileal cannulated dog in one study. Amino acid digestibility of dry dog foods made from different meat meals was evaluated using both precision feed roosters and ileal cannulated dogs (Johnson et al., 1998). Although some amino acids such as cysteine showed SID 11-41% higher for roosters than the dog, other amino acids such as methionine showed only 0 to 3% difference in SID. Overall, the authors concluded there was good correlation for amino acids digestibility between the 2 species (r=0.87-0.92). Together, this data has been used to validate the use of the cecectomized rooster for quantification of digestibility of protein and amino acids in dogs and stands today as the preferred translational animal model.

Despite these positive results for correlation between the two models, digestive physiology of the two species has notable differences. While dogs are mammals and facultative carnivores (Deschamps et al., 2022), chickens are more closely related to reptiles and are classified as omnivores (Klasing, 2005). The canine digestive tract is adapted to a high protein diet, different from a chicken's natural diet of largely cereal grains and oilseeds. Where the canine stomach comprises of one large organ (National Research Council, 2006), the avian stomach is composed of two parts, the proventriculus (glandular stomach) and the gizzard (muscular stomach) (Bell, 2002). The proventriculus of chickens is most similar to the canine stomach, where pepsin and acid are secreted to initiate protein digestion. After the proventriculus the feed moves to the gizzard where grinding and mixing occurs at an average fed pH of 2.6 (Farner, 1942). In comparison the canine stomach can reach a much lower pH of 1.26 during digestion (Sagawa et al., 2009). In addition to these differences in stomach structure and pH, chickens and dogs also have differences in intestinal physiology.

Both chickens and dogs have a relatively short digestive tract; however, broiler chickens have a longer small intestine relative to body size compared to dogs. For broiler chickens, small

intestine length ranges from approximately 85 to 94 cm per kg of body weight (Kokoszyński et al., 2017). In dogs, small intestine length is correlated to body size, ranging from 20 - 48 cm per kg for 33 kg and 5 kg dogs, respectively (Albors et al., 2011). Although chickens have a longer small intestine per kg of BW compared to a dog, they do have a narrower diameter and small surface area per cm of small intestine. In dogs the jejunum and ileum have 54 cm² and 38 cm² of surface area, respectively, for every cm of length (National Research Council, 2006). In chickens this surface is lower, with only approximately 8 cm² per cm of jejunum, and 3.4 cm² per cm of ileum (Mitjans et al., 1996). While the lower surface area of the avian small intestine may make digestive capacity more like the dogs despite the larger length to body weight ratio, these differences in digestive physiology may lead to differences in digestive capacity between the two species. While Johnston et al. (1998) found good correlation between amino acid digestibility of chickens and dogs, more research is needed to evaluate the accuracy of cecectomized roosters as a model for canine digestion with respect to all dietary nutrients of interest among different ingredients and complete diets.

1.6.2   Ileal cannulated pigs

Being mammals, pigs share more similarities in digestive tract structure with dogs than chickens. Additionally, ileal cannulated pigs may offer some benefits for research over cecectomized roosters. Despite this, pigs are rarely used as a model for canine digestion and digestibility. However, both dogs and pigs have been used historically as models for human digestion and digestive diseases (Ziegler et al., 2016).

With pigs, the most accurate method to collect data on food digestion is the use of cannulation. For the most common method of cannulation, pigs have a plastic T-cannula surgically placed in the distal ileum of the small intestine (Wubben et al., 2001). Similar to cecectomy in roosters, the cannula allows for the collection of digesta from the end of the small intestine that can be used to calculate the AID and SID of nutrients before fermentation occurs in the large intestine. While cannulation can be maintained in dogs for up to 14 months, post-operative infections and other complications are common (Hill et al., 1996). Due to the high prevalence of complications and welfare concerns cannulation surgery is no longer common practice for dogs, however it is still a technique used for swine research. Although cannulation in pigs is typically performed on growing pigs, the technique has historically been used in all ages of pigs, from piglets to adult sows (Petry et al., 2020). In growing pigs, the cannula is typically maintained only for the time the animal is used for study, after which the animal is euthanized. Unlike cecectomized roosters, the large intestine of the cannulated pig is not removed, allowing collection of both digesta and feces. Because of this, ileal cannulated pigs can be used to study both ileal and total tract digestibility, while roosters can only be used to evaluate ileal digestibility. Furthermore, collection of feces could allow for evaluation of the effects of dog foods on fecal consistency, intestinal microbiome, and intestinal health, which is not possible when using roosters. These extra measures possible with pigs could help increase understanding of how dog food formulation and treatments affect digestibility and health. Since cannulations are still used in swine research it would be possible to use ileal cannulated pigs as a model for canine digestion.

Like dogs, pigs are classified as mammals and have more similarities in GI tract structure compared to chickens (Henze et al., 2021). Like dogs, pigs have a single compartment stomach

reaching a pH of 1.15 (Henze et al., 2021), which is close to the 1.26 pH recorded for dog's (Sagawa et al., 2009). Gastric emptying time (GET) between the two species may also overlap. While the GET does depend in part on feed composition, the average GET was 2.94 hours for the dog (Koziolek et al., 2019) and 1.5 to 6 hours for pigs (Davis, Illum and Hinchcliffe, 2001). This overlap may mean similar digestive capacity particularity for protein that begins digestion in the stomach.

In the small intestine stomach acid is neutralized, the pH rises and both species can reach pH values of up to 8 (Henze et al., 2021, Koziolek et al., 2019). Small intestinal transit time (SITT) is also longer in the pig than in the dog, with mean fasted and fed SITT of 1.37 and 1.94 hours, respectively for dogs (Koziolek et al., 2019), while fasted pigs SITT for pigs ranged from 2.6 to 4 hours, and fed SITT ranged from 2.3 to 3.8 hours (Henze et al., 2021). This longer transit time for pigs occurs despite the pig's lower small intestinal length to body weight ratio. Pigs of 200-300 kg have less than 10 cm of small intestine per kilogram of body weight (Gonzalez et al., 2015) compared to the dog's 20-48 cm per kg (Albors et al., 2011). The slower motility and longer SITT of the pig may make up for their shorter small intestine relative to body weight by allowing more time for the nutrients to be digested and absorbed.

As result of these differences in transit time and small intestinal length, how comparable ileal digestibility of food in pigs and dogs might be is difficult to predict. Currently, studies directly comparing digestibility of the same diets in ileal cannulated pigs and dogs have not yet been conducted, although comparisons have been made between roosters and pigs. When fed casein protein cecectomized roosters had lower AID and SID of amino acids than ileal cannulated pigs (Chung, and Baker, 1992). The AID and SID for the methionine for roosters was 89.3% and 93.6% respectively while the AID and SID of methionine for pigs was 97.2% and 99.2%

respectively. However, with no similar study having been done with dogs, therefore accurately determining which model would be most similar to the digestive capacity of dogs is not possible. Studies will need to compare digestibility of the same diet in both pigs and dogs to determine if swine would be a good translational model for canine digestibility studies.

1.7   Conclusions

Vegetarian and vegan pet foods have been growing in popularity among dog owners. The main protein sources in these diets are typically pulses including peas, chickpeas, beans and lentils and their protein concentrates. While these ingredients provide more protein than cereal grains, they also contain more insoluble fiber in addition to other ANF. Due to this, high levels of pulses in vegan pet foods could reduce nutrient digestibility. The addition of exogenous enzymes to dog food could help solve these problems, however results for their use are mixed. While protease, phytase and fiber degrading enzymes have shown consistent increases in digestibility of cereal grain-based livestock diets, their results are not as consistent when used in pulse-based livestock diets. The use of exogenous enzymes in canine diets have also shown mixed results and none so far have used pulse-based diets. Therefore, there is a need for more research examining the use of exogenous enzymes in dog diets, especially pulse based vegetarian and vegan diets.

Conducting these studies may be difficult as canine studies face many regulations and cannulation studies are no longer done with dogs. Livestock species can be used as models for canine digestibility studies. Cecectomized roosters are the most common model currently used in place of data generated using dogs, but there is little evidence to show their accuracy given the

differences in the digestive anatomy among chickens and dogs. Cannulated pigs are another possible model, having more similar anatomy however differences in digestive physiology should be considered. Comparative studies will need to be conducted to determine the accuracy of swine as a model for canine digestion.

1.8   Knowledge Gaps

Consumer demand for plant-based dog foods has been growing, leading to increasing production of vegetarian and vegan dog foods by pet food manufacturers. However, to the authors' knowledge no studies have yet been conducted to assess the digestibility of pulse-based vegan dog foods. Digestibility of these dog foods could potentially be aided by the use of exogenous enzymes which are commonly used in livestock feed. Some studies have been conducted concerning enzymes added to dog foods, however, to the authors knowledge none have yet been conducted using enzymes in dog foods high in pulses. While more research on these topics is needed, restrictions around the use of companion animals in research have resulted in some research practices no longer being accepted in canine nutrition research. Given their similarities in digestive tract physiology, pigs have the potential to be a translational model for canine nutrition studies. In order to determine if pigs can be a good model for dog digestibility studies, research will need to be done comparing results from both species fed the same diets.

1.9   Thesis Hypotheses

The null hypothesis of the present thesis were a) that extrusion and the addition of an enzyme blend either before or after extrusion would not affect the ATTD and SID of field pea and lentil flour or protein concentrate based vegan dog foods in growing pigs; b) the addition of an enzyme blend either before or after extrusion would not affect the ATTD, fecal metabolites or fecal quality from field pea and lentil flour or protein concentrate based vegan dog foods in adult dogs; c) comparison of ATTD results from dogs and pigs fed the same diets would not have any relation.

1.10   Thesis Objectives

Based on these null hypotheses, the objectives of the present thesis were

a) To determine and compare the ATTD of OM, CP, and GE, and SID of CP and AA for ileal cannulated pigs fed pulse-based vegan dog foods that were raw or extruded, with or without an enzymes blend added before or after extrusion.

b) To determine and compare the ATTD of OM, CP, crude fat, ash and GE, fecal fermentation products and fecal quality for dogs fed the same diets.

c) To compare the ATTD values from the dogs and pigs fed the same diets to determine if pigs can be used as a model for dog food digestibility studies.

1.11 Tables

Table 1.1. Proximate composition of common pet food ingredients on dry matter basis. Adapted from National Research Council Nutrient Requirements for Swine (2012)[1].

Item, % DM	Lentil	Chickpea	Field pea	Corn, dent	Rice	Hulled barley
Crude protein	28.9	22.7	25.2	9.33	8.97	14.3
Crude Fat	1.44	4.47	1.36	3.94	1.26	1.23
Neutral detergent fiber	19.3	17.6	14.6	10.3	1.46	14.0
Starch	46.4	49.9	49.3	70.8	85.7	60.9
Moisture	10.0	10.3	11.9	11.7	12.2	10.4

[1] Values converted to dry matter basis from as fed.

Table 1.2. Essential amino acid (AA) composition of common pet food ingredients on dry matter basis. Adapted from National Research Council Nutrient Requirements for Swine (2012)[1].

AA, %	Lentil	Chickpea	Field pea	Corn, dent	Rice	Barley, hulled
Arg	2.27	2.50	1.35	0.40	0.50	0.75
His	0.87	0.93	0.60	0.27	0.38	0.45
Ile	1.11	1.01	1.06	0.32	0.36	0.39
Leu	2.04	1.79	1.77	1.09	0.64	0.83
Lys	1.90	1.57	1.85	0.28	0.40	0.57
Met	0.20	0.33	0.24	0.20	0.28	0.22
Phe	1.43	1.37	1.16	0.44	0.50	0.60
Thr	0.93	1.01	0.94	0.32	0.26	0.41
Try	0.23	-	0.24	0.07	0.13	0.15
Val	1.41	1.13	1.17	0.32	0.48	0.61

[1] Values Converted to dry matter basis from as fed.

Table 1.3. Fiber and antinutritional factor composition of some pulse flours and protein concentrates (dry matter basis)

Item, g/100g	Lentil flour	Chickpea flour	Field Pea flour	Pea protein concentrate
TDF	11.5[2]	22.7[2]	8.16[7]	16.1[7]
SDF	2.0[2]	5.5[2]	1.19[7]	1.59[7]
IDF	9.5[2]	17.2[2]	6.98[7]	14.5[7]
Phytate	0.41[3]	2.12[5]	0.88[7]	2.25[7]
TIA	0.28[3]	1.46[5]	0.25[7]	0.60[7]
Total phenolics	0.77[4]	0.22[6]	0.25[5]	0.25[8]

[1] Total dietary fiber (TDF), soluble dietary fiber (SDF), insoluble dietary fiber (IDF), trypsin inhibitor activity (TIA)

[2] Khan et al., 2007

[3] Hefnawy, 2011

[4] Yeo and Shahidi, 2017

[5] Adamidou et al., 2011

[6] Han and Baik, 2008

[7] Fenn et al., 2022

[8] Çabuk et al., 2018

Table 1.4. Comparison of methods and results for studies involving exogenous enzyme use in pulse-based diets for livestock

Species	Diet ingredients	Enzyme, U/kg	Application method	Results	Citation
Broiler chickens	Soybean and corn with 300g/kg pea meal of 6 different cultivars	Cellulase, 2000 Xylanase, 3000 α-amylase, 2000	Added to mash diets at mixing	One cultivar showed increased ATTD of DM	Cowieson, Acamovic and Bedford, 2003
Broiler chickens	Corn with 300 g/kg hulled peas	Cellulase, 120 Xylanase, 1000 Glucanase, 400 Pectinase, 1000 Mannase, 200 Galactanase, 180	Added to mash diets at mixing	No increase to AID of starch or protein	Meng and Slominski, 2005
Grower/ finisher pigs	Barley and wheat with 350 g/kg hulled or dehulled peas	Cellulase, xylanase, α-amylase, β-glucanase, protease. Quantities not specified	Added after steam pelleting	Increased ATTD of DM, CP and GE	Thacker and Racz, 2001
Growing pigs	Hulled field pea only	Phytase, 500	Added to mash diets at mixing	Increased SID of Essential amino acids	Kahindi, Thacker and Nyachoti, 2015

Broiler turkeys	Corn and soybean with 80, 160 or 240 g/kg Chickpea meal	Phytase, 1000	Added to mash diets at mixing	Increased growth performance	Ciurescu, Vasilachi and Grosu, 2020
Broiler chickens	Corn starch and 115, 230 or 345 g/kg black-eyed peas	Phytase, 1000	Added to mash diets at mixing	Increased ATTD of phosphorous and growth performance	Iyayi, Fru-Nji and Adeola, 2013
Broiler chickens	Corn, wheat and soybean with 100, 200, or 300 g/kg pea meal	Protease, 15,000 α-galactosidase, 115 β-glucanase 10	Mixed with water and pea meal then incubated at 30 for 24h	No increase to AID of nutrients	Boroojeni et al., 2017
Broiler chickens	810 g/kg white flower pea or 710g/kg colored	Protease 15,000	Added to mash diets at mixing	Increased SID of essential amino acids	Szczurek and Świątkiewicz, 2020
Broiler chicken	Corn and soyabean with 300g/kg hulled field pea, lupin or faba bean	Protease 300,000	Added to mash diets at mixing	Increased AID of essential AA for pea and faba bean	Hejdysz et al., 2020

Table 1.5. Summary of studies involving exogenous enzymes in canine diets

Diet ingredients	Enzyme, U/kg	Application method	Results	Citation
Barley, wheat and animal by-product meal with 11, 16, or 20 g/kg NSP	Xylanase, 340 β-Glucanase, 300 Amylase, 1	Mixed with water and sprayed on feed at feeding	Increased ATTD of DM, starch, CP and GE. Increased stool quality	Twomey et al., 2003
Corn, rice and poultry by-product meal with 250 g/kg wheat bran	β-glucanase, 4.5 Xylanase, 16 Cellulase, 1.5 Phytase, 1.9 a-amylase, 9000	Added to ingredients prior to extrusion, or as a dry coating after extrusion	No increase to nutrient digestibility	Sa et al., 2013
Corn and poultry by-product meal with 60, 120 or 180 g/kg DDGS	Xylanase, 80,000	Dry coating after extrusion	Increased ATTD of DM, CP and OM	Silva et al.,2016
Corn and poultry offal meal with 200 g/kg DDGS	Xylanase, 80,000 Protease, 170	Coating after extrusion dispersed in soybean oil	No increase to nutrient digestibility	Risolia et al., 2019
Corn, soybean meal and poultry offal meal with 100g/kg wheat bran	Phytase, 5, 10 or 15 Or Carbohydrase enzyme complex, 50	Dry coating after extrusion	No increase to nutrient digestibility, carbohydrase addition increased fecal SCFA and moisture	Machado et al., 2021

1.12   Literature cited

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CHAPTER 2. EXTRUSION AND ENZYME ADDITION ENHANCED ILEAL AND TOTAL TRACT DIGESTIBILITY OF PULSE GRAIN-BASED DIETS FED TO GROWING PIGS

2.1   Introduction

Pulses crops are defined as non-oilseed legumes that are grown for their dried seed, and include lentil, chickpea, field pea, faba bean, and dried beans. Preferring cool agronomic conditions, pulse crops are suited for cultivation in the Canadian Prairies, US Northern Plains, northeastern India and southern Australia (Endres and Kandel, 2021; Laskar et al., 2019). At 210 and 250 g/kg crude protein (CP) respectively, field pea and lentil contain more protein than cereal grains (Bell and Wilson, 1970; Landero et al., 2012). With up to 558 g/kg CP (Fenn et al., 2022), pulse protein concentrates can also contribute more protein in pulse inclusive swine diets. However, pulses and pulse protein concentrates do contain fiber, resistant starch, and anti-nutritional factors (ANF) that may reduce nutrient digestibility.

Whole pulse grains contain ANF such as tannins, trypsin inhibitors, phytate, and phenolic compounds, and insoluble dietary fiber (IDF). Consisting of structural plant fibers like cellulose, hemicellulose, and lignin, IDF is not readily fermentable in monogastric species such as pigs and has a relatively rapid passage rate (Jha and Berrocoso, 2015). While IDF does offer some benefits for intestinal health, the IDF in pulses acts as a bulking diluter, and encapsulates protein and other nutrients. Consuming large quantities of IDF is associated with reduced nutrient digestibility (Brummer et al., 2015). Heat treatment can increase digestibility of field pea and lentil by gelatinizing starch and inactivating trypsin inhibitors and phenolic compounds (Adamidou et al., 2011; Hugman et al. 2021a; Hugman et al. 2021b). Heat treatment may also reduce IDF content, however, the effects of cooking on IDF depends on treatment conditions,

pulse type, and cultivar (Berrios, et al., 2010; Adamidou et al., 2011; Cargo-Froom et al., 2022). Exogenous enzymes such as cellulase and xylanase can hydrolyze IDF to some extent into soluble sugars and may increase nutrient digestibility for pigs (Zhao et al., 2020). Therefore, supplementation of exogenous enzymes in addition to heat treatment may further increase digestibility of diets containing pulses and their protein concentrates for growing swine.

The null hypothesis of the present study was that extrusion and the addition of an enzyme blend either before or after extrusion would not affect digestibility of CP, amino acids (AA), organic matter (OM), ash, or gross energy (GE) in growing pigs. The objective was to determine and compare the apparent total tract digestibility (ATTD) of DM, OM, GE, CP, and ash, and standardized ileal digestibility (SID) of GE, CP, and AA of field pea and lentil flour or protein concentrate based diets that were either raw, extruded only, or extruded with enzymes added before or after extrusion in growing pigs.

2.2 Materials and Methods

Experimental procedures were reviewed and animal use was approved by the Animal Care and Use Committee for Livestock of the University of Alberta and followed principles established by the Canadian Council on Animal Care (CCAC, 2009). The study was conducted at the Swine Research and Technology Centre of the University of Alberta.

2.2.1 Diets

Two pulse-based diets were formulated: a low protein (L) diet containing mainly field pea and red lentil flours and a high protein (H) diet containing mostly field pea and lentil protein concentrates produced by air classification (Table 2.1). For both diets, four treatments were applied: raw, not extruded and did not contain enzymes (R); extruded without enzymes (E), extruded with an enzyme blend added prior to extrusion (E+P), or an enzyme blend added after extrusion (E+A), resulting in a 2 × 4 factorial arrangement. The enzyme blend contained alkaline protease, phytase, cellulase, and xylanase activity (Bio-cat, Troy, VA). The quantity of enzymes added was determined by *in vitro* studies prior to the present study. Quantity added varied for the H and L diets, with protease, phytase, cellulase and xylanase added at 960,000 U/kg, 12,000 U/kg, 480,000 U/kg, and 360,000 U/kg, respectively for the LE+P and LE+A diets, and 1,360,000 U/kg, 17,000 U/kg, 510,000 U/kg, and 680,000 U/kg, respectively for the HE+P and HE+A diets. More enzyme blend was added to the H diets because pulse protein concentrates contain more ANF and IDF than pulse flours (Fenn et al., 2022). Finally, the N-free diet was fed to measure basal ileal endogenous losses of CP and AA (Stein et al., 2007).

To create the LE+P and H E+P diets, two pulse ingredients with the highest inclusion for each diet were mixed in five batches in a 50-kg horizontal paddle mixer (model PB35, A & M Process Equipment Ltd. Ajax, ON, Canada) with 15% water and the enzyme blend for 10 minutes to achieve homogeneity. Subsequently, the mixture was spread in aluminum pans and incubated in a forced air oven at 50°C for 3 h and the temperature then increased to 60°C to remove excess moisture. First, the H diet was mixed and dried at 60°C overnight. Subsequently, the L diet was mixed and dried at 60°C for 2 d to achieve better drying. The enzyme-treated ingredients were then rolled and mixed with the remaining ingredients in a 300-kg horizontal

paddle mixer (model SPC2748, Marion Mixers Inc., Marion, IA) in three batches. The ingredients for the R and E diets were also combined and mixed in the same 300-kg horizontal paddle mixer in 3 batches.

The E and E+P mixes were extruded using a single screw extruder (model X-115; Wenger, Sabetha, KS) at 275 kg/h and the preconditioner set at 3% water and 1% steam. The extruder barrel contained 5 zones, with the first set at 95°C and each subsequent zone increased by 5°C to reach 115°C in the fifth zone. The extrudate exited through a die with the extruder speed set to 400 rpm, and knife cutting speed set to 1400 rpm, and then cooled using a fluidized bed cooler (model 200; Wenger, Sabetha, KS).

After extrusion, the kibble was crushed using a twin roller mill (Model CHD 8.5 × 12, Iowa Farm Automation Ltd., Stanly, IA) to reduce particle size to be similar to the R diets and ensure that remaining ingredients (canola oil, liquid preservatives, and 0.4% titanium dioxide) added post extrusion were mixed homogeneously into the final E and E+P diets. Following extrusion and crushing, a portion of the LE and HE mixes were mixed in the 300-kg mixer with the enzyme blend to create the LE+A and HE+A diets. Maltodextrin was the carrier for enzymes added; therefore, an identical quantity of maltodextrin was added to R and E diets as enzyme blend in the E+P and E+A diets. After final mixing, diets were transferred to rodent-proof plastic tubs and transported to the barn.

2.2.2 Experimental design and management

Ten Duroc × Large White/Landrace growing barrows (F1) were selected at 22–23 kg body weight (BW), with 8 pigs for the study and 2 pigs as backup. Pigs were socialized and

acclimated to housing in individual pens for 10 d prior to cannulation surgery. Each pig had a simple T-cannula surgically inserted at the distal ileum so that the ostomy of the cannula was between the last two false ribs on the pig's right flank. Post-surgery, pigs recovered for 10 d while gradually transitioning to test diets during the last 3 d of recovery.

For the duration of the study, pigs were housed in individual pens raised 0.4 m off the floor on steel frames. Pen comprised of polyvinyl chloride (PVC) walls with plastic slatted floors, and measured 1.2 m wide × 1.5 m long × 1 m tall. Each pen was fitted with plexiglass windows (0.35 × 0.35 m) on all sides to facilitate eye contact between pigs in adjacent pens, and a stainless-steel cup drinker and a stainless-steel feeder were attached to the front wall. Enrichment was provided using non-consumable toys. A single chimney exhaust fan in the ceiling created negative pressure ventilation that maintained the room temperature at 22°C ± 2.5°C. Room lights were automatic, set to be on from 07:00 to 19:00. Pens were scraped clean of feces twice daily and washed with water once daily.

The study was conducted as 8 × 8 Latin square. After the eighth period, pigs were fed the N-free diet for one period. Each period consisted of 5-d diet acclimation followed subsequently by 2-d of feces collection and 2-d of digesta collection. Pigs were fed at 2.8 × maintenance energy requirement (110 kcal DE/kg of BW$^{0.75}$; NRC, 1998) split into two meals fed at 8:00 and 15:00. After digesta collection, pigs were weighed so that feed allowance could be adjusted for the next period. To collect feces, plastic bags were snapped between a leather ring and two Velcro rings glued to the skin around the pig's anus (van Kleef et al., 1994). Digesta was collected continuously from 08:00 to 15:00 using plastic bags attached to opened cannula with rubber bands (Li et al., 1993). Digesta collection bags contained 15 mL of 5% formic acid to prevent microbial fermentation.

2.2.3 Sample preparation and laboratory analysis

Feces and digesta samples were stored at -20°C after collection. At the end of each period, samples from each pig were thawed, homogenized, and stored again at -20°C. At the end of the experiment, feces and digesta were freeze dried and then ground through a 1-mm screen in a centrifugal mill (Model ZM200, Retch GmbH, Haan, Germany).

Feed and lyophilized digesta and feces were analyzed at the University of Alberta for DM and ash (method 930.15; AOAC, 2006), GE using a bomb calorimeter (model 5003; Ika-Werke, Staufen, Germany), and TiO_2 using methods described by Myers et al. (2004) with slight modification (digestion of samples for 24 h at 120°C in 10 mL tubes, dilution in 100 mL volumetric flasks followed by filtration and H_2O_2 addition). Feces were analyzed for CP at the University of Alberta by Leco (N × 6.25; method 990.03). Diet and pulse ingredients were analyzed at the Experimental Station Chemical Laboratories (ESCL, University of Missouri, Columbia, MO) for DM and ash, neutral detergent fiber (NDF) assayed without a heat-stable amylase and expressed inclusive of residual ash (Holst, 1973), acid detergent fiber (ADF) inclusive of residual ash (method 973.18), soluble and insoluble dietary fiber (method 991.43), starch (American Association of Cereal Chemists Approved Methods of Analysis, 2010, method 76-13.01), crude fiber (method 934.01), crude fat (method 954.02), CP (N × 6.25, method 990.03), Ca (method 968.08), P (method 946.06), AA [method 982.30E (a–c)], chemically available Lys (method 975.44) and phytic acid (method 986.11). Digesta was analyzed at ESCL for DM, CP (method 990.03), and AA (method 982.30E [a–c]).

2.2.4 Calculations

Calculations for AID of DM, CP, and AA, and ATTD of DM, OM, CP, ash, and GE for the test diets were conducted using the index method (Adeola, 2001)

$$ATTD\ or\ AID = 100 - (100 * \frac{Mdiet * Ndf}{Mdf * Ndiet})$$

Where N_{diet} was the concentration of the nutrient in the diet, N_{df} was the concentration of the nutrient in the digesta or feces, M_{diet} was the concentration of TiO_2 in the diet, and M_{df} was the concentration of TiO_2 in the digesta or feces.

Basal ileal endogenous loss (I_{end}) of CP and AA (g/kg DM intake) were determined and SID for CP and AA of the test diets was calculated as follows (Stein et al., 2007):

$$Basal\ Iend = AAdigesta * \frac{Mdiet}{Mdigesta}$$

$$SID = AID + \frac{Basal\ AA\ Iend}{AAdiet}$$

Diet DE was calculated by multiplying diet GE by its ATTD. Diet NE values were calculated using equation 5 of Noblet et al. (1994) adopted by NRC (2012) using calculated DE and analyzed starch, EE, CP, and ADF values for the diets.

2.2.5 Statistical analyses

Data was analyzed using the GLIMMIX procedure of SAS (2016) initially as a 2 × 4 factorial arrangement including protein level (H and L) and treatment (R, E, E+P, and E+A) as

fixed effects, and pig and period as random effects. However, interactions were observed for all variables between protein level and treatment ($P < 0.05$) that were associated solely with the two E+P treatments (with unequal drying) that prevented detection of differences among other main factors. Thus, data were re-analyzed as a 2 × 3 factorial arrangement without the two E+P treatments. Separately, diet LE was compared to LE+P and diet HE was compared to HE+P using contrasts. Prior to analyses, carry-over effects, normality, and homogeneity of variance of residuals were tested for each variable. Differences were considered significant if $P < 0.05$.

2.3   Results

One pig was euthanized in the third period because of medical reasons unrelated to the study. This pig was replaced with one of the extra pigs, starting sample collections in period 5.

The two pulse protein concentrates contained double the CP and essential AA, 4 times more crude fiber, more IDF, double the phytic acid, and 3 to 8 times more ether extract than the corresponding pulse flours (Table 2.2).

The HR diet contained the highest ADF and NDF whereas the LE+P diet contained the most IDF (Table 2.3). The H diets contained less IDF than L diets. The Lys availability was lower for the E+P diets than the other diets and was lowest for the LE+P diet. The E diets contained less phytic acid than R diets; and the E+P diets contained more phytic acid than E and E+A diets.

The ATTD of OM was greater ($P < 0.001$; Table 2.4) for L than H. The ATTD of OM and GE was greater ($P < 0.001$) for E than R. Interactions between protein level and treatment were observed ($P < 0.05$) for ATTD of CP and ash. Specifically, ATTD of CP was greater ($P <$

0.05) for E than R for L diets (LR vs LE) but not H diets. However, ATTD of CP was greater (P < 0.05) for E+A than E for both L and H diets. Moreover, ATTD of ash for both L and H diets did not differ between R and E, and was greater (P < 0.05) for E+A than E, with a large increase for H than L. The DE value was greater (P < 0.001) for H than L diets and was 85 kcal/kg greater (P < 0.001) for E than R. An interaction between protein level and treatment was observed (P < 0.001) for NE value. Specifically, NE value was greater (P < 0.05) for E than R for H diets (HR vs HE) but not for L diets and did not differ between E and E+A diets.

The ATTD of OM, CP, and GE was lower (P < 0.05; Table 2.5) for E+P than E diets for both L and H. However, ATTD of CP was 7.5% lower (P < 0.001) for LE+P than LE but was only 1.2% lower (P < 0.05) for HE+P than HE. In contrast, the ATTD of ash was greater (P <0.05) for E+P than E diets for both L and H. The DE and NE values were lower (P < 0.05) for E+P than E diets for both L and H.

For essential AA, SID was 2% greater on average for E diets compared with R diets (P < 0.05; Table 2.6) and was an additional 2% greater on average for E+A diets compared with E diets (P < 0.05). For dispensable AA, E had greater SID compared with R for all AA (P < 0.05) except for taurine, and E+A had greater SID compared with E for all dispensable AA (P < 0.05) except for Cys and Glu. Protein level and treatment interactions were observed for SID of Met and taurine (P < 0.05), with SID of Met being greater (P < 0.05) for E than R and E+A greater than E for H diets but not L diets. The SID of total AA was 4.7% higher for E than R (P < 0.05), and an additional 1.9% higher for E+A than E (P < 0.05).

The SID of CP and AA was lower (P < 0.05; Table 2.7) for E+P than E diets. The SID of essential AA averaged 10% lower (P < 0.05) for LE+P than LE and was 20% lower for SID of

Lys. The SID of essential AA averaged 6% lower for HE+P than HE. The SID of CP was 14.2% and 5.7% lower, respectively, for LE+P and HE+P diets than their respective E diets ($P < 0.05$). Total AA digestibility was also lower ($P < 0.05$) for E+P than E diets.

2.4   Discussion

In the present study, the extrusion of pulse flour or protein concentrate diets increased ATTD of OM, CP, and GE, and SID of CP and AA. The addition of enzymes after extrusion further increased ATTD of CP and ash, and SID of CP and AA. Addition of enzymes prior to extrusion reduced digestibility of nearly all values of ATTD and SID except ATTD of ash.

2.4.1   Ingredient and diet composition

Available information regarding nutrient content of field pea, lentil, and especially their protein concentrates is limited, and nutrient content results differ between publications. Compared to previously reported values for raw field pea and lentil, the pea and lentil flours fed in the present study contained similar CP and AA but contained less crude fiber, EE, and phytic acid, and more starch (NRC, 2012; Landero et al., 2012). The pea protein concentrate fed in the present study contained similar CP but less IDF and phytic acid than previously reported (Fenn et al., 2022). The lentil protein concentrate fed contained similar CP but more IDF and less phytic acid than previously reported (Elkowicz and Sosulski, 1982). Differences between studies are likely due to different pea and lentil cultivar being fed, and differences in fractionation processing conditions, highlighting the importance of processing through the ingredient supply chain.

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In the present study, treatment affected the analyzed total nutrient content of the diets. Reductions in dietary available Lys content likely occurred because of Maillard reactions during heat processing, specifically prolonged drying during enzyme pre-treatment. Maillard reactions occur when proteins interact with reducing sugars, such as glucose, during heat processing, and result in the formation of Maillard reaction products MN-ε-2-furoylmethyl-L-lys and N-ε-(carboxymethyl)-Lys (Hofmann et al., 2020). Crosslinking with Lys in these Maillard reaction products may hinder protein digestion by enzymes. Studies in humans have estimated that up to 10% of Maillard reaction products may be absorbed; however, such bound Lys compounds are not bioavailable so are not used by animals for protein synthesis (van Rooijen et al., 2013; Hofmann et al., 2020). While Lys can be damaged during extrusion, the E and E+A diets in the present study contained similar levels of chemically-available Lys as the R diets, indicating that extrusion alone did not cause much protein damage. However, chemically-available Lys and Lys/CP was less for the E+P diets than E and E+A diets indicating that Maillard reactions did occur for the E+P diets. The LE+P diet contained the least sulfur AA Met and Cys that can become oxidized during heat processing (Tran et al., 2008); also indicating heat damage. The E and E+A diets contained less phytic acid than the R diets, likely due to phytic acid being heat labile (Daneluti and Matos, 2013). Phytic acid forms complexes with minerals and proteins that inhibit digestion. As such, reduced phytic acid content of livestock feeds is associated with increased mineral and protein digestibility (Selle et al., 2009). Overall, these changes demonstrate how different processing conditions can both positively and negatively affect the composition and nutritional quality of animal feeds.

2.4.2 Digestibility

In addition to inactivating heat labile ANF, extrusion can increase digestibility through starch gelatinization and protein denaturation that allow better access for digestive enzymes to their substrates (Ali et al., 2017; Sun et al., 2019). In the present study, extrusion increased ATTD of OM, CP, and GE, and SID of CP and AA for both pulse flour and protein concentrate based diets. The increases in digestibility were consistent with results of previous studies examining extrusion of field pea and lentil fed to swine, where extrusion increased SID of AA (Hugman et al. 2021a; Hugman et al. 2021b). The ATTD of CP and ash, and SID of CP and essential AA for both pulse flour and pulse protein concentrate based diets was increased further by addition of exogenous enzymes after extrusion. These increases in digestibility can be attributed to the activity of these supplemental enzymes within the pig intestine after feed ingestion. Cellulase enzymes hydrolyze cellulose β-bonds, converting insoluble fiber into soluble, or readily utilizable disaccharides and glucose (Ejaz et al., 2021). Xylanase works similarly, hydrolyzing arabinoxylans into soluble xylo-oligosaccharides and xylose, which are fermentable by gut microbes (Singh et al., 2021). Solubilization of these fibers can increase access by enzymes to nutrients that were previously entrapped within the fiber (Liu et al., 2016). In the present study, the enzyme blend also included phytase, which is well known for its ability to hydrolyze phytate, thereby increasing digestibility of P, other minerals, and protein (Singh et al., 2018). Protease included in the enzyme blend may also increase protein digestibility by hydrolyzing proteins into smaller peptides that may be more easily digested by the pig (Cowieson and Roos, 2013). Overall, the activities of the enzymes fed in the present study increased digestibility of the pulse-based diets for the growing pigs and could potentially increase feed efficiency.

Few studies have previously examined enzyme addition for pulse-based swine diets. One previous study involved an enzyme blend including cellulase, xylanase, α-amylase, β-glucanase, protease, and phytase (Thacker, and Racz, 2001), and another involved phytase alone (Kahindi et al., 2015). Both studies used raw pea inclusive diets fed to growing pigs and increased ATTD of CP and DM, and SID of essential AA with enzyme addition, however animal growth performance and feed efficiency was not different (Thacker, and Racz, 2001; Kahindi et al., 2015). Increases in digestibility and growth performance with enzyme addition may depend on type, concentration and source of enzymes, methods of diet preparation and enzyme application, and feed intake. Results of one study involving amylase and xylanase addition to raw or extruded pea-based diets fed to early weaned pigs, did increases ATTD of CP and SID of AA when the enzymes were added to extruded diets compared to when the enzymes were added to raw diets (Owusu-Asiedu et al., 2002). Digestibility increases for the diets in this previous study and the present study were likely aided by extrusion reducing the integrity of the plant cell wall structures, thereby allowing better access by the added enzymes (Redgwell et al. 2011). As far as we are aware, there are no previous reports feeding extruded lentil or pulse flour or protein concentrate based diets with added enzymes to growing pigs. The results of the present study indicate that extrusion and subsequent addition of exogenous enzymes can increase nutrient digestibility of pea and lentil flours and protein concentrates fed to growing swine.

Although the current study did not examine pig growth performance, given the positive results of the present study, future research involving growth performance of pigs fed pea and lentil flour and protein concentrates with extrusion and exogenous enzymes added after extrusion are warranted. Future studies should also examine the possible intestinal health benefits of adding enzymes to pulse based swine diets. The use of celullases, xylanase, protease and phytase

in swine feeds can support bacterial fermentation, reduce populations of pathogenic bacteria, and support the health of the intestinal epithelium though increased villi high (Duarte et al., 2019; Li et al., 2021; Moita et al., 2022). Positive impacts on intestinal health with enzymes addition to pulse-based swine diets may further increase the usefulness of these ingredients and technologies for swine producers.

The additive combination of steam heat applied during extrusion and screw friction denatures and inactivates enzymes, reducing their activity (Sa et al., 2013). In the present study, for the E+P diets, the main pulse ingredients in each diet were incubated with enzymes prior to extrusion, giving the enzymes time to work on hydrolyzing IDF, phytate, and protein in the feedstuffs before being denatured in the extruder. The Maillard reactions that reduced digestibility and chemically-available Lys content in the E+P diets likely occurred because of this process. Soluble carbohydrates resulting from enzymatic hydrolysis of IDF can increase viscosity of the feedstuff matrix (Ognean et al., 2011). Maillard reactions can be influenced by viscosity of the material in the barrel of the extruder, where greater viscosity increases shear force and heat in the extruder leading to more Maillard reactions (Guerrero et al., 2012). If the enzymes in the oven pre-treatment hydrolyzed some of the cellulose and hemicellulose into soluble carbohydrates, it may have increased the viscosity of the material in the extruder. However, the E+P diets did not have reduced IDF or ADF content or increased SDF or NDF content to indicate an enzyme effect, making this theory unlikely. The chemically-available Lys was lower for the LE+P diet than HE+P diet and likely because of extended drying time for the LE+P diet. Given that the LE+P ingredients were dried longer, more Maillard reactions may have occurred. Maillard reactions and the different extent of heat damage also explain the

reduced nutrient digestibility for E+P diets, and the greater reduction in digestibility for LE+P diet than HE+P diet.

In conclusion, extrusion increased the ATTD of OM, GE and CP, and SID AA of both pea and lentil flour and protein concentrate based diets for growing pigs. Addition of enzyme blend after extrusion further increased ATTD of CP and ash, and SID of CP and AA. In the present study, treatment of pulse flours and protein concentrates with enzymes prior to extrusion decreased nutrient digestibility because of protein heat damage during oven drying before extrusion. However, extrusion alone or with addition of exogenous enzymes after extrusion, can increase the nutritional quality of diets containing both pea and lentil flours and protein concentrates fed to growing pigs.

2.5   Tables

Table 2.1. Ingredient composition of the high/low protein and N-free diets.

Ingredient, %	Low protein	High protein	N-free
Corn starch	–	–	71.50
Field pea protein concentrate	5.20	50.00	–
Field pea flour	35.00	1.30	–
Lentil protein concentrate	5.20	20.00	–
Sugar	–	–	15.10
Lentil flour	15.00	1.30	–
Hulled barley	12.20	6.00	–
Oat flour	12.20	6.00	–
Canola oil	8.51	8.86	3.00
Solka floc[1]	–	–	5.00
Vegetable digest	4.00	4.00	–
Mono-/di-calcium phosphate	–	–	2.10
Limestone	1.00	1.20	1.25
Vitamin premix	0.20[3]	0.20[3]	0.50[4]
Mineral premix	0.10[5]	0.10[5]	0.50[6]
Calcium monophosphate	0.50	0.10	–
Salt	0.20	0.20	0.50
Choline chloride	0.20	0.25	0.05
K_2CO_3	–	–	0.40
DL-Met	0.20	0.20	–
MgO	–	–	0.10
KCl	0.10	0.10	–
Taurine	0.10	0.10	–
Naturox dry[2]	0.05	0.05	–

Naturox liquid[2]	0.03	0.03	–
L-carnitine	0.02	0.02	–

[1]Solka-floc, International Fiber Corp., North Tonawanda, NY.

[2]Kemin Industries Inc., Des Moines, IA.

Table 2.2. Analyzed nutrient profile of field pea and lentil flours and pea and lentil protein concentrates included in experimental diets (dry matter basis).

Nutrient, %	Field pea flour	Red lentil flour	Pea protein concentrate	Lentil protein concentrate
Moisture	7.81	7.67	6.04	6.74
Starch	49.2	43.6	12.8	13.2
CP	23.1	26.1	52.2	50.8
NDF	4.73	9.26	16.4	13.3
ADF	1.82	2.19	9.76	10.2
IDF	8.27	9.26	11.1	10.3
Ash	3.25	2.67	5.95	4.97
Crude fiber	1.21	1.34	5.26	2.12
EE	0.59	0.17	1.55	1.32
SDF	0.13	0.44	0.52	0.49
Indispensable AA				
Arg	1.82	2.24	5.01	4.37
His	0.56	0.69	1.35	1.35
Ile	1.00	1.22	2.45	2.45
Leu	1.70	2.03	4.05	4.2
Lys	1.78	1.92	4.21	3.96
Available Lys	1.71	1.85	3.93	3.71
Met	0.21	0.21	0.48	0.41
Phe	1.13	1.37	2.70	2.86
Thr	0.84	0.99	1.95	1.95
Trp	0.24	0.21	0.46	0.41
Val	1.12	1.39	2.17	2.76
Dispensable AA				
Ala	1.00	1.12	2.30	2.21

Asn	2.58	3.11	6.01	5.9
Cys	0.36	0.30	0.67	0.53
Glu	4.00	4.60	9.07	8.8
Gly	1.01	1.10	2.27	2.14
Hyp	0.03	0.01	0.05	0.05
Ser	0.99	1.20	2.28	2.39
Tau	0.31	0.29	0.17	0.08
Tyr	0.85	0.84	1.83	1.84
Total AA	22.5	26.1	52.2	50.8
Minerals				
Ca	0.041	0.020	0.081	0.068
P	0.40	0.38	0.82	0.67
Phytic acid	0.64	0.53	1.46	1.10

Table 2.3. Analyzed nutrient content of experimental diets (dry matter basis)

Nutrient, %	LR	LE	LE+P	LE+A	HR	HE	HE+P	HE+A	N-free
Proximate									
Moisture	9.12	7.38	7.86	7.66	7.11	6.43	0.96	6.42	7.68
Starch	44.6	39.7	35.8	39.1	18.0	20.7	18.0	19.1	84.4
Crude protein	24.2	24.3	25.1	24.7	43.7	43.3	43.5	43.8	0.40
Crude fat	10.0	7.43	7.39	6.58	10.8	10.63	8.03	10.47	0.76
IDF	< 0.05	8.37	9.80	8.65	6.33	5.84	5.71	7.06	< 0.05
Ash	5.74	5.47	6.24	5.66	6.99	6.69	6.68	6.77	4.74
NDF	5.41	4.69	4.60	4.6	13.94	2.97	5.14	3.32	2.52
ADF	3.48	2.26	2.01	2.77	9.97	2.26	4.25	3.19	1.18
Crude fiber	3.48	1.52	1.50	1.65	1.34	1.91	2.17	2.35	1.53
SDF	< 0.05	0.33	0.20	0.24	0.19	0.26	0.3	0.25	< 0.05
Indispensable AA									
Arg	1.78	1.78	1.69	1.78	3.61	3.61	3.51	3.69	0.01
His	0.57	0.57	0.54	0.57	1.03	1.03	1.01	1.06	0.01
Ile	0.99	1.01	1.03	1.01	1.87	1.83	1.85	1.91	0.01
Leu	1.72	1.71	1.73	1.73	3.08	3.1	3.07	3.16	0.08
Lys	1.62	1.59	1.31	1.61	3.06	3.06	2.71	3.15	0.04
Available Lys	1.55	1.53	1.18	1.53	2.9	2.97	2.52	3.01	0.04
Lys availability	95.7	96.2	90.1	95.0	94.8	97.1	93.0	95.6	100
Lys/CP	6.69	6.54	5.21	6.52	7.00	7.07	5.79	6.87	1.00
Met	0.70	0.50	0.33	0.45	0.49	0.59	0.67	0.69	0.01
Phe	1.13	1.14	1.15	1.13	2.05	2.06	2.05	2.09	0.02
Thr	0.86	0.85	0.86	0.86	1.51	0.15	1.52	1.58	0.10
Trp	0.15	0.21	0.19	0.81	0.23	0.33	0.34	0.34	< 0.02
Val	1.15	1.17	1.2	1.19	2.11	2.08	2.11	2.16	0.02

Dispensable AA

Ala	1.15	1.03	1.06	1.06	1.76	1.76	1.78	1.83	0.03
Asn	2.45	2.44	2.47	2.45	4.47	4.48	4.48	4.63	0.03
Cys	0.37	0.35	0.33	0.34	0.49	0.52	0.49	0.53	0.01
Glu	3.92	4.01	3.95	3.95	6.74	6.97	6.69	6.86	0.09
Gly	1.08	1.05	1.09	1.08	1.74	1.76	1.75	1.81	0.02
Pro	1.04	1.03	1.06	1.03	1.63	1.62	1.66	1.66	0.03
Ser	1.00	0.97	0.97	0.98	1.76	1.88	1.77	1.83	0.02
Tau	0.39	0.36	0.29	0.32	0.2	0.21	0.28	0.26	0.24
Tyr	0.72	0.72	0.75	0.73	3.08	3.1	1.34	1.32	0.03
Total AA	22.8	22.6	22.1	22.6	39.2	39.9	39.2	40.7	0.72
Minerals									
Ca	0.55	0.58	0.83	0.70	0.81	0.56	0.47	0.49	0.73
P	0.55	0.54	0.63	0.59	0.69	0.67	0.68	0.67	0.28
Phytic acid	1.10	0.85	1.28	0.93	1.23	0.80	1.17	1.04	0.23
GE, Mcal/kg	4.44	4.45	4.43	4.44	4.69	4.77	4.64	4.78	3.72

Low protein (L), High protein (H), raw (R), extruded without enzymes (E), extruded with
enzymes added prior to extrusion (E+P), extruded with enzymes added after extrusion (E+A).

Table 2.4. Apparent total tract (ATTD) digestibility of dry matter (DM), organic matter (OM), crude protein (CP), ash, and gross energy (GE), and digestible energy (DE) and net energy (NE) values of diets by protein level and treatement[1]

Variable	Protein level			Treatment				P-value			
	L	H	SEM[2]	R	E	E+A	SEM[3]	L vs. H	R vs E	E vs. E+A	Protein × treatment
ATTD, %											
OM	93.1	92.0	0.227	92.1	92.8	92.7	0.194	< 0.001	0.018	0.951	0.128
CP[4]	90.6	92.4	0.341	90.5	91.6	93.8	0.307	< 0.001	0.011	< 0.001	0.019
Ash[5]	62.7	62.7	1.13	57.7	57.2	73.2	1.17	0.952	0.661	< 0.001	< 0.001
GE	92.4	91.4	0.224	91.0	92.0	92.1	0.199	0.139	0.001	0.685	0.216
Energy, Mcal/kg DM											
DE	4.09	4.34	7.36	4.16	4.24	4.25	9.02	< 0.001	< 0.001	1.00	0.305
NE[6]	2.94	2.86	5.15	2.86	2.93	2.91	9.47	< 0.001	< 0.001	0.160	< 0.001

Low protein (L), High protein (H), raw (R), extruded without enzymes (E), extruded with enzymes added after extrusion (E+A)

[2] Least squared means based on 17 observations per formulation

[3] Least squared means based on 7 to 8 observations per diet

[4] Protein × treatment interaction means: 88.9^d, 91.0^c, 93.4^{ab}, 92.1^{bc}, 92.3^{bc}, and 94.2^a for LR, LE, LE+A, HR, HE, and HE+A, respectively. a-d: means without a common superscript differ ($P < 0.05$).

[5] Protein × treatment interaction means: 61.7^c, 57.2^c, 69.3^b, 53.8^c, 57.2^c, and 77.1^a for LR, LE, LE+A, HR, HE, and HE+A, respectively. a-c: means without a common superscript differ ($P < 0.05$).

[6] Protein × treatment interaction: 2.96^a, 2.95^{ab}, 2.93^{abc}, 2.77^d, 2.91^{bc}, and 2.90^c for LR, LE, LE+A, HR, HE, HE+A respectively. a-c: means without a common superscript differ ($P < 0.05$).

Table 2.5. Apparent total tract (ATTD) digestibility of dry matter (DM), organic matter (OM), crude protein (CP), ash, and gross energy (GE), and digestible energy (DE) and net energy (NE) values for E and E+P diets[1]

Variable	Low protein			High Protein			P-value	
	LE	LE+P	SEM[2]	HE	HE+P	SEM[2]	LE vs LE+P	HE vs HE+P
ATTD, %								
OM	93.3	90.8	0.391	92.2	91.3	0.377	< 0.001	0.019
CP	91.0	83.5	0.621	92.3	90.8	0.599	< 0.001	0.018
Ash	57.2	64.6	1.65	57.2	64.5	1.64	< 0.001	< 0.001
GE	92.4	89.5	0.398	91.6	90.4	0.383	< 0.001	0.004
Energy, Mcal/kg DM								
DE	4.14	3.99	13.0	4.29	4.22	12.6	< 0.001	< 0.001
NE	2.97	2.84	9.12	2.91	2.72	8.80	< 0.001	< 0.001

[1]Low protein (L), high protein (H), extruded only (E), extruded with enzymes added prior to extrusion (E+P).

[2] Least squared means based on 7 to 8 observations per diet.

Table 2.6. Standardized ileal digestibility (SID) of crude protein (CP) and amino acids (AA) of diets by protein level and treatment[1,2]

SID	Protein level			Treatment				P-value			
	L	H	SEM[3]	R	E	E+A	SEM[4]	L vs H	R vs E	E vs E+A	Protein × treatment
CP	88.7	89.5	0.442	88.0	92.0	94.2	0.569	0.149	< 0.001	0.004	0.4929
Indispensable AA											
Arg	97.5	96.5	0.259	94.5	97.5	99.0	0.305	0.009	< 0.001	0.002	0.8415
His	91.9	91.9	0.345	88.3	92.8	94.5	0.396	0.913	< 0.001	0.003	0.682
Ile	90.1	90.1	0.380	85.2	91.7	93.5	0.444	0.998	< 0.001	0.008	0.737
Leu	90.9	90.6	0.375	86.1	92.1	94.0	0.432	0.554	< 0.001	0.003	0.503
Lys	93.2	93.7	0.343	90.4	94.1	95.8	0.401	0.308	< 0.001	0.006	0.347
Met[5]	94.2	91.5	0.333	90.7	93.1	94.7	0.371	< 0.001	< 0.001	0.001	< 0.001
Phe	91.1	91.0	0.377	85.8	92.7	94.5	0.432	0.790	< 0.001	0.004	0.476
Thr	87.0	87.7	0.420	82.6	88.7	90.7	0.509	0.191	< 0.001	0.025	0.521
Try	86.5	87.5	0.671	75.4	90.3	93.0	0.755	0.500	< 0.001	0.008	0.215
Val	88.7	88.8	0.405	83.8	90.2	92.3	0.479	0.934	< 0.001	0.004	0.588
Dispensable AA											
Ala	90.9	90.0	0.389	86.3	91.3	93.8	0.454	0.072	< 0.001	< 0.001	0.354
Asp	90.0	90.0	0.475	86.2	90.8	93.0	0.553	0.952	< 0.001	0.005	0.865
Cys	78.4	72.7	1.21	69.2	77.2	80.3	1.34	< 0.001	< 0.001	0.114	0.093

Glu	94.0	90.0	0.958	88.7	92.9	94.4	1.08	< 0.001	0.003	0.546	0.911
Gly	94.7	91.7	0.779	89.5	93.3	96.9	0.895	0.003	0.005	0.009	0.675
Pro	139	119	0.718	124	130	133	0.777	< 0.001	< 0.001	0.002	0.408
Ser	89.3	89.5	0.394	85.0	90.8	92.4	0.460	0.697	< 0.001	0.028	0.121
Tau[6]	84.1	65.4	1.93	74.4	71.7	78.2	2.02	< 0.001	0.207	< 0.001	< 0.001
Tyr	90.8	91.1	0.327	86.9	92.2	93.8	1.14	0.403	< 0.001	0.006	0.438
Total AA	91.7	91.4	0.405	89.4	94.1	96.0	0.519	0.512	< 0.001	0.006	0.586

[1] Low protein (L), High protein (H), raw (R), extruded without enzymes (E), extruded with enzymes added after extrusion (E+A).

[2] Mean basal ileal endogenous losses (g/kg, DM): CP (3.77), Arg (2.89), His (2.08), Ile (1.80), Leu (1.92), Lys (1.53), Met (0.93), Phe (1.74), Thr (4.28), Tyr (2.54), Val (2.66), Ala (3.97), Asp (1.94), Cys (3.64), Glu (1.52), Gly (11.4), Pro (44.5), Ser (3.20), Tau (12.4), total AA (2.58).

[3] Least squared means based on 17 observations per formulation.

[4] Least squared means based on 7 or 8 observations per diet.

[5] Protein × treatment interaction: 94.1[ab], 93.7[ab], 94.9[a], 87.3[c], 92.5[b], and 94.6[a] for LR, LE, LE+A, HR, HE, and HE+A respectively. a-c: means without a common superscript differ ($P < 0.05$).

[6] Protein × treatment interaction: 82.2[a], 85.7[a], 84.5[a], 66.5[b], 57.6[c], and 72.0[b] for LR, LE, LE+A, HR, HE, and HE+A respectively. a-c: means without a common superscript differ ($P < 0.05$).

Table 2.7. Standardized ileal digestibility (SID) of crude protein (CP) and amino acids (AA) for E and E+P diets[1,2]

SID	Low protein			High protein			P-value	
	LE	LE+P	SEM[3]	HE	HE+P	SEM[3]	LE vs LE+P	HE vs HE+P
CP	92.5	78.3	0.756	91.6	85.9	0.756	< 0.001	< 0.001
Indispensable AA								
Arg	97.9	90.7	0.453	97.2	93.3	0.439	< 0.001	< 0.001
His	92.5	81.2	0.791	93.0	87.2	0.767	< 0.001	< 0.001
Ile	91.5	82.9	0.804	91.8	86.6	0.779	< 0.001	< 0.001
Leu	91.9	85.4	0.741	92.3	87.8	0.718	< 0.001	< 0.001
Lys	93.5	73.5	0.755	94.7	82.5	0.731	< 0.001	< 0.001
Met	93.7	83.6	0.621	92.5	88.9	0.603	< 0.001	< 0.001
Phe	92.4	86.4	0.714	93.0	88.9	0.692	< 0.001	< 0.001
Thr	87.9	75.0	1.05	89.5	82.5	1.02	< 0.001	< 0.001
Try	90.1	80.9	1.11	90.5	84.5	1.07	< 0.001	< 0.001
Val	89.9	80.4	0.874	90.5	84.8	0.843	< 0.001	< 0.001
Dispensable AA								
Ala	91.3	82.2	0.921	91.1	86.0	0.888	< 0.001	< 0.001
Asp	90.6	78.6	1.07	91.1	84.7	1.03	< 0.001	< 0.001
Cys	78.9	52.6	2.41	75.4	54.23	2.35	< 0.001	< 0.001

Glu	94.6	844.2	1.51	91.1	86.2	1.46	< 0.001	0.073
Gly	94.3	77.0	1.53	92.3	83.8	1.47	< 0.001	0.001
Pro	139	126	1.09	121	114	1.05	< 0.001	< 0.001
Ser	90.0	77.7	0.926	91.5	84.7	0.894	< 0.001	< 0.001
Tau	85.7	83.5	2.36	57.6	61.5	2.31	1.00	0.731
Tyr	91.7	84.5	0.662	92.7	88.2	0.638	< 0.001	< 0.001
Total AA	94.8	84.3	0.692	93.4	89.0	0.692	< 0.001	< 0.001

[1] Low protein (L), high protein (H), extruded with enzymes added prior to extrusion (E+P).

[2] Mean basal ileal endogenous losses (g/kg DM): CP (6.43), Arg, (2.96), His (1.68), Ile (1.79), Leu (1.94), Lys (1.68), Met (1.07), Phe (1.73), Thr (4.34), Tyr (2.49), Val (2.66), Ala (4.05), Asp (1.94), Cys (3.74), Glu (1.50), Gly (11.6), Pro (44.4), Ser (3.19), Tau (12.4), total AA (5.26)

[3] Least squared means based on 7 to 8 observations per diet.

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CHAPTER 3. APPARENT TOTAL TRACT DIGESTIBILITY, FECAL METABOLITES, AND FECES QUALITY OF PULSE-BASED VEGAN DOG FOOD WITH OR WITHOUT ADDED ENZYMES IN ADULT DOGS AND COMPARISON TO DIGESTIBILITY FROM A PIG MODEL

3.1   Introduction

Demand for plant-based dog foods has been growing, driven largely by vegan and vegetarian owners who wish to feed their dogs a food that coincides with their own lifestyle or that they believe will reduce their environmental footprint (Pet Food Industry, 2021, Dodd et al., 2019, Vanderhoydonck, 2022). These specialty diets rely on the use of pulses, the dried seeds of non-oilseed legumes such as field pea, chickpea, and lentil, to provide protein without the use of animal derived ingredients. However, concerns exist from owners and veterinarians that vegan dog foods may not be nutritionally adequate (Dodd et al., 2019), especially considering the FDA investigations regarding pulses and nutritionally mediated DCM in dogs. One concern regarding the use of pulses in pet food is their content of anti-nutritional factors (ANF).

Pulses contain phytate, tannins, and trypsin inhibitors that can inhibit the activity of digestive enzymes and reduce nutrient absorption (Adamidou et al., 2011). Even dehulled pulses contain insoluble fiber, which consists mainly of cellulose and other cell wall structures that are not digestible by monogastric species such as dogs and pigs. Insoluble fiber can help maintain gastrointestinal regularity and helps reduce the glycemic response (Kimmel et al., 2000, McRorie and McKeown, 2017), but may also reduce digestion and absorption of protein, starch, and minerals (de-Oliveira et al., 2012; Wehrmaker et al., 2022). During pulse protein concentrate production, some ANF including phytate, trypsin inhibitors, and insoluble fiber are concentrated together with protein (Fenn et al., 2022). Supplemental enzymes such as phytase, xylanase, and

protease are already used commercially to reduce effects of ANF and increase digestibility of livestock feed (Zhao et al., 2020; Zouaoui, et al., 2018; Lee et al., 2020). These enzymes may also increase digestibility and improve the nutritional quality of pulse-based vegan dry dog foods. Therefore, the hypothesis of the present study was that the addition of an enzyme blend either before or after extrusion would increase digestibility of nutrients in extruded kibble where pea and lentil flour or protein concentrates are used.

To test this hypothesis, digestibility studies are required; however, requirements for studies with dogs are more restrictive than for livestock. Procedures that are acceptable to assess digestibility of livestock feed are not accepted practices in companion animal research. Translational animal models can provide data for the development of foods for companion animals. Cecectomized roosters have historically been used as model for canine protein and amino acid digestibility (Johnson et al., 1998), however ileal cannulated swine may also offer a suitable model for dog food digestibility studies, and have already been used to calculate digestible indispensable amino acid score (DIAAS) like values for dog food ingredients (Templeman and Shoveller, 2022). The present study was conducted with the same diets in both dogs and ileal cannulated growing pigs. Apparent total tract digestibility (ATTD) of organic matter (OM), crude protein (CP), crude fat (EE), ash and gross energy (GE), fecal metabolites, and fecal consistency were measured in the dogs. The ATTD of OM, CP, ash and GE, and standardized ileal digestibility (SID) of CP and indispensable amino acid (AA) were measured in the pigs (Van Straten, 2023). The ATTD in pigs and dogs was compared to determine if swine can be a good translational model for canine digestibility studies.

3.2 Materials and Methods

Experimental procedures were reviewed and animal use was approved by the Institutional Animal Care and Use committee (IACUC) at the University of Guelph (AUP# 4648) that followed principles established by the Canadian Council on Animal Care (CCAC, 1989). The study was conducted at the University of Guelph Central Animal Facility.

3.2.1 Diets

Test diet production occurred at the University of Alberta (Edmonton, AB, Canada). All diets were formulated to meet or exceed the Association of American Feed Control Officials (AAFCO) nutrient recommendations for adult dogs at maintenance (AAFCO, 2018). Two pulse based vegan formulations were created, low protein (L) containing mainly pea and lentil flours, and high protein (H) containing mostly pea and lentil protein concentrates (table 1). For each of the formulations three treatments were produced: Control diets without supplemental enzymes (C), and diets with a blend of enzymes added prior to extrusion (P) or enzymes added after extrusion (A), resulting in a 2 × 3 factorial arrangement and 6 treatments. The enzyme blend used consisted of protease, phytase, cellulase, and xylanase (Bio-cat, Troy, VA). The quantity of enzymes used was determined by in vitro experimentation prior to the animal studies. Quantity added varied for the H and L diets, with protease, phytase, cellulase and xylanase added at 960,000 U/kg, 12,000 U/kg, 480,000 U/kg, and 360,000 U/kg, respectively for the LE+P and LE+A diets, and 1,360,000 U/kg, 17,000 U/kg, 510,000 U/kg, and 680,000 U/kg respectively for the HE+P and HE+A diets. More enzymes were added to the H diets because pulse protein concentrates contain more ANF and IDF than pulse flours (Fenn et al., 2022).

To create the LP and HP diets, the two pulse ingredients with greatest inclusion for each diet were mixed in five batches in a 50-kg horizontal paddle mixer (model PB35, A & M Process Equipment Ltd. Ajax, ON, Canada) with 15% water and the enzyme blend for 10 minutes to achieve homogeneity. Subsequently, the mixture was spread in aluminum pans and incubated in a forced air oven at 50°C for 3 h and the temperature then increased to 60°C to remove excess moisture. First, the H diet was mixed and dried at 60°C overnight. Then the L diet was mixed and then dried at 60°C for 2-d to achieve better drying. The enzyme-treated ingredients were then mixed with the remaining ingredients in a 300-kg horizontal paddle mixer (model SPC2748, Marion Mixers Inc., Marion, IA) in three batches. The ingredients for the C diets were also combined and mixed in the 300-kg horizontal paddle mixer in 3 batches.

The C and P mixes were extruded using a single screw extruder (model X-115; Wenger, Sabetha, KS) at 275 kg/h and the preconditioner set at 3% water and 1% steam. The extruder barrel contained 5 zones, with the first set at 95°C and each subsequent zone increased by 5°C to reach 115°C in the fifth zone. The extrudate exited through a die with the extruder speed set to 400 rpm, and knife cutting speed set to 1400 rpm, and then cooled using a fluidized bed cooler (model 200; Wenger, Sabetha, KS).

After extrusion, the kibble was crushed using a twin roller mill (Model CHD 8.5 × 12, Iowa Farm Automation Ltd., Stanly, IA) to reduce particle size and ensure that ingredients added post extrusion (canola oil, liquid preservative, and vegetable digest) were mixed homogeneously into the final diets. Following extrusion and crushing, a portion of the LC and HC mixes were mixed in the 50-kg mixer with the enzyme blend to create the LA and HA diets. Maltodextrin was the carrier for enzymes added; therefore, an identical quantity of maltodextrin was added to

the C diets as enzyme blend in the E+P and E+A diets. After final mixing, diets were bagged in double layer plastic sacks, transported to the University of Guelph, and stored at 4°C until used.

3.2.2 Experimental design and management

Ten neutered male mixed breed hounds (21-30 kg) between 1-1.5 years of age were used for the study. Dogs were housed individually or paired in indoor kennels (121.9 cm × 190.5 cm) with stainless steel walls, and raised Tenderfoot flooring grids (Tandem Products, Inc., Minneapolis, MN). Each kennel included a divider used to separate pair housed dogs during feeding and fecal collection. Kennel rooms were temperature controlled (20-22°C and 30-50% relative humidity), with automatic lighting set to 12h light:12h dark. Kennels were washed with water once daily and disinfected with Peroxiguard (Bayer, Mississauga, ON, Canada) once every 10-d. Kennels included a canvas bed, two stainless steel water bowls and non-consumable toys. Dogs were exercised in pairs for a minimum of 20 min 6 times per week either by walking outdoors (weather permitting) or playing in an indoor exercise space. Dogs were health checked daily by an animal technician separate from the research team, with a plan in place for any dog exhibiting signs of illness (weight loss, bloody stool, or dehydration) to be removed from the study, treated, and returned to the study once the issues were resolved.

During the study, the dogs were fed once daily in the morning in stainless steel bowls placed in holders attached to the kennel door. An indigestible TiO_2 marker (0.5%) was mixed into the crushed kibble for each dog individually at feeding to measure ATTD. To ensure that all digestibility marker was consumed, the dog food was mixed with equal parts water directly before feeding. On the first day of each period, dogs were weighed after an overnight fast using a

standardized walk on scale. Feed allowance was adjusted at the start of each period to maintain

BW, based on historical feed allowance (27.60 ± 8.59 kcal ME/kg BW), and current BW.

The study was conducted as a replicated incomplete 6×6 Latin square design. Four dogs

housed in pairs and were fed the same diets to ensure no cross contamination due to coprophagy.

Each period consisted of 10-d, 6-d of acclimation to the new test diet followed by 4-d for feces

collection.

3.2.3   Sample preparation and laboratory analyses

At collection, fecal samples were scored for consistency on a scale from 1 to 7, with 1

being extremely dry and 7 being extremely watery diarrhea. Fecal samples were collected fresh

in Whirl-Pak bags (Thermo Fisher Scientific, Waltham, MA) then frozen and stored at -20°C. At

the end of each period, fecal samples were pooled by dog and homogenized prior to being dried

in a forced air oven at 45°C and subsequently finely ground through a 1-mm screen (Wiley mill

model 4, Thomas Scientific, Swedesboror, NJ).

Diets and dried feces were analyzed in the Department of Animal Biosciences at the

University of Guelph for dry matter and ash (method 930.15; AOAC, 2006), crude fat (method

920.39A) using a XT-15 Extractor (Ankom Technology, Fairport, NY) and crude protein by

Leco ($N \times 6.25$; method 990.03). Fecal samples were analyzed for TiO_2 as described by Myers et

al. (2004) with slight modification (digestion of samples for 24 h at 120°C in 10 mL tubes,

precipitation in 100 mL volumetric flasks followed by H_2O_2 addition). Fecal samples were

analyzed for metabolites including short chain fatty acids (SCFA), branched chain fatty acids

(BCFA), lactic acid, glucose, arabinose, and lactose using an Agilent HP1000 series high-

performance liquid chromatography system (HPLC; Agilent Technologies, Santa Clara, CA; Templeman et al., 2020). At the University of Alberta, feces and diets were analyzed for gross energy using a bomb calorimeter (model 5003; Ika-Werke, Staufen, Germany).

3.2.4 Calculations and statistics

Calculations for ATTD of OM, ash, EE, CP, and GE of the test diets were completed using the index method (Adeola, 2001)

$$ATTD = 100 - (100 * \frac{Mdiet * Nf}{Mf * Ndiet})$$

Where N_{diet} was the concentration of the nutrient in the diet, N_{df} was the concentration of the nutrient in feces, M_{diet} was the concentration of TiO_2 in the diet, and M_{df} was the concentration of TiO_2 in feces.

Data analysis was completed using the GLIMMIX procedure of SAS (2016) in a 2×3 factorial with contrasts, with diet as fixed effect and square, period, and dog as random effect. The carry-over effect, normality, and homogeneity of variance of residuals was tested for each variable prior to analysis. Comparisons between dog and pig ATTD data were analyzed in SAS (2016) using linear regression. Outliers were determined using Cook's D and removed before R^2 values were calculated. Differences were considered significant if $P < 0.05$.

3.3   Results

During the study one dog refused to consume the test diets and was removed from the study after the first period. One dog showed signs of illness and was removed from the study for one period before re-entering the study in the following period. The effected the number of observations per diet, with 9 observations for all diets except HA and HC, that had 8 and 10 observations respectively.

The ATTD of OM and GE was lower ($P < 0.05$; Table 3) for H than L diets, while ash ATTD was 6.3% greater ($P < 0.05$) for H diets than L diets. The sole difference for A vs C was 1% lower ($P < 0.05$) ATTD of EE for A than C. An interaction between protein level and treatment was observed ($P < 0.05$) for ATTD of CP. The ATTD of CP was lower ($P < 0.05$) for enzyme pretreatment (P) than controls, however, the LP diet had lower ATTD of CP compared to the HP ($P < 0.05$).

Interactions between protein level and treatment were observed ($P < 0.05$; Table 4) for arabinose, glucose, and xylose. Fecal glucose and xylose concentrations were greater ($P < 0.05$) for A than C and for H diets, but not L diets. Fecal arabinose concentrations were lower ($P < 0.05$) for P than C and for L diets but not H diets. Fecal acetic and propionic acid concentrations were greater ($P < 0.05$) for P and A than C. Fecal lactic acid concentrations were lower ($P < 0.05$) and n-butyric acid concentrations were greater ($P < 0.05$) for P than C. Fecal lactose and lactic acid concentrations were greater ($P < 0.05$) for H than L diets, while acetic and iso-butyric acid concentrations were lower ($P < 0.05$) for H than L diets.

An interaction between protein level and treatment was observed for fecal consistency score ($P < 0.05$; Table 5). While fecal consistency score was greater ($P < 0.05$) for A than C for

both L and H diets, fecal consistency score was greater ($P < 0.05$) for the HA than the LA diet. Fecal consistency score was greater ($P < 0.05$) for P than C. Fecal moisture was lower ($P < 0.05$) for L than H diets. Fecal moisture was 2.5% greater ($P < 0.05$) for A than C diets.

The ATTD of OM, CP, ash, and GE was greater for pigs than dogs (Table 6; Figure 1), with the intercepts of OM, CP and GE being negative. The association between ATTD of OM, CP, and GE for pigs and dogs was strong ($R^2 > 0.7$) and was weak for ATTD of ash ($R^2 < 0.7$).

3.4   Discussion

In the present study, the addition of enzymes after extrusion did not increase ATTD but changed fecal metabolites including glucose, xylose, and acetic and propionic acids, and increased fecal moisture. The addition of enzymes before extrusion reduced ATTD and increased fecal metabolites. Pigs fed the same diets were a good model to predict ATTD of nutrients for dogs in the present study, despite ATTD being greater in pigs than dogs.

3.4.1   Digestibility

To the authors' knowledge, the present study is the first to report *in vivo* digestibility and fecal metabolites of vegan pulse-based dog foods using a canine total tract digestibility model. In the present study, the ATTD of OM was lower for the HC diet than LC diet, likely due to the greater ANF content of the pea and lentil protein concentrates. The ANF such as fiber and phytate, are concentrated together with protein during the production of pulse protein concentrates (Fenn et al., 2022). These ANF reduce digestion of nutrients through, e.g., nutrient

encapsulation and inhibition of digestive enzymes (Blow et al., 1974, Selle at al., 2012, Brummer et al., 2015) that likely reduced OM digestibility for the H diet.

The average ATTD of OM, and CP for the control diets in the present study was 7.8 and 2.0% lower respectively than values reported for premium grain free dry dog food that contained animal protein (Cargo-Froom et al., 2019). The ATTD values for the present control diets were similar to those reported for traditional dry dog foods containing cereal grain and animal protein (Hendriks et al., 2013), and for a wheat and rice based vegan dog food (El-Wahab et al., 2021) which had ATTD of CP ranging from 78-84%, and ATTD of OM ranging from 82-88%. Extrusion of pulses can reduce or eliminate ANF including phytic acid, trypsin inhibitors, and IDF (Berrios et al., 2010, Adamidou et al., 2011, Hugman et al. 2021a, Hugman et al. 2021b). Vegan extruded dog food with high pulse inclusion can be exposed to sufficient heat during extrusion to increase digestibility and achieve similar ATTD as traditional dry dog foods, even without the addition of enzymes. Notably, diets in the present study were formulated to test the study hypotheses and were not commercial dog foods. Both L and H diets included more pulse flour or protein concentrates, respectively, than commercial extruded vegan dog food is likely to contain. As such, our results may not be representative of what could be achieved with a commercially available vegan dog food that uses less pulse ingredients. Longer term studies to examine the long-term effects of different extruded vegan dog foods is warranted as unlike agricultural animals, dogs may consume a similar formula for the majority of their life and as such, studies that are at least 26 weeks long may be warranted.

Feed enzymes have been studied extensively in livestock, with results of most studies indicating biologically significant increases in feed digestibility and animal performance (Cowieson and Roos, 2013, Zouaoui, et al., 2018). In the present study, enzymes added after

extrusion did not increase ATTD of OM, CP, EE, ash, or GE. These results are consistent with findings of other studies examining the use of enzymes in dog diets with various ingredients that did not observe an effect of enzymes on digestibility (Sá et al., 2013; Risolia et al., 2019; Machado et al., 2021). However, ATTD of DM, CP, ash, and GE were greater when enzyme blends were added to dry dog food after extrusion in two studies (Twomey et al., 2003; Silva et al., 2016). In these, enzymes used were a blend of xylanase (340 U/kg), β-glucanase (300 U/kg), and amylase (1 U/kg) that was sprayed on the kibble at feeding (Twomey et al., 2003), or solely xylanase (80,000 U/kg) added as dry coating on the kibble after extrusion (Silva et al., 2016). A lack of effect on digestibility was observed when diets were supplemented with little enzyme (1.5-15 U/kg) which may have been insufficient to increase digestibility (Sa et al., 2013; Machado et al., 2021). However, in the present study, more enzymes were added than in previous studies where ATTD was greater following enzyme supplementation; thus, lack of enzyme activity is an unlikely reason for not observing enzyme effects on ATTD. Increased SCFA in feces in the present study does imply enzyme efficacy; however, enzyme activity may have increased bacterial fermentation and changed fecal consistency without observable increases to ATTD.

Heat can denature protein and therefore inactivate exogenous enzymes during extrusion (Sa et al., 2013). To increase the likelihood of effects from enzymes added prior to extrusion on digestibility, the main pulse ingredients were incubated with enzymes prior to extrusion in the present study. During this process, reactions likely occurred that reduced chemically available Lys content in the E+P diets. Heat can stimulate Maillard reactions and oxidation of sulfur AA and heat sensitive vitamins (Tran et al., 2008). The Maillard reaction occurs when proteins interact with reducing sugars during heat processing to form Maillard reaction products such as

M*N*-ε-2-furoylmethyl-L-lysine and *N*-ε-(carboxymethyl) lysine that hinder protein digestion and reduce Lys bioavailability (Hofmann et al., 2020). Recently, extrusion of pulses at 110 to 150°C with 18 to 22% moisture did not reduce reactive lysine or sulfur AA (Cargo-Froom et al., 2022). However, the P diets in the present study did have lower chemically available lysine content than the C and A diets (Van Straten et al., 2023). These effects observed with the P diets in the present study likely occurred during enzyme treatment and subsequent drying prior to extrusion. The greater reduction in ATTD of CP in LP than HP diets might be due to longer drying during enzyme treatment of the LP diet.

3.4.2 *Fecal metabolites and feces quality*

Analysis of fecal metabolites can provide insights into effects of dietary treatments on dog intestinal health and digestion (Suchodolski, 2022). In the present study, fecal fermentation product concentrations were different between control and enzyme supplemented diets, especially for the H diets. Greater fecal short chain fatty acid (SCFA) acetic and propionic acid concentrations for A than C diets indicate increased bacterial fermentation. In addition, fecal glucose and xylose were also greater for A than C diets. The supplemental enzymes cellulase and xylanase hydrolyze the bonds of cellulose and hemicellulose (arabinoxylans), thereby converting these insoluble fibers into soluble sugars including glucose, xylooligosaccharides, and xylose (Puls, 1997; Ejaz et al., 2021). These released soluble sugars are fermentable by gut bacteria (Singh et al., 2021). Combined, greater fecal glucose, xylose, and propionic and acetic acids concentrations in the present study indicated that enzymes added to the dog food after extrusion did hydrolyze some dietary cellulose and hemicellulose into glucose and xylose that were then

available for bacterial fermentation, resulting in more SCFA in feces. Our results are consistent with previous research using carbohydrase and xylanase enzymes added to canine and poultry diets that also increased SFCA production, particularly acetate and propionate (Dale et al., 2020; Machado et al., 2021; Singh et al., 2021). Fermentation by intestinal bacteria uses and creates compounds which may affect ATTD calculations. Changes in digesta consistency due to fiber content and enzyme activity may also have affected the ATTD values.

Dog foods with IDF:SDF ratios of 3:1 or higher may increase fecal moisture, creating undesirably wet feces (Burkhalter et al., 2001). The control diets in the present study did result in feces with greater than desirable fecal consistency score and moisture content, likely resulting from IDF:SDF ratios of up to 49:1 due to the IDF content of the pulse ingredients (Van Straten et al., 2023). Dietary supplementation of enzymes after extrusion further decreased fecal quality by increasing fecal moisture content. The sugars resulting from endogenous enzyme hydrolysis of insoluble fiber are hygroscopic, and can reduce digesta viscosity, increase passage rate, increase fecal moisture, and may reduce nutrient absorption (Machado et al., 2021). Increased bacterial fermentation and SCFA production have been associated with improved intestinal health and fecal consistency in dogs (Pilla and Suchodolski, 2020). Despite increased fermentation and SCFA concentration, enzyme addition to dry dog foods increased fecal moisture and decreased fecal quality in the present and other studies (Machado et al., 2021). While enzyme addition did not affect fecal consistency in some studies (Sá at al., 2013; Silva et al., 2016; Risolia et al., 2019), the present study used high quantities of enzyme activity that may have caused greater effects on fecal consistency. Dogs have, relatively, a shorter large intestine than monogastric omnivorous species such as humans and pigs (Kararli, 1995). The quantity of sugars produced by

the enzymes in the present study was likely too much for the bacteria in the dog's large intestine to metabolize. These effects may be mitigated by using less enzyme activity in future studies.

3.4.3 Dog and pig comparison

While regulations exist for using dogs in research to ensure that their basic needs are met (National Research Council, 2010), the public often opposes companion animal research (Ormandy and Schuppli, 2014). As welfare concerns over laboratory companion animals grow, the use of dogs in invasive research is becoming difficult, necessitating the use of livestock as translational models for dog nutrition studies. The ileal cannulated growing pig model may offer some benefits over the cecectomized rooster model. Unlike cecectomized roosters, both digesta and feces can be collected from ileal cannulated pigs, allowing for evaluation of both ileal and total tract digestibility, fecal quality, and intestinal microbiome and health. To make an accurate comparison, both dogs and pigs studied were fed the same diet formulations produced from the same batches of extruded dog food. When compared directly, pigs had on average 10.5, 7.23, 27.1 and 8.85% greater ATTD of OM, CP, ash, and GE, respectively, than dogs. Pigs have a bigger large intestine than dogs (Ormandy and Schuppli, 2014), which may explain the greater ATTD values for pigs. A bigger large intestine allows for greater bacterial fermentation of undigested nutrients that may cause overestimation of nutrients digested and absorbed in the small intestine.

Despite potential differences in pig and dog digestive capacity and physiology, regression analyses from the present study indicated that pigs may be a good model for canine digestion. Both canine and swine models followed a similar trend, with lower ATTD of OM for H than L

diets in both pigs and dogs, and reduced ATTD of CP for P diets for both species (Van Straten et al., 2023). Regression analysis indicated a strong association between swine and dog ATTD of CP, OM, and GE. In contrast, ATTD of ash had a weak association between the two species. In dogs, few studies have measured mineral digestibility. Most canine digestibility studies do not measure ATTD of ash; however, dog's true mineral digestibility may be greater than ATTD as the dog's endogenous losses of minerals does not appear to adapt to changes in mineral content of the dog food (Cargo-Froom et al., 2019). While ATTD values may be greater for pigs than dogs, overall, this comparison does demonstrate that pigs may be an accurate model to determine trends and predict ATTD of various dog food treatments.

In the pig model, standardized ileal digestibility (SID) of amino acids for the vegan pulse-based diets ranged from 70 to 99% (Van Straten et al., 2023). However, in the present study pig ileal digestibility could not be compared to dogs, because it was not measured in dogs. Therefore, conclusions cannot be drawn from the present study regarding the suitability of pigs as a model for canine ileal digestibility of CP and AA. However, gently cooked vegan dog foods fed recently in cecectomized roosters ranged from 70 to 100% in ileal digestibility of amino acids (Roberts et al., 2023). Cecectomized roosters have been used as models for dog food research, with some studies finding good correlation between rooster and dog ileal digestibility (Johnson et al., 1998). Given similarities in results between these pig and rooster models fed vegan dog foods, pigs may also be an accurate model for canine ileal digestibility.

In conclusion, ATTD of CP, OM, and GE for pulse based vegan dog food was similar to previously reported values for dog foods containing meat and cereal grain. The addition of an enzyme blend after extrusion did not increase ATTD of CP, OM, GE, or ash. Nonetheless, increased glucose, xylose, propionic and acetic acid in dog feces did indicate enzymatic

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hydrolysis of cellulose and hemicellulose and increased microbial fermentation. Addition of enzymes prior to extrusion decreased ATTD of CP, likely due to overheating during diet processing. However, comparing dog ATTD with pig ATTD for the same diets, a strong association was detected between the two species, indicating that pigs could be an accurate translational model for testing ATTD of dog foods.

3.5 Tables and figures

Table 6.1. Ingredient composition and calculated nutritional analysis of experimental diets

Variable	Low protein diet	High protein diet
Ingredient, % as is		
Field pea protein concentrate	5.20	50.0
Field pea flour	35.0	1.30
Lentil protein concentrate	5.20	20.0
Lentil flour	15.0	1.30
Hulled barley	12.2	6.00
Oat flour	12.2	6.00
Canola oil	8.51	8.86
Vegetable digest	4.00	4.00
Limestone	1.00	1.20
Calcium monophosphate	0.50	0.10
Choline chloride	0.20	0.25
Vitamin premix	0.20	0.20
Salt	0.20	0.20
DL-methionine	0.20	0.20
Mineral premix	0.10	0.10
Potassium chloride	0.10	0.10
Taurine	0.10	0.10
Naturox dry[1]	0.05	0.05
Naturox liquid[1]	0.03	0.03
L-carnitine	0.02	0.02
Estimated analysis, % DM		
Crude protein	22.0	41.4

Crude fat	11.2	11.1
Soluble carbohydrates	58.2	42.3
Ash	4.73	4.68
Metabolizable energy (Mcal/kg)	3.76	3.87

[2]Kemin Industries Inc., Des Moines, IA.

Table 3.2. Analyzed nutrient profile of experimental diets (dry matter basis)[1]

Variable	LC	LP	LA	HC	HP	HA
CP	24.6	25.4	24.5	44.1	44.3	44.3
EE	9.55	9.50	9.36	11.6	9.84	11.8
Ash	5.55	6.18	5.56	6.83	6.84	6.79
GE (Mcal/kg)	4.99	4.99	4.88	5.19	5.24	5.15

[1]Low protein (L), High protein (H), enzymes added prior to extrusion (P), enzymes added after extrusion (A).

Table 3.3. Apparent total tract digestibility (ATTD) of organic matter (OM), crude protein (CP), crude fat (EE), ash, and gross energy (GE) of experimental diets[1]

ATTD	Protein level			Treatment				P-value			
	L	H	SEM[2]	C	P	A	SEM[3]	L vs H	C vs P	C vs A	Protein × treatment
OM	85.1	78.3	0.637	81.4	81.0	82.8	0.760	< 0.001	0.922	0.353	0.962
CP[4]	81.4	85.9	0.537	86.3	78.4	86.3	0.643	< 0.001	< 0.001	0.998	0.015
EE	94.3	94.2	0.286	95.2	94.7	94.2	0.334	0.649	0.017	0.001	0.562
Ash	34.7	41.0	2.39	34.6	40.4	38.6	2.60	0.006	0.071	0.282	0.125
GE	85.2	79.8	0.617	82.6	81.6	83.2	0.724	< 0.001	0.519	0.818	0.862

[1] Low protein (L), High protein (H), enzymes added prior to extrusion (P), enzymes added after extrusion (A).

[2] Means based on 26-30 observations per formulation.

[3] Means based on 18-20 observations per treatment.

[4] Protein × processing interaction: 84.9^{ab}, 74.6^{c}, 84.7^{ab}, 87.7^{a}, 82.2^{b}, and 87.8^{a} for LC, LP, LS, HC, HP, and HS respectively. a-c: means without a common superscript differ ($P < 0.05$).

Table 3.4. Fecal metabolites for experimental diets (dry matter basis)[1]

Metabolite, µmol/g	Protein level			Treatment				P-value			
	L	H	SEM[2]	C	P	A	SEM[3]	L vs H	C vs P	C vs A	Protein × treatment
Arabinose[4]	18.7	34.6	3.13	32.1	21.9	26.0	3.59	< 0.001	0.024	0.347	0.024
Glucose[5]	41.2	50.2	4.59	35.6	31.8	69.6	5.03	0.040	0.728	< 0.001	< 0.001
Lactose	31.4	48.0	2.36	43.5	36.9	38.6	2.54	< 0.001	0.019	0.104	0.960
Xylose[6]	35.6	110.5	8.45	64.8	42.4	111.9	9.78	< 0.001	0.157	0.001	0.003
Lactic acid	849	1216	119	1105	826	1165	123	< 0.001	0.004	0.749	0.677
Acetic acid	295	248	20.7	201	344	270	21.8	0.002	< 0.001	< 0.001	0.747
Propionic acid	137	99.2	7.64	91.2	141	122	8.05	< 0.001	< 0.001	< 0.001	0.051
Iso-butyric acid	15.7	10.2	2.05	12.8	11.1	14.9	2.16	< 0.001	0.325	0.201	0.145
N-butyric acid	11.1	10.2	2.12	6.78	17.0	8.04	2.39	0.688	<0.001	0.645	0.287

[1] Low protein (L), High protein (H), enzymes added prior to extrusion (P), enzymes added after extrusion (A).

[2] Least squared means based on 26-30 observations per formulation.

[3] Least squared means based on 18-20 observations per treatment.

[4] Protein × processing interaction: 31.1[ab], 9.18[c], 15.8[bc], 33.1[ab], 34.6[a], and 36.1[a] for LC, LP, LS, HC, HP, and HS respectively. a-c: means without a common superscript differ ($P < 0.05$).

[5] Protein × processing interaction: 39.9[b], 31.6[b], 52.0[b], 31.4[b], 31.9[b], and 87.3[a] for LC, LP, LS, HC, HP, and HS respectively. a-b: means without a common superscript differ ($P < 0.05$).

[6] Protein × processing interaction: 28.8[c], 26.9[c], 51.1[bc], 100.9[b], 58.0[bc], and 172.6[a] for LC, LP, LS, HC, HP, and HS respectively. a-c: means without a common superscript differ ($P < 0.05$).

Table 3.5. Fecal moisture content and fecal consistency scores (1-7) for experimental diets, with 1 being extremely dry, and 7 being extremely wet diarrhea[1]

Variable	Protein level			Treatment				P-value			
	L	H	SEM[2]	C	P	A	SEM[3]	L vs H	C vs P	C vs A	Protein × treatment
Fecal score[4]	5.13	5.92	0.224	4.89	5.57	6.13	0.234	< 0.001	< 0.001	< 0.001	< 0.001
Moisture, %	79.0	80.4	0.978	78.7	79.0	81.5	1.03	0.034	0.664	0.001	0.720

[1] Low protein (L), High protein (H), enzymes added prior to extrusion (P), enzymes added after extrusion (A).

[2] Means based on 26-30 observations per formulation.

[3] Means based on 18-20 observations per treatment.

[4] Protein × processing interaction: 4.19^c, 5.74^b, 5.48^b, 5.59^b, 5.41^b, and 6.78^a for LC, LP, LS, HC, HP, and HS respectively. a-c: means without a common superscript differ ($P < 0.05$).

Table 3.6. Comparison of apparent total tract digestibility (ATTD) of organic matter (OM), crude protein (CP), ash, and gross energy (GE) for adult dogs and growing pigs fed the same experimental diets[1]

Item, %	OM		CP		Ash		GE	
	Dog	Pig	Dog	Pig	Dog	Pig	Dog	Pig
LC	84.9	93.3	84.9	91	28.8	57.2	85.4	92.4
LP	84.2	90.8	74.6	83.5	37.1	64.6	84.1	89.5
LA	86.3	93.6	84.7	93.4	38.2	69.3	86.2	92.7
HC	77.9	92.2	87.7	92.3	40.5	57.2	79.9	91.6
HP	77.7	91.3	82.2	90.9	43.6	64.5	79.2	90.4
HA	79.2	91.9	87.8	94.2	38.9	77.1	80.2	91.5
R^2	0.894[2]		0.893		0.097[3]		0.807[2]	
Intercept	-260		-26.0		46.8		-223	
Slope	4.01		1.21		0.189		3.34	

[1] Low protein (L), High protein (H), enzymes added prior to extrusion (P), enzymes added after extrusion (A).

[2] LP removed due to being an outlier.

[3] LC was removed due to being an outlier.

Figure 1. Linear regression of apparent total tract digestibility (ATTD) of organic matter (OM) for adult dogs and growing pigs fed the same diets
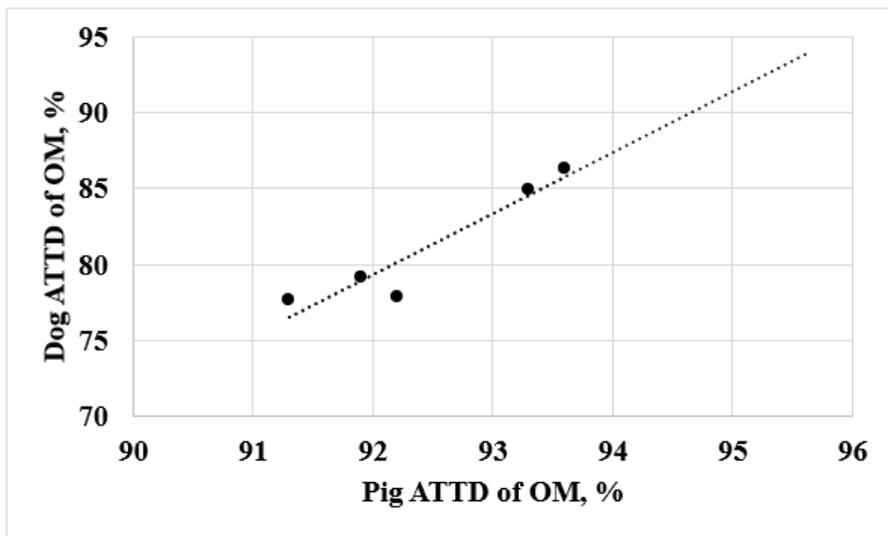
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CHAPTER 4. GENERAL DISCUSSION

4.1   Project Summary and Conclusions

The main objectives of this thesis were to determine if extrusion and the addition of an enzyme blend either before or after extrusion could increase the digestibility of pulse flour or protein concentrates based vegan dog foods in pigs and dogs. We first used ileal cannulated growing pigs to demonstrate that extrusion, and the addition of an enzyme blend after extrusion, can increase the ATTD of CP, GE, and ash, and SID of CP and AA (Van Straten et al., 2023a; chapter 1). We also demonstrated that the process of incubating enzymes with pulse-based vegan dog foods prior to extrusion can reduce digestibility due to Maillard reactions during heat processing. Large breed mixed hounds were then fed the same diets, and while no increases in ATTD were observed for the dogs when enzymes were added to the food, there were increased concentrations of glucose, xylose, propionic and acetic acid in the dog's feces, which does indicate the enzymes added after extrusion were successful at hydrolyzing cellulose and hemicellulose in the foods (van Straten et al., 2023b; chapter 2). However, the addition of enzymes after extrusion also resulted in higher fecal moisture and loose stools. For the dogs we also demonstrated that the incubation of enzymes with pulse-based vegan dog foods prior to extrusion can reduce ATTD of OM, CP, and GE. Based on these results we can conclude that extrusion and the addition of enzymes after extrusion can increase the digestibility of pulse-based diets for growing pigs, and that enzymes added to pulse-based vegan dog foods after extrusion can impact dog fecal quality.

The secondary objective of this thesis was to compare ATTD results of pigs and dogs fed the same diets to determine if pigs can be used as a translational model for dog food digestibility studies. While the ATTD values from the pigs were greater than from the dogs, the results

followed similar trends with good fit (Van Straten et al., 2023b; chapter 2). From this comparison we can conclude that pigs can be used as a model to study the total tract digestibility of different dog food treatments. Future swine studies can have translational value to dogs, a species where use in controlled animal studies is becoming more challenging. Future studies could conduct similar research, but compare this to digestibility studies using client owned dogs and in home collection methods being developed.

4.2   Implications

Both animal trials completed in the present study have several practical implications for industry. In addition to implications for the pet food industry, the swine portion of this study also has value for pork producers. By using extrusion, the ATTD and SID of field pea and lentil flour and protein concentrate based diets was greater when measured in growing pigs ($P < 0.05$; Table 2.4 and Table 2.6), thereby improving the nutritional quality of these ingredients for growing swine. The addition of an enzyme blend further increased ATTD and SID of the diets ($P < 0.05$), further increasing the nutritional value of these ingredient for growing swine. Depending on the costs of extrusion and enzymes, these treatments could be used by the Canadian swine industry to better utilize locally grown pulse crops in their herds.

With respect to the pet food industry, the digestibility of pulses is also a concern, especially for vegan dog foods due to their high inclusion of pulses. In the canine portion of the present study (Chapter 3), the control diets containing 60-73% pulse ingredients resulted in 81.4% ATTD of OM and 86.3% ATTD of CP (Table 9). These values are similar to values previously reported for traditional cereal grain and animal protein inclusive dog foods (Hendriks

et al., 2013; Corsato Alvarenga and Aldrich, 2018). While more research is needed to ensure the nutritional adequacy of vegan dog foods, the data collected in the present study is a good start for the validation of vegan dog foods as an option for dog owners. Vegan dog foods are an appealing option for many dog owners, especially vegan dog owners, due to perceived benefits for the environment, animal welfare, and animal health (Dodd et al., 2019; Vanderhoydonck, 2022). They are also an important option for owners whose dogs may have allergies to animal derived protein rich ingredients such as chicken. Surveys of dog owners have determined that many owners would consider feeding their dogs a plant-based food but are concerned about the nutritional adequacy of the plant-based dog foods (Dodd et al., 2019). While the diets used in the present study were not commercially available dog foods, the formulas resulted in fecal consistency scores which were not ideal (Table 3.5). The present study does suggest that vegan dog foods containing high levels of pulse flour and protein concentrate can achieve relatively high ATTD values, and when properly formulated with added sulfur AA may provide nutrients in surfeit for dogs. This is especially important considering the uncertainty surrounding the use of pulses in pet foods due to the FDA investigation regarding pulses in dog food and the hypothesized development of nutritionally mediated dilated cardiomyopathy in dogs (FDA, 2019). The result of this study may help reduce pet owners and veterinary professionals concerns about pulses in dog food, especially with regards to high pulse content vegan dog foods. This could not only help pet owners, but also the pet food industry and pulse crop growers who have been negatively impacted by the uncertainty caused by the FDA investigation.

Although the addition of enzymes to dog foods in the present study did not increase any measures of ATTD (Table 3.3), the knowledge gained from this work is nonetheless valuable. Gut health and fecal quality are an issue of concern for dog owners, as foods that cause softer

feces can make clean up difficult. In the present study, differences in fecal quality and fecal metabolites occurred for diets with different processing. Less desirable soft tools with higher fecal consistency scores (P <0.05) occurred when the dogs were fed diets with enzymes added after extrusion compared to the other diets (Table 3.5). Higher fecal consistency scores and fecal moisture (P < 0.05) also occurred for the high protein diet compared to the low protein diet. These results also corresponded to differences in fecal metabolites (table 3.4). These insights into the relationship between dog food ingredients, processing, microbial fermentation, and fecal quality may be beneficial to the pet food industry and future research.

The final part of this project, the comparison of results between species, is also an important aspect of the present study. Despite guidelines being in place for the care of laboratory dogs, people may view dog research as inhumane, leading to the introduction of more restrictions on canine nutrition research. In the present study, comparison of ATTD of the same diets in dogs and pigs resulted in a model with good fit (R^2 > 0.7; table 3.6). This research opens the possibility for more research using pigs for dog food studies. Using pigs allows for ileal digestibility to be assessed via cannulation, which would otherwise not be possible using dogs alone due to restrictions on research. Furthermore, with pigs it is possible to collect both feces and digesta samples, allowing for greater insights into particularly the protein and amino acid digestibility of dog foods and their effects on intestinal health than what would be possible with a cecectomized rooster model. Pigs are already heavily used for human nutrition research and should be considered more for canine research, as additional information gained regarding dog food by using pigs as a translational model would be beneficial for researchers, consumers and the pet food industry.

4.3   Limitations

Despite this thesis research being carefully designed and implemented, some limitations do exist due to unexpected events during the experiments, external restrictions, and the study design itself.

During both studies, pig and dog, animals were removed from study either temporarily or permanently, thereby reducing the number of observations for some treatments. For the swine experiment (chapter 2), one of the pigs fell ill during the third period. Despite the research team and animal care staff's best efforts, the pig continued to decline in health and was euthanized. Postmortem examination by a veterinarian determined the reason for the pig's poor health was likely congenital, unrelated to the experiment being conducted. Due to the pig being on medication before being euthanized, any sample collected during the treatment was not used in the statistical analyses. The euthanized pig was replaced with one of the extra cannulated pigs, however while the extra pig was being transitioned to the test diet it stopped eating. Due to the refusal to eat, a longer transition was required, and the extra pig missed taking part in the 4th period of the study. Due to these events, two of the treatments missed one observation each, a reduction that may have impacted the ability to detect significant treatment differences or be confident in the lack of differences.

For the dogs (chapter 3), unforeseen circumstances also resulted in fewer samples being collected than the study design planned for. One of the dogs refused to eat the test diets after the first period despite efforts to encourage the dog to eat by adding extra palatants or changing the food consistency. Due to refusal to eat, the dog was permanently removed from the study, resulting in one less observation for all but one of the treatments. One other dog was removed from the study for one period due to illness. This dog was returned to the study after treatment,

however this also resulted in the loss of one observation. Again, this reduction in observations may have impacted the ability to detect significant treatment differences.

The swine experiment and beginning of the canine experiment occurred while restrictions were still in place for the COVID-19 pandemic. While these restrictions had little effect on the swine study, changes were made for the dog study. Originally the study design planned for blood samples to be collected from the dogs to analyze amino acid bioavailability. Bioavailability of amino acids can be predicted by measuring the quantity of amino acids circulating in the blood and is partially dependent on the digestion of the food proteins and absorption of the resulting amino acids (Gaudichon and Calvez, 2021). Analysis of amino acid bioavailability would have been extremely useful for this study on plant-based dog food since plant amino acid bioavailability is impacted by the structure of the plant's chemical matrix and anti-nutritional factors (Capuano and Pellegrini, 2019). Unfortunately, due to the close contact with other people required to do blood draws from the dogs as one person needs to hold the dog while the other samples, the decision was made to cancel the blood sampling.

External restrictions also impacted the study through the types of dogs which were available for the study. The dogs used in Chapter 3 were acquired before the present study from a research animal breeding facility, with a high probability of some of the dogs being closely related. Digestive tract length, transit time and permeability can vary between different sizes and breeds of dogs (Fleischer et al., 2008). In particular, large breed dogs have been observed to have greater ATTD of CP and wetter feces compared to smaller dogs fed the same diets, especially when those diets contain higher levels of crude protein (Nery et al., 2010). This may be due to large dogs having greater gut permeability leading to greater electrolyte and water loss from the intestinal epithelium (Nery et al., 2010). While a relatively homogenous sample population

means that there are fewer confounding variables, it also means that the sample population may not be an accurate representation of the entire population of dogs that could be fed a pulse-based vegan diet. Additionally, due to being bred and raised in a research facility with minimal handling, the dogs in the present study displayed some behaviors consistent with poor early socialization. Poor early socialization may cause stress which impacts digestion by accelerating gastric emptying and intestinal transit time, resulting in stress induced diarrhea, and potentially reducing the digestion and absorption of nutrients (Dong et al., 2018; Fan et al., 2023). Therefor the dogs being stressed and the homogeneity of the sample population in the current study may have impacted the results of the study, especially in regard to fecal consistency.

The ages of dogs and pigs created a limitation for the present thesis research. Animal age impacts digestion, for example, juvenile animals produce fewer digestive enzymes and having lower absorptive capacity compared to adult animals (Lindemann et al., 1986). Senior animals also typically have reduced digestion and absorption of nutrients, especially protein, compared to younger adult animals (Laflamme, 2005). Given these age dependent differences, the studies in the present thesis have limited application depending on the ages of the animal used. For the swine study (chapter 2) this means the results may only be applicable for growing pigs. And the canine results (chapter 3) may only be applicable for young adult dogs. Results of the pigs and dogs were compared to determine if pigs could be a model for dogs despite the difference in age of the animals. A fully grown adult domestic pig is more difficult to perform cannulation surgery on and maintain due to their large size and increased body weight, therefore cannulation is made easier by using growing pigs. Despite this difference in age, results of the comparison indicate that growing pigs can be a good model for testing dog food treatments.

The inability to perform cannulation surgery on the dogs created a limitation for the current research. While comparing ATTD can at least provide some indication regarding the feasibility of using pigs as a model for canine nutrition studies, this method is not as accurate as comparing ileal digestibility. Pigs have a bigger large intestine than dogs (Ormandy and Schuppli, 2014). Differences in fermentation capacity mean ATTD may not be an accurate representation of actual digestibility of nutrients in the small intestine between the two species, therefore no inferences can be drawn from the current study regarding ileal amino acid digestibility for dogs. Comparing standardized ileal digestibility between growing pigs and dogs would give a better indication if the two species are similar enough for pigs to be used as a model for dogs. However, comparison if SID is not currently possible due to restrictions on canine research. When using pigs as a model, future researchers should acknowledge that the ATTD values acquired from pig research will be higher than what would be achieved with a dog. This needs to be clear or else the public and pet food industry may incorrectly interpret the information and assume dogs will have the same high digestibility percentage as the pigs. While the pig model can be useful for dog nutrition research, differences between the two species still need to be considered when interpreting results.

Finally, the diets themselves created some limitations. The diets in the present research were formulated specifically to test the effects of enzymes on pea and lentil flours and protein concentrates. As such, these ingredients made up most of the diets, in higher quantity than may be found in a commercially available vegan dog food. Table 4.1 presents the nutrient compositions of some commercially available vegetarian and vegan dog kibbles acquired prior to the work of this thesis research. The CP of these commercial diets is similar to the L diets in the current research; however, they are much lower in CP than the H diet. These commercial plant-

based diets often also include cereal grain ingredients such as rice and oats, as well as potato and potato protein (Natural Balance, 2023) in comparison to the diets used in the present research. As such, the result in these present studies may not be the same as what would be achieved with commercial vegan dog food. While the present results are still valuable for vegan pet owners, veterinarians and manufacturers, more research is needed to determine the nutritional adequacy of commercially available vegan dog foods.

Table 4.1. Chemical composition of commercial vegan dog foods purchased locally (dry matter basis)[1]

Brand	CP	Starch	EE	Ash	TDF	Crude fiber
Natural Balance	21.3+/-0.7	42.3+/-1.0	9.0+/-0.2	5.8+/-0.0	13.8+/-0.8	3.4+/-0.2
Halo	23.0+/-0.1	30.5+/-0.6	9.8+/-0.1	6.1+/-0.1	23.7+/-1.1	5.3+/-0.0

[1] data collected by Dr. Thava Vasanthan and Jun Goa, presented as mean +/- standard deviation.

Additionally, while these diets were formulated to meet AAFCO recommended nutrient levels for adult dogs, they were not formulated with fiber quantities to support ideal fecal quality. In dogs some research has indicated that an IDF:SDF ratio higher than 3:1 result in more wet feces and lower digestibility compared to lower ratios (Burkhalter et al., 2001). In the present studies the diets had ratios of up to 49:1 for IDF:SDF. The high IDF:SDF ratio of these diets may have resulted in digestibility results and fecal quality which are different from what could be achieved with a commercial vegan dog food. Starch cook was also not measured during the chemical analysis of the test diets. The degree of starch gelatinization and amount of resistant starch in dog kibbles can vary depending on extrusion conditions (Corsato Alvarenga et al., 2021). The amount of resistant starch in dog foods can impact dog intestinal health and may also

have an impact on fecal quality (Peixoto et al., 2018). Again, this highlights the limitation of these results for application in vegan dog food production.

4.4   Future research

Based on the results and limitations of the present research, there is opportunity for future research on the topics of vegan dog food and enzymes added to the extruded pulse-based diets for both pigs and dogs. Firstly, given the positive effects of the enzymes on increasing digestibility of the diets for pigs, and the effects on canine fecal quality, it may be beneficial for future research using enzymes in pulse-based diets to examine effects on gut health. Increased fermentation due to enzymatic breakdown of insoluble fiber into substrates usable by large intestinal bacteria may have benefits for pig intestinal and overall health (Singh et al., 2021). -based diets impact gut health for both pigs and dogs. In dogs these studies may be better carried out using a lower quantity of enzymes added to the food. Since there was an increase in digestibility for the pigs, it may also be possible to achieve increased digestibility for the dogs. However, a lower quantity of enzymes should be used to potentially help prevent negative effects on fecal quality. Overall, there is still potential for more research using enzymes for both dog and pig diets.

Given that the diets used in this present research were not commercial diets, it would also be beneficial for more research to be conducted using commercially available vegan dog foods. Especially considering that evaluations of some commercially available vegan dog food have found them to not meet all AAFCO recommended minimum nutrient levels (Zafalon et al., 2020). To date there have been few studies published regarding the nutritional adequacy of

commercial vegan dog food. The published studies are either only laboratory analyzed nutrient values (Zafalon et al., 2020), or digestibility studies using roosters fed fresh gently cooked foods (Roberts et al., 2023). To the author's knowledge, no studies have yet been published that examine the digestibility and bioavailability of commercial vegan dog kibble in an in vivo canine study. Blood sampling to assess nutrient bioavailability and indicators of cardiac health would be extremely beneficial for this future research, especially given the ongoing controversy regarding pulses and DCM in dogs. The sulfur AA methionine is a limiting AA in pulses (Table 2). This AA is essential for the production of taurine, an AA that is important for cardiac health (Sanderson et al., 2001). With proper formulation, high pulse inclusion diets can meet AA requirements and support good cardiac health in dogs (Singh, 2023). However more research is needed to ensure commercially available vegan dog foods are meeting the physiological requirement for nutrient for the maintenance of good health in dogs. Studies using different ages and breeds of dogs would also be beneficial. These future evaluations of commercially available vegan dog foods may give valuable information to help improve the nutritional quality of these foods, as well as increase professional and consumer confidence in commercial vegan dog food options.

A final area of future research could be to conduct more comparison studies between dogs and pigs to further evaluate the accuracy of pigs as a model for canine nutrition. The present study found pigs to be an accurate ATTD model using vegan dog kibbles, however this is no guarantee that pigs would be an accurate model for other types of dog food. Therefore, further testing should be done using a variety of dog food types. Additionally, since cecectomized roosters are more commonly used and accepted as a model for dog food studies, it may be beneficial to compare results from cecectomized roosters and ileal cannulated pigs fed the same

dog foods. More evidence in support of pigs being an accurate model for dog food studies would help increase industry and public confidence in studies that may use this model and fully utilize translational animal models.

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