THE EFFECTS OF FEEDING PULSE-BASED, GRAIN-FREE, DIETS ON DIGESTIBILITY, GLYCEMIC RESPONSE, AND CARDIOVASCULAR HEALTH IN DOMESTIC DOGS

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ABSTRACT

The following thesis will focus on the effects of feeding pulse-based, grain-free, diets on digestibility, glycemic response, and cardiovascular health in dogs. In July of 2018, the United States Food and Drug Administration reported a link between grain-free diets and dilated cardiomyopathy (DCM) in dogs. Despite this, there is no definitive evidence confirming or demonstrating that grain-free diets are unsafe to feed to dogs. Due to this statement, there is currently major controversy surrounding whether or not pet owners should be feeding grain-free diets to dogs.

The first study conducted was a 7-day short-term feeding trial that involved six different experimental diets. All diets were formulated to include 20% available carbohydrate using either rice (grain-containing) or pulse flours from either smooth pea, fava bean, red lentil, or wrinkled pea varieties (4140-3 or Amigold). It was hypothesized that dog diets with higher levels of dietary fiber would produce a lowered glycemic response due to decreased rates of digestion and lowered bioavailability of macronutrients, as well as increased fecal bile salt excretion. This in turn was hypothesized to produce lowered plasma concentrations of cystine, cysteine, methionine, and taurine. This study demonstrated that pulse-based diets have the potential to reduce postprandial glucose response, which is beneficial for dogs. After 7-days of feeding each diet, there was a trend of reduced fasted plasma taurine levels in the dogs consuming pulse-based diets, with exception to the lentil diet. However, all plasma taurine levels remained within normal limits. Decreased apparent total tract digestibility of macronutrients and amino acids were also observed and associated with increasing levels of both amylose and dietary fiber, however, a specific causative agent for this could not be determined due to the nature of this

study. Surprisingly, increases in dietary fiber within the diets led to decreased fecal bile acid excretion.

The second study was a longer 28-day feeding study, that tested the pulse-based diets with the highest and lowest levels of amylose from study one and compared them to an experimental rice-based diet and a commercial grain-containing diet. For the second study, it was hypothesized that dogs fed pulse-based, grain-free diets for 28 days would cause decreased apparent total tract digestibility of macronutrients, increased fecal bile acid excretion, and decreased plasma levels of cystine, cysteine, methionine, and taurine, resulting in sub-clinical cardiac or blood changes indicative of early stages of DCM. After conducting echocardiography, it was observed that the high amylose wrinkled pea (Amigold) diet in study two increased left ventricular size and volume, as well as increased plasma levels of NT-ProBnP, in a sub-clinical manner that was reversible. These cardiac changes representative of early-stage DCM are possibly associated with the increased level of amylose within the wrinkled pea (Amigold) diet. The second study confirmed from study one that grain-free diets did not cause plasma taurine levels to fall below normal limits. After consuming all diets (both grain-containing and grain-free), plasma levels of taurine were unchanged and remained within normal limits. In contrast, all grain-containing and grain-free diets caused reduced plasma methionine levels. Similar to study one, apparent total tract digestibility of macronutrients and amino acids, excluding taurine, was reduced when the dogs consumed the pulse-based diets possibly due to higher levels of fiber. Both studies showed pulse-containing diets decreased fecal bile acid excretions.

Overall, slight DCM-like changes from the wrinkled pea diet, but not the lentil diet, demonstrated that not all grain-free diets can be treated the same. In addition to this, if diet-induced DCM-like changes are in fact associated with increased levels of dietary amylose, this could pose an issue to both grain-free and grain-containing diets. This complicates the original claim made by the FDA regarding grain-free diets, as diet-induced DCM in dogs may not be exclusively associated with grain-free diets.

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LIST OF ABBREVIATIONS

AAFCO - Association of American Feed Control Officials

ALP - Alkaline phosphate

ALT - Alanine aminotransferase

AMY2B - Alpha amylase 2B

AOAC - Association of Official Agricultural Chemists

ATTD – Apparent total tract digestibility

AUC – Area under the curve

CBC – Complete blood count

CK - Creatine kinase

CO – Cardiac output

DCM - Dilated cardiomyopathy

DM – Dry matter

EDTA - Ethylenediaminetetraacetic acid

EF – Ejection fraction

FDA - Food and Drug Administration

GGT - Gamma-glutamyl transferase

GLDH - Glutamate dehydrogenase

HMWDF - High molecular weight dietary fiber

HR – Heart rate

LMWDF - Low molecular weight dietary fiber

LVID - Left ventricular internal diameter

LVIDD - Left ventricular internal diameter diastolic

LVIDS - Left ventricular internal diameter systolic

LVEDV - Left ventricular end diastolic

LVESV - Left ventricular end systolic volume

ME - Metabolizable energy

MGAM - Maltase-glucoamylase

NFC - Non-fiber carbohydrates

NT-ProBNP - N-terminal pro-brain natriuretic peptide

PCA - Principal components analysis

RDS - Rapidly digested starch

RS - Resistant starch

SDS - Slowly digested starch

SGLT1 - Sodium-glucose cotransporter 1

SV – Stroke volume

TDF - Total dietary fiber

TDN - Total digestible nutrients

V Max – Maximum velocity

VTI A-wave - velocity time integral A-wave

VTI E-wave - velocity time integral e-wave

1 CHAPTER 1: INTRODUCTION

1.1 Overall Preface

The following thesis examines the impacts of grain-free compared to grain-containing diets on domestic dog health regarding glycemic response, apparent total tract digestibility, and cardiovascular health. Other end points such as fecal bile acid excretion, fasted plasma levels of cystine, cysteine, methionine and taurine, and blood markers associated with well-being are to be examined. Chapter 3 of the following document is currently published in the *Frontiers in Veterinary Science* journal. Chapter 4 has also been submitted to the journal *PLOS One*.

1.2 Rationale of the Research

The following thesis will evaluate the detriments that pulse-based diets may have on dogs after consumption. This will be done to determine if pulse-based diets are a safe diet to feed to dogs, or if they pose more risk than reward for dogs to consume. For an initial 7-day analysis, 5 pulse-based diets using red lentil, fava bean, smooth pea, and 2 wrinkled pea varieties (4140-4 and Amigold), and 1 grain-based diet using rice were created. Pulse-based diets were compared to a grain-containing diet to determine if there were any differences between them regarding glycemic response, digestibility, and fasted plasma amino acid levels when fed to dogs. All diets were formulated to include approximately 20% available carbohydrate for this study to have similar starch fractions across the diets in order to compare the glycemic response of each diet observed in the dogs. In addition to this, formulating all diets to 20% available carbohydrate allowed a dietary gradient of amylose and fiber content, to study how each pulse ingredient differs from one another. While the grain-containing rice diet consisted largely of animal proteins, the pulse-based diets contained lowered animal proteins with larger amounts of plant proteins from the pulse flours. This could be beneficial for commercial pulse-based diets, as it could reduce overall costs by reducing the use of animal protein. The diets formulated for this thesis, however, are not commercially available on the market and should only be used in a laboratory setting. Despite being formulated to meet canine adult maintenance requirements, some of these diets were comprised of mainly pulse-flour (up to 58%) to achieve 20% available carbohydrate, which is not something one would typically see in a commercial diet. These formulated diets would be viewed as extreme from a commercial stand-point, but are beneficial in a laboratory setting as we can better observe the long-term effects of these pulse-based diets in a shorter amount of time.

After the first study, a second study was performed using 2 pulse-based diets from study one containing the highest and lowest levels of amylose. The rice diet as well as a commercial diet was fed to the dogs to use as grain-containing control diets. The formulated diets from study one underwent very slight modifications in study two to include slightly higher levels of protein, as a basic blood chemistry prior to the start of study two demonstrated low blood protein levels in all of our research dogs. The cause for this remains unknown. These diets were formulated to have 15% enzymatic starch and again, varying levels of dietary amylose and fiber. Each diet was fed to the dogs for 28 days in a repeated measures, cross-over, blinded study design to determine their impacts on the dogs cardiac function, plasma taurine levels, and macronutrient and amino acid digestibility.

This research is critical to better understand the role of amylose and dietary fiber, if any, in the reported link between grain-free diets and heart failure in dogs. Whether amylose and fiber content of pulse-containing diets influence sulfur-containing amino acid and taurine levels in altering cardiac or vascular function is unknown, it will be addressed by this thesis. It is important to determine if there is a cause-and-effect between dilated cardiomyopathy (DCM) and reduced taurine concentrations in dogs fed grain-free diets. This research can be utilized to establish these gaps in research and provide insight to pet food industry leaders, veterinarians, and pet owners.

In addition, this research will determine which pulse-based crops are safest to be best utilized within the pet food industry to create pet foods that promote a healthy, low-glycemic response, yet minimize the potential for developing heart failure and nutrient deficiencies. This will be optimal for pulse farmers across Canada, as it can create more markets for their crops to be utilized in and influence which varieties are prioritized for crop growth.

1.3 Study 1 Objectives

1) Evaluate and compare the digestibility between all tested diets based on their varying levels of fiber and amylose content
2) Assess the glycemic response of each diet to determine which diets promote lower glycemic responses in dogs
3) Determine short-term impacts of pulse-based diets on plasma levels of cystine, cysteine, methionine, and taurine
4) Determine the short-term impacts of dietary fiber and amylose on fecal bile acids

1.4 Study 2 Objectives

1) Evaluate and compare the digestibility between all tested diets based on their varying levels of fiber and amylose content
2) Determine long-term impacts of pulse-based diets on plasma levels of cystine, cysteine, methionine, and taurine
3) Determine the short-term and long-term impacts of dietary fiber and amylose on fecal bile acids
4) Observe general health markers in blood samples to determine if pulse-based diets are causing any negative impacts on the dog's health
5) Determine if pulse-based diets result in cardiac changes in dogs
6) Link differences in dietary fiber and amylose content to long-term effects on cysteine, methionine, taurine, and early, reversible signs of DCM.

1.5 Overall Hypothesis

Pulse-based, grain-free diets with higher levels of dietary fiber and amylose will produce a low glycemic response in dogs due to decreased rates of digestion and lowered bioavailability of nutrients for absorption, which will lower plasma concentrations of cystine, cysteine, methionine, and taurine, resulting in early indications of DCM.

1.5.1 Study 1 Hypothesis:

- After 7 days of feeding, dog diets with higher levels of dietary fiber will produce a low glycemic response due to decreased rates of apparent total tract digestibility of all macronutrients and increased fecal bile acid excretion.

1.5.2 Study 2 Hypothesis:

- In dogs, feeding pulse-based, grain-free diets for 28 days will cause a decrease in apparent total tract digestibility of macronutrients, an increase in the excretion of fecal bile acids, and a decrease in plasma levels of cystine, cysteine, methionine, and taurine, resulting in sub-clinical cardiac and blood changes consistent with early signs of DCM.

2 CHAPTER 2: LITERATURE REVIEW

2.1 The Pet Food Industry

The pet food industry has been steadily growing on a global scale and is projected to reach a value of $91 billion USD by 2022 (Olatunde et al, 2018). Within the United States (U.S.) alone, it is estimated that approximately 82 million homes have a pet, which equates to approximately 68% of all households in the U.S. The most commonly owned pets are cats and dogs, with approximately 56 million households owning dogs (Fox & Kenagy, 2015).

In the majority of homes, pet owners feed their beloved pets commercially prepared diets that are formulated to meet nutritional requirements of pets based on standards set by the Association of American Feed Control Officials (AAFCO) (Dzanis, 1994). These diets are relatively inexpensive and are optimal to feed since they are complete diets that meet the nutritional needs of domestic pets. Commercial pet foods are a convenient feeding option for pet owners as opposed to homemade diets. In 2014, sales of commercial pet foods within the U.S. were estimated to be approximately $23 billion, which is a substantial increase compared to the pet food sales of $14 billion in 2005 (Fox & Kenagy, 2015).

Pet owners have an active role in choosing diets for their pets. Owners prefer to feed commercial diets as they provide proper nutrition, contributing to longer and healthier lives of pets. Factors that can influence the feeding habits of owners are their knowledge as to what comprises a healthy diet, their perception of their pet's nutritional requirements and their overall perception of the pet food industry. Personal beliefs of owners regarding their own human diet are also a determinant of the diets that will be fed to their pets. In other words, owners will change their pets' diet to match their own (Suarez et al, 2012).

Based on these influencing factors, a variety of formulated pet foods have been made and are marketed in strategic ways to pet owners. Trendy diet claims such as "organic", "natural" and “grain-free” can be found on premium pet foods that are specialized to attract the attention of pet owners. These premium diets are believed by pet owners to have higher nutritional value and are increasingly preferred diets to feed to their pets (Pirsich et al, 2017). Of all current premium pet foods, diets that are advertised as grain-free are some of the most highly preferred. In competition with grain-free is another trend advertised as a diet that mimics the domestic dog’s ancestral diet i.e. high protein diets (Conway & Saker, 2018).

Grain-free diets exclude the use of grains such as wheat, corn, or rice flours and instead incorporate pulses such as peas and lentils (Prantil et al, 2018). In 2011, pulses represented approximately 6% of Canada’s field crop production. The use of pulse crops is seen to be increasing with Canada being one of the leading producers and exporters worldwide. Explanation for this market growth is correlated with profitability and research into new crop varieties and uses including pet food (Bekkering, 2014).

2.2 The Pulse Crop Industry

In general, the Prairie provinces of Canada are advantageous locations to grow pulse crops, due to having optimal environments such as cooler temperatures and ideal soil types for pulse crop production. Pulse crops are also an ideal crop to incorporate into crop rotations due to their nitrogen fixing properties. Pulses contribute to soil organic matter and provide nitrogen to the soil for future crops to utilize (Peoples et al, 2009). In 2010, Saskatchewan had the largest sales of pulses accounting for 78% of Canada’s national pulse farm cash receipts. On average, farm cash receipts in Canada for pulses have increased from $56 million in 1980 to $1.5 billion in 2010 (Bekkering, 2014).

With pulse flours being used more commonly in pet foods to produce grain-free diets, trends have shown continued increases in production to meet demands (Tyler et al, 2017).

However, there is currently controversy in regards to grain-free diets, based on a statement released by the U.S. Food and Drug Administration (FDA) claiming that grain-free diets are causing heart failure in dogs (Mansilla et al, 2019). However, the original claim was based on nine case studies with no controls and direct proof to support this hypothesis remains sparse.

2.3 Digestive System of Domestic Dogs

The domestic dog (*Canis lupus familiaris*) co-evolved with ancestral humans, originating from the gray wolf (*Canis lupus*) (Morell, 1997). As a result of domestication, a variety of physiologic changes occurred (Honeycutt, 2010). Using both artificial and natural selective measures, phenotypic changes such as size, coat colour, positioning of ears and more were observed (Clutton-Brock, 1995). In addition to external physical changes, the domestication of the wolf to dog resulted in physiological adaptations to changes in nutrition (Clutton-Brock, 1995). Currently, both the dog and the wolf are classified under the family *Canidae* and fall under the order *Carnivora* (Clutton-Brock, 1995). However, changes in lifestyle and diet have led many to question the conclusion of dogs belonging to the order Carnivora as opposed to being classified under the order Omnivora (Bosch et al, 2015).

The domestic dog has undergone a variety of genetic changes, specifically with increased expression of the alpha amylase 2B, maltase-glucoamylase, and sodium-glucose cotransporter 1 (AMY2B, MGAM, SGLT1) genes. These adaptations provide dogs with improved starch digestion (Bosch et al, 2015). Through the process of selection, a duplication affected the gene AMY2B in the dog, which is a gene that codes pancreatic amylase (Arendt et al, 2016). This gene duplication event is what led to increased canine AMY2B and pancreatic amylase expression levels. Pancreatic amylase is an enzyme involved in the breakdown of starch into maltose, which is then used to release glucose (Arendt et al, 2016; Arendt et al, 2014).

Selection also targeted the genes that are responsible for converting maltose into glucose by the enzyme MGAM through the process of hydrolysis (Arendt et al, 2016; Axelsson, 2014). The gene for MGAM can be found within a region on chromosome 16 and is also expressed at higher levels in dogs than wolves (Axelsson, 2014). Once the process of hydrolysis of starch is completed, glucose monomers are then absorbed through the brush border membrane of the small intestinal wall with the assistance of SGLT1 (Axelsson, 2014).

Recent studies have shown evidence that diet can regulate the expression of SGLT1 (Batchelor et al, 2010). For example, chronic feeding with diets high in carbohydrate results in an increase in the expression of SGLT1 (Batchelor et al, 2010). Since domestic pet dogs consume diets similar to humans with a higher carbohydrate content, the expression of SGLT1 will be increased (Batchelor et al, 2010). Overall, the combination of the three genetic mutations (AMY2B, MGAM, SGLT1) suggests that dogs are more capable of digesting, absorbing, and metabolizing starch in an efficient manner. This in turn enables dogs to consume diets with increased levels of starch and amylose (Arendt et al, 2016; Arendt et al, 2014), such as commercialized dog food containing grains or pulses.

2.4 Carbohydrates

Carbohydrates are a vast group of organic compounds utilized in diets with an impact on satiety, energy metabolism, blood glucose levels, insulin response, lipid metabolism, and gut microbiota (Cummings & Stephen, 2007; Moon et al, 2018). Dietary carbohydrates are observed in many different physical, physiological, and chemical forms and can be classified based on their molecular size, degree of polymerization, linkage types, and monomers (Cummings & Stephen, 2007). There are 3 different size classes of dietary carbohydrates which are: sugars, oligosaccharides, and polysaccharides (Cummings & Stephen, 2007).

Firstly, the sugar class consists of monosaccharides, disaccharides, and polyols, for example glucose, fructose, and maltitol (Cummings & Stephen, 2007). These carbohydrates are broken down and absorbed quickly and are a major source of available energy when consumed (Cummings et al, 1997). The rapidly available energy observed in the blood after carbohydrate consumption is free glucose (Cummings et al, 1997). Secondly, oligosaccharides are known as short-chain carbohydrates such as stachyose and inulin (Cummings & Stephen, 2007). Thirdly, polysaccharides contain subgroups of starches (amylose and amylopectin) and non-starches (cellulose, hemicellulose, pectin, etc.).

Starches are a common carbohydrate seen in many different diets and are the storage carbohydrate of plants. The structural form of starch is partially crystalline and contains varying mixtures of the two glucose polymers: amylose and amylopectin. Amylose is a helical chain of linear, non-branching glucose residues with α-1,4 glucosidic linkages, while amylopectin is highly branched glucose residues linked by both α-1,4 and α-1,6 glucosidic bonds (Cummings & Stephen, 2007). Based on structural differences, amylose can behave as a flexible coil and can fold into a more compact structure, making it more difficult to digest (Lopez et al, 2012). In contrast, branching of amylopectin allows for better and quicker digestion rates of starch (Benmoussa et al, 2007).

Depending on the composition as well as quantity consumed, in human studies, carbohydrates containing starch aid in suppressing hunger and promote satiety. This is due to the metabolic response that occurs after the ingestion of starches. Diets that contain higher amounts of amylose can increase satiety for a sustainable amount of time (more than 6 hours) when compared to diets that are high in amylopectin (Anderson & Woodend, 2003). In humans, it is ideal to consume carbohydrates that contain higher levels of amylose to decrease the rate of digestion. This is beneficial as it will reduce long-term appetite and reduce total feed intake (Anderson & Woodend, 2003). Studies in dogs however are limited in regards to addressing how starch can impact satiety and digestion. This is why it is crucial to conduct appropriate studies to gain further knowledge on how diets, specifically grain-free, could be utilized to promote satiety in pets.

Carbohydrates are frequently used in a wide variety of dog foods, with starch being the most abundant feed ingredient in most dog food formulations for both economic reasons and supplemental dietary energy (Hilton, 1990). These starches are supplied in pet foods in a variety of forms based on different uses of feedstuffs. For example, corn, rice, and wheat grains are all commonly used in dog foods at high inclusion rates as a carbohydrate source (Hilton, 1990). Different feedstuffs are seen to have different starch structures, which alters the digestibility of the feedstuff provided (Hilton, 1990). Due to this insight, starches in pet feed are cooked in order to increase their levels of digestibility and utilization once consumed (Hilton, 1990).

2.5 Starch Digestibility & Fiber Fermentability

In vitro tests of starch digestibility can be categorized into three fractions known as rapidly digested starch (RDS), slowly digested starch (SDS), and resistant starch (RS) (Englyst et al, 1992). In humans, RDS has been defined as the amount of starch that is digested within 20 minutes of consumption and represents a rapid release of glucose into the blood (Singh & Kaur, 2010). SDS is the fraction of starch that is digested within 20-120 minutes after consumption and results in a steady and moderate release of glucose into the bloodstream (Singh & Kaur, 2010). RS is the portion of starch that is not digested after 120 minutes and is excreted from the body (Singh & Kaur, 2010). A major determining factor in regards to starch digestibility is the amylose:amylopectin ratio (Lehmann & Robin, 2007). Diets that contain higher amounts of amylose are seen to have higher levels of RS, whereas lower levels of amylose are classified as RDS (Lehmann & Robin, 2007).

Based on in vitro human nutrition models studied, SDS is typically the most preferred form of dietary starch as it is fully digested within the small intestine, but at a slower rate. This causes a reduction in the postprandial plasma glucose and insulin response as well as an increase in satiety (Sandhu & Lim, 2008). Comparatively, RDS has a quick and high peak and a rapid decline in blood glucose levels, which ultimately results in decreased levels of satiety (Lehmann & Robin, 2007). Studies in regards to starch digestibility in dogs are few, however, there are some recent studies that demonstrate that diets composing of higher levels of SDS and RS in dogs reduces the rate of digestion (Sandri et al, 2020). The following research to be conducted aims to further address the concept of starch digestibility in dogs, which has yet to be studied in great detail.

In canine diets, the addition of plant-based ingredients has resulted not only in the addition of starch, but the increase in different kinds of fiber (Donadelli et al, 2021). Fiber can be beneficial to dog diets as it promotes weight management through increased satiety (Brennan & Cleary, 2005; Jenkins et al., 2008; Skinkė & Januškevičius, 2015; de Godoy et al., 2013). Another benefit of fiber is that is can be fermented by colonic bacteria, which provides short chain fatty acids to be utilized by the animal (Sunvold et al., 1995). Increases in fiber however have also been observed to decrease nutrient digestibility within dogs (Carciofi et al., 2008). Despite this, fiber found within diets appears to alter post-prandial insulin and glucose responses (Wolever, 1990; Graham et al., 1994), demonstrating that increased fiber fraction found within plant-based grain-free diets might have more positive trade-offs than negatives.

2.6 Glycemic Index & Glycemic Response

Glycemic index is a measurement of how available carbohydrates affect blood glucose levels developed and validated for humans. This glycemic index is primarily determined by the type and structure of the starch, but is also impacted by postprandial insulin responses (Jones, 2013). This index can be determined by integrating the area under the glucose response curve (AUC) of the test food, generally within a 2-hour time frame for humans, and then presenting it as a percentage of the AUC for pure glucose which is used as a standard for glycemic index testing (Thomas et al, 1991). This is beneficial as it standardizes glycemic responses of different test foods or diets to glucose, and corrects for any between subject variations, to allow for food and diet comparisons (Anderson & Woodend, 2003). The glycemic index is commonly considered a characteristic intrinsic to specific foods, and is not to be confused with the term "glycemic response". Glycemic response is the change in blood glucose levels as a result of ingesting food (Roberts, 2003).

Blood glucose responses can vary based on the type of carbohydrate and the rate of digestion (Thomas et al, 1991). In dogs, diets with different levels of starch cause variations in postprandial glucose responses (Nguyen et al, 1998). In human studies, foods with a low glycemic index, such as legumes, are slowly digested and provide a slow and sustained release of glucose into the bloodstream (Thomas et al, 1991). Alternatively, foods that are quickly digested, such as white bread, have a high glycemic index and result in rapid and high glucose levels (Thomas et al, 1991). These trends are also true in dogs, as legumes resulted in a slowed postprandial glucose response, and grains resulted in an immediate postprandial glucose response (Carciofi et al, 2008; Adolphe et al., 2012; Briens et al., 2021). Previous studies in humans have also demonstrated that when a diet is comprised of high glycemic foods, the elevated blood glucose, and insulin responses are detrimental to overall health (Englyst et al, 1999). For this reason, it has been suggested that diets with a low glycemic response might be a protective measure to prevent chronic illness in both humans and dogs (Englyst et al, 1999; Adolphe et al, 2014).

Diets that result in a low glycemic response are generally seen to promote either weight loss or maintenance of weight through increased satiety (Jones, 2013). What this means is that diets with a low glycemic index aids to make individuals feel full for longer periods of time than high glycemic foods would (Jones, 2013). Alternatively, high glycemic foods result in a quick response of fullness, which only lasts for a short amount of time (Anderson & Woodend, 2003). This as a result is a possible reason as to why high glycemic, rapidly available carbohydrate containing diets are a cause of obesity due to overeating and a lack of long-term satiety effects (Anderson & Woodend, 2003).

In addition, in humans, there have been many epidemiological and interventional studies that display an association between postprandial hyperglycemia with an increase in risk of cardiovascular disease for individuals with diabetes and the general public (Aziz, 2009; Ludwig, 2002). There are currently several known factors that determine postprandial hyperglycemia such as insulin sensitivity, quantity and compositions of the meal, and the amount of dietary carbohydrates present (Aziz, 2009). Through the use of the glycemic index, it appears that there may be optimal health benefits of consuming low glycemic index diets in regards to better managing postprandial glucose levels (Aziz, 2009).

2.7 Pulse Crops & Manufacturing

Pulse crops are a member of the Leguminosae family and are among the most extensively used foods worldwide. These crops, in addition to food uses, are also used for animal feed. Pulse crop production is sustainable as pulse production has a lower carbon footprint compared to many other crops (Mudryj et al, 2014).

Pulses, such as peas and lentils are a commonly used source of nutrition for both humans and animals due to their high levels of protein, carbohydrates, fiber, vitamins, and minerals (Singh, 2017). Based on their dietary protein, pulses contain high amounts of the amino acid lysine, which is seen to be lower in cereal grains (Mudryj et al, 2014). However, compared to cereal grains, pulses contain limited amounts of the non-essential amino acid cysteine and reduced amounts of the essential amino acids methionine and tryptophan (Singh, 2017).

Moreover, despite pulses having a high carbohydrate content of approximately 50-65%, they are slowly digested (Mudryj et al, 2014). This is because pulse crops contain high amounts of amylose and fiber, as well as plant proteins, which can vary across different pulse crops. This characteristic of increased fiber and protein content in pulses can be utilized to optimize satiety through a lowered glycemic response and glycemic index (Singh, 2017). Other than amylose and fiber content, additional factors that influence the glycemic index of pulse-based feeds are the manufacturing methods of cooking and processing. For example, canned beans are seen to have a higher glycemic index than dried and cooked beans (Mudryj et al, 2014).

In regards to conventional dog foods that are kibbles, they undergo an extrusion process. This manufacturing process can impact the glycemic index of the whole diet that is created. Extrusion is a process in which ingredients are subjected to high temperatures, moisture, and high pressure to produce a kibble extrudate. Extrusion is frequently used to manufacture pet foods as it increases nutrient digestibility and availability of diets. Due to the high temperatures, structural modifications occur which impacts the structure of amylose and amylopectin. As a result of extrusion, retrogradation of starch occurs, which decreases starch digestibility by creating RS which in dogs can result in increased fecal bulk (Tran et al, 2008).

Extrusion also results in the formation of starch and lipid complexes. These complexes consist of a hydrophobic core of amylose, which traps hydrocarbon chains of lipids. These lipid-amylose complexes inhibit the actions of amylase during digestion. Another inhibiting factor of amylose digestion is proteins, which limits hydrolysis. These are all implicating factors that impact feed digestibility, which in turn impacts glycemic response and index. Thus, there are processing factors that can either increase or decrease starch digestibility, leading to variable effects depending on conditions used. This area of study requires further research to better assess whether starch digestibility is increased or decreased, compared to unprocessed pulse starches and how this can impact canine health (Tran et al, 2008).

In addition to starch, fibrous fractions found within plant-based, grain-free diets impact the process of extrusion. For example, fiber can produce increased kibble density and decreased expansion of the kibble (Monti et al., 2016). Insoluble fiber can cause weak spots in expanding cells due to its inability to mix with the molten matrix, causing the expanding cells to burst prematurely (Wang et al., 2017; Donadelli et al., 2021). Soluble fiber however, does mix with the molten matrix, but does not contribute to structural properties. This produces a weaker cell wall that does not expand but collapses (Parada et al., 2011). Ultimately, fiber found in plant-based diets impacts the processes of extrusion which has a direct effect on kibble characteristics.

The following studies will examine different pulse-based diets comprising of either fava bean (CDC Snowdrop), red lentil (CDC Maxim), round pea (CDC Inca), or wrinkled pea (4140-4 and Amigold varieties). Multiple pulse varieties were selected to be used in order to further study and determine the feeding efficacy of different pulse-based diets in dogs. These pulses were also selected because they have varying levels of fiber and amylose, that would be used to create experimental test diets with a gradient of fiber and amylose content. This amylose gradient across the pulses is important to study, as it is important to determine how dietary fiber and amylose can impact biological functions of the dogs.

2.8 Macronutrient Digestibility & Metabolism

The digestion of carbohydrates occurs in both the upper and lower regions of the gastrointestinal tract. Absorption of metabolizable carbohydrates occurs in the upper region, while fermentation that results in the production of short-chain fatty acids occurs in the lower region. Carbohydrates that are absorbed within the small intestine are referred to as available carbohydrates and provide energy for metabolism (Englyst et al, 2007). The digestive tract of the dog is seen to have a rather large absorptive surface area, which ultimately increases nutrient digestion (National Research Council (NRC), 2006). Dietary carbohydrates that are digested within the small intestine are absorbed in the forms of monosaccharides, glucose, fructose, and galactose. Within both food and circulation, glucose is the predominant monomer and is utilized by all tissues (Aziz, 2009; Englyst et al 2007).

The rate of digestion of carbohydrates is highly dependent on the rate in which carbohydrates become available for absorption at the epithelium of the small intestinal region (Englyst et al, 2007). This can be influenced by a variety of factors that occur within the gastrointestinal tract. One influential factor is the rate of passage of the carbohydrate through the stomach, which is dependent on particle size of the food bolus that has been ingested. Another factor is the rate in which carbohydrates are released from the ingested bolus of food (Englyst et al, 2007). Carbohydrates are released from foods through the actions of amylase on starch (RDS and SDS) but are also impacted by food processing measures (Englyst et al, 2007).

There are currently four known factors that impact the values of digestibility in dogs. First, is the effect of food processing that modifies ingredient particle size through mechanical processes such as grinding, as well as extrusion/cooking that alter starch structure. Second, is human feeding practices, such as amounts of feed fed. Third, is biological animal factors such as age, gender, breed, metabolic requirements. Fourth, is housing and environment, such as kennel-type, temperature, circadian rhythm (de Godoy et al, 2016).

In vivo nutrient digestibility is used to provide insight on the relative amount of nutrients that are utilized by the dog and can provide information on the quality of diets fed to dogs (NRC, 2006). In general, nutrient digestibility is the amount of nutrient that has been absorbed by an individual. Any digestibility values that are calculated in the following studies should be viewed as “apparent” total tract digestibility (ATTD) (de Godoy et al, 2016). During total tract digestibility studies, there is no clear distinction between the endogenous nutrients that are secreted and not reabsorbed in the digestive tract and undigested dietary nutrients. This is also known as endogenous losses and can be determined in dogs via ileal digestibility studies. Ileal digestibility quantifies nutrients before reaching the large intestinal region of the gastrointestinal tract. However, in order to perform ileal digestibility studies, animals must be cannulated at the terminal ileum, which can have ethical and welfare issues, especially in canine studies (de Godoy et al, 2016).

AAFCO has published ethical protocols to be used to measure the nutritive value of both dog and cat foods through the means of fecal collection. This protocol consists of a 5-day dietary adjustment feeding period, followed by a 5-day fecal collection. This is done to ensure that digestibility measurements are accurate (de Godoy et al, 2016). To better determine digestibility through fecal collection, the use of external, non-digestible dietary markers is advantageous.

Markers such as acid-insoluble ash (Celite) consist of indigestible minerals that can be incorporated into test diets and used to measure feed digestibility (Sales & Janssens, 2003). This method of study can be used in replacement of the total collection method, removing the need to subject animals to the stress of being confined in metabolic cages for 72 hours or more. This in turn can improve animal welfare and behaviour to produce more reliable measurements. The use of an external Celite marker is a simplistic method that is also cost effective, making it an ideal way to measure diet digestibility (Sales & Jannsens, 2003).

2.9 Cardiovascular disease and Sulfur-Containing Amino Acids in Dogs

In July 2018, there was a statement released by the Food and Drug Administration (FDA) connecting DCM in dogs to the consumption of grain-free (which are largely pulse-based) diets (U.S. FDA, 2019; Mansilla et al, 2019). DCM is one of the most common heart diseases in domestic dogs (Backus et al, 2006). Canine DCM is viewed as a myocardial disorder and can cause an array of issues such as systolic dysfunction, reduced ventricular wall thickness, and increased cardiac size (myocyte enlargement) (Dutton & Lopez-Alvares, 2018). The development of DCM in canines is generally slow and end-stage disease can present with clinical symptoms of lethargy, shallow breathing, sudden collapse, and possible death. Early DCM that often goes undetected is characterized by increased ventricular volume and diameter in diastole along with reduced ejection fraction. Based on previous diagnoses, there appears to be a predisposition to DCM in medium to large dog breeds (Newfoundlands, Irish Wolfhounds, King Charles Cavalier Spaniels, Scottish Terriers, Dalmatians, Boxers, Cocker Spaniels, and Golden Retrievers; (Fascetti et al, 2003; Kaplan et al, 2018). In addition to genetic predisposition DCM, another suspected cause in dogs is taurine deficiency (Backus et al, 2006).

Unlike cats, dogs are able to synthesize taurine from dietary sulfur-containing amino acids: cysteine and methionine (Backus et al, 2006). Due to this, dogs do not have a minimum requirement for taurine (Torres et al, 2003). In dogs, normal reference ranges for both whole blood and plasma taurine have been established through studies. One study by Torres et al (2003) collected blood from the jugular vein of 12 healthy beagles. Results of this study reported mean plasma taurine concentrations of blood to be 109 nmol/ml and mean whole blood taurine levels to be 291 nmol/ml (Torres et al, 2003; Kaplan et al, 2018). Another study performed by Delaney et al, (2003) collected blood samples of 131 dogs from the jugular or cephalic vein for analysis. Results of this study reported mean plasma taurine levels to be 77 nmol/ml and mean whole blood taurine concentrations to be 266 nmol/ml (Delaney et al, 2003; Kaplan et al, 2018). It is important to study both plasma and whole blood concentrations in dogs as they are indicators of short-term and long-term feeding effects on amino acid concentrations, respectively (Sanderson et al, 2001). While it is ideal to measure changes in both whole blood and plasma, for this thesis it was not financially feasible as resources were limited. In addition, while plasma and/or whole blood taurine levels are commonly measured, it is unknown how these concentrations correlate with myocardial taurine levels in canines (Kaplan et al, 2018). This is because endomyocardial biopsies, although the most biologically relevant to cardiac pathology, are challenging and are ethically not performed in research dogs (Kaplan et al, 2018). Due to budget constraints for this thesis, we decided to run plasma samples to analyze cystine, cysteine, methionine, and taurine. Plasma samples were selected over whole blood for this study as many early studies evaluating taurine deficiencies and DCM used primarily plasma (Sanderson, 2006).

Since dogs are able to synthesize taurine, it is not considered to be a dietary essential nutrient (Backus et al, 2006). Animal protein products are a natural source of dietary taurine.

However, grain-free diets contain a high percentage of protein from pulses that lack taurine, leading to lower dietary taurine supplied by the diet. At the same time, pulse proteins are limiting in the two amino acids that are needed to produce taurine, namely cysteine and methionine. Despite dog diets being formulated to be nutritionally complete and balanced, taurine is not an essential nutrient in dogs. Combined, this could be a factor leading to a detrimental decrease in the blood and plasma taurine concentrations (Fascetti et al, 2003). Diets with increased levels of fiber (i.e. beet pulp) may also be a contributing factor to decreased taurine levels in dogs. This is because fiber can increase the excretion of fecal bile acids, with taurocholate being the predominant bile acid in dogs, leading to increased fecal loss of taurine while at the same time also decreases dietary protein digestion (Pezzali et al, 2020; Ko & Fascetti, 2016).

Taurine is associated with bile acid metabolism (Enright et al., 2018) and in dogs is used to exclusively conjugate bile acids (Czuba & Vessey, 1981). Primary bile acids such as cholic acid and chenodeoxycholic acid conjugate with taurine in the liver and are then released into the small intestine (Pezzali et al., 2020). Of this, 95% of conjugated bile acids are reabsorbed in the distal ileum and transported to the liver (Ajouz et al., 2014). However, fiber can interrupt this process and bind the bile acids within the intestinal lumen leading to the fecal loss of bile acids and taurine (Garcia-Diez et al., 1996; Stratton-Phelps et al., 2002). It is important to study bile acid metabolism and excretion as it impacts overall taurine status in dogs and could be a contributing factor to taurine loss and DCM in dogs

Not all fiber has the same biological effect. It is unclear whether soluble or insoluble fiber, high or low molecular weight dietary fiber, or even amylose which can contribute bulk-forming or fiber-like properties are the links to altered bile acid secretion and taurine loss in dogs. The fiber effect to decrease protein digestibility results in decreases in the bioavailability of cysteine and methionine (precursors of taurine). Again, while the slow carbohydrate digestibility of pulses has some beneficial health effects such as low glycemic index, pulses are also high in dietary fiber which may exacerbate the low taurine, cysteine, and methionine that grain-free diets provide. Overall results of this are a decrease in taurine levels, which could be the link to adverse cardiac health and DCM in dogs (Fascetti et al, 2003; Ko & Fascetti, 2016). Previous studies have also suggested that large breeds of dogs may also have a different taurine biosynthetic rate, compared to smaller breeds. Thus, making large breeds more sensitive to diets that are low in either taurine or precursors of taurine (cysteine and methionine) (Vollmar et al, 2013).

Aside from the susceptibility of large dog breeds, breeds not genetically predisposed to DCM are also being diagnosed with increasing frequency. Canine nutrition is a suspected cause of this leading to the FDA warning in July 2018 (Mansilla et al, 2019). A similar previous example of this are lamb-based diets, which were also associated with DCM and taurine deficiencies in dogs (Torres et al, 2003). The explanation at the time was that lamb-meal is poorly digested in dogs, resulting in limited availability of sulfur-containing amino acids for taurine synthesis (Freeman et al, 2018; Torres et al, 2003). Early DCM-like changes as a result of taurine deficiencies in lamb-based diets (left ventricular enlargement and reduced ejection fraction) were reversed through methionine supplementation (Backus et al, 2003). This condition was also reversible through either taurine supplementation or changing the dog's diet to a protein source other than lamb (Freeman et al, 2018).

As of November 2020, the FDA released more information to the public stating that DCM has been associated with both grain-free and grain-containing diets and that they are unsure about the connection between diet and non-hereditary DCM (U.S. FDA, 2020). While some recent studies show some initial evidence that grain-free diets are a potential cause of heart failure in dogs (Adin et al, 2019; Kaplan et al, 2018), the exact mechanistic link and the possible culprit dietary component within pulses remains unclear. It is important to conduct further research to better support or refute this possible connection between DCM and grain-free diets. It is also crucial to address how the digestibility of these diets might impact cysteine, methionine, and taurine availability. This issue can be addressed through studying how increased amylose, a contributor to RS versus different fiber fractions impact the bioavailability of sulfur-containing amino acids cysteine and methionine, as well as blood levels of taurine in short-term and long-term feeding trials. This in turn will aid to indicate if increased levels of amylose in a diet can lead to a predisposition for DCM, due to decreased levels of blood and plasma taurine levels.

3 CHAPTER 3: THE EFFECTS OF 7 DAYS OF FEEDING PULSE-BASED DIETS ON DIGESTIBILITY, GLYCEMIC RESPONSE AND TAURINE LEVELS IN DOMESTIC DOGS

3.1 Chapter 3 Preface

The following chapter is the first study of two that was conducted on research Beagles in the Weber Lab. This was a short-term feeding study conducted to observe the impacts of feeding multiple types of pulse-based diets to dogs. This chapter focuses on the impacts of diet on glycemic response, digestibility, and fasted plasma taurine levels in dogs after 7 days of feeding.

This chapter has already been published in the *Frontiers in Veterinary Science* journal in the Animal Nutrition and Metabolism section (Quilliam, C., Ren, Y., Morris, T., Ai, Y., & Weber, L. P. (2021). The Effects of 7 Days of Feeding Pulse-Based Diets on Digestibility, Glycemic Response and Taurine Levels in Domestic Dogs. *Frontiers in veterinary science*, 8, 408.). The following authors associated with this publication were: C. Quilliam: formulated and created test diets, designed and performed experiments, and analyzed data, and co-wrote the paper. Y. Ren: material preparation, starch analyses, data curation, validation, and review & editing. T. Morris: performed experiments and review & editing. Y. Ai: Conceptualization, funding acquisition, investigation, project administration, resources, and review & editing. L. Weber: Supervised animal research, experimental design, funding acquisition, investigation, resources, project administration, review & editing, and co-wrote the paper.

3.2 Abstract

Grain-based carbohydrate sources such as rice comprise 30–50% of commercial pet foods. Some pet foods however have removed the use of grains and have instead incorporated pulses, such as peas and lentils, resulting in grain-free diets. The hypothesis was dog diets with higher levels of dietary fiber will produce a low glycemic response due to decreased rates of digestion and lowered bioavailability of all macronutrients and increased fecal bile salt excretion. This in turn was hypothesized to produce lower fasting plasma concentrations of cysteine, methionine, and taurine after 7 days of feeding each test diet in dogs. Six diets were formulated at an inclusion level of 20% available carbohydrate, using white rice flour (grain) or whole pulse flours from smooth pea, fava bean, red lentil, or 2 different wrinkled pea varieties (CDC 4,140–4 or Amigold) and fed to beagles in a randomized, cross-over, blinded design. After 7 days of feeding each diet, fasting blood glucose was the lowest in the lentil (3.5 ± 0.1 mmol/L) and wrinkled pea (4,140–4; 3.6 ± 0.1 mmol/L) diet periods, while peak glucose levels was lowest after feeding the lentil diet (4.4 ± 0.1 mmol/L) compared to the rice diet. Apparent total tract digestibility of all macronutrients, as well as taurine, differed among diets yet plasma taurine was not outside normal range. Decreased macronutrient and amino acid digestibility was associated with increasing amylose and dietary fiber content but the specific causative agent could not be determined from this study. Surprisingly, digestibility decreases were not due to increased bile salt loss in the feces since increasing dietary fiber content led to decreased fecal bile salt levels. In conclusion, although pulse-based canine diets have beneficial low glycemic properties, after only 7 days, these pulse-based diets decrease macronutrient and amino acid digestibility. This is likely related at least in part to the lower animal protein and increased fiber content, but on a long-term basis could put domestic dogs at risk for low taurine and DCM.

3.3 Introduction

The global pet food industry has been steadily growing and is projected to reach a value of $91 billion USD by 2022 (Olatunde, 2018). In a majority of homes with pets, pet owners feed them commercially prepared diets as they are affordable and nutritionally complete with the belief that they promote animal health (Dzanis, 1994). Pet diets in North America are formulated to meet nutritional requirements based on the standards set by AAFCO.

Pet owners have an active role in choosing diets to feed their pets and generally make their decisions based on: (i) true knowledge of what comprises a healthy diet, (ii) perception of nutritional requirements, (iii) human diet trends, and (iv) overall opinions of the pet food industry. Importantly, owners will often change their pet's diet to match the diet consumed by the human owner (Suarez et al., 2012). Pet foods are marketed strategically to owners, with trendy claims such as “organic,” “natural” and “grain-free” often found with premium pet foods (Pirsich et al., 2017). Grain-free diets exclude the use of grains such as wheat, corn, or rice flours and instead incorporate pulses such as peas, lentils, and fava beans as the major carbohydrate source (Prantil et al., 2018). While pulses are common dietary ingredients for both humans and animals, they are considered to be highly nutritious primarily due to their high levels of protein, in addition to carbohydrate, fiber, vitamins, and minerals (Singh, 2017). Pulse crops are slowly digested due to relatively high amylose, resistant starch, and dietary fiber content (Mudryj et al., 2014; Ren et al., 2021). This characteristic of pulses can be utilized to optimize satiety through a lowered glycemic response and glycemic index (Singh, 2017), a feature that is also in dogs (Adolphe et al., 2012; Adolphe et al., 2014; Briens et al., unpublished).

Postprandial blood glucose responses can vary based on the type of carbohydrate and the rate of digestion (Thomas et al., 1991). In dogs, diets with different levels of starch cause variations in postprandial glucose responses (Nguyen et al., 1998). In human studies, foods with a low glycemic index, such as legumes or pulses, are slowly digested, which provides a slow and sustained release of glucose into the bloodstream (Thomas et al., 1991). Alternatively, foods that are quickly digested, such as white bread, have a high glycemic index, which result in rapid and high postprandial blood glucose levels (Thomas et al., 1991). Similarly, in both dogs and cats, legumes result in a slow or negligible postprandial glucose response while grains result in a faster, higher postprandial blood glucose response (Adolphe et al., 2012; Carciofi et al., 2008; Briens et al., unpublished). Thus, pulse-containing diets promote glucose control, insulin sensitivity, satiety, weight control, and longer-term health in humans and dogs (Adolphe et al., 2014; Englyst et al., 1999).

While high-protein pulses can decrease the cost to produce a high protein pet food due to a lower need for animal protein, a downside to pulses is that plant protein lacks taurine. Moreover, compared to cereal grains, pulses contain limited amounts of the non-essential amino acid cysteine and reduced amounts of the essential amino acids, methionine, and tryptophan (Singh, 2017). Cysteine and methionine are used by the dog liver and the central nervous system to synthesize taurine via the transsulfuration pathway. Thus, taurine is not considered to be an essential amino acid in dogs. While DCM is common in dogs (Backus et al., 2006), some cases are associated with low plasma taurine and can be reversed with taurine supplementation (Kaplan et al., 2018). Canine DCM was reported by the US Food and Drug Administration (FDA), based initially on 9 case studies, to be associated with feeding grain-free diets in July 2018. This led to a decrease in grain-free dog food sales, decreased use of pulses in pet food, and losses to the pulse-growing agriculture sector. Confusion among veterinarians and pet owners as to whether grain-free diets are healthy for dogs was further exacerbated by a recent acknowledgment by the FDA in November 2020 that causes of DCM in dogs may be more complicated than just a single ingredient such as pulses and is instead likely multi-factorial. To begin to address some of these questions, this study aimed to explore whether grain-free diets lead to taurine deficiency and if this is associated with simultaneous low cysteine and methionine levels. Moreover, mechanisms by which pulses could deplete taurine, cysteine, or methionine require experimental confirmation.

One possible explanation is dietary fiber which was linked to decreased taurine levels in dogs (Sanderson et al., 2001; Mansilla et al., 2019). Fiber increases the excretion of fecal bile acids, and since taurocholate is the most common bile salt in dogs, this leads to increased fecal loss of taurine (Pezzali et al., 2020; Ko & Fascetti 2016). In addition to taurine loss, high dietary fiber decreases protein digestibility, resulting in decreased cysteine and methionine (precursors of taurine) bioavailability. Again, while the slow carbohydrate digestibility of pulses has some beneficial health effects such as low glycemic index, high dietary fiber of pulses may exacerbate the already low taurine, cysteine, and methionine found in grain-free diets (Ko & Fascetti 2016; Fascetti et al., 2003). What is unclear is what component of dietary fiber is responsible for the increased fecal bile salt loss, decreased protein digestibility, and decreased sulfur-containing amino acid bioavailability. As a first exploration, this study aims to use diets with increasing levels of dietary fiber and amylose content using different pulses as well as to explore its role in these processes in dogs.

The hypothesis was that dog diets with higher levels of dietary fiber will produce a low glycemic response due to decreased rates of digestion and lowered bioavailability of all macronutrients and increased fecal bile salt excretion. This in turn is hypothesized to produce lower plasma concentrations of cysteine, methionine, and taurine after 7 days of feeding each test diet in dogs. In order to investigate these hypotheses, whole and complete diets formulated to include 20% available carbohydrate using a grain (white rice flour) compared to whole pulse flours from smooth pea (CDC Inca), fava bean (CDC Snowdrop), red lentil (CDC Maxim) or 2 different wrinkled pea varieties (CDC 4,140–4 or Amigold) were fed to beagles in a randomized, cross-over, blinded design. After 7 days of feeding each diet, macronutrient digestibility and glycemic responses were examined along with fasted plasma concentrations of cysteine, methionine, and taurine as well as fecal bile salt concentrations in beagle dogs.

3.4 Experimental Methods

All procedures and handling of the dogs were conducted following protocols approved by the University of Saskatchewan's Animal Research Ethics Board according to guidelines that were established by the Canadian Council on Animal Care (Animal Utilization Protocol #20190055).

3.4.1 Animals

Adult Beagle dogs (n = 8; 4 spayed females, 4 neutered males; 8.87 ± 0.90 kg, 2–4 years old) were obtained from certified scientific breeders (Marshal Bioresources, North Rose, NY, USA, and King Fisher International, Stouffville, ON, Canada). Beagles were housed at the Animal Care Unit (ACU) in the Western College of Veterinary Medicine at the University of Saskatchewan, Saskatoon, SK, Canada. Beagles were group-housed during the day in a large enclosure to allow for daily socialization but were individually kenneled during feedings and overnight. Dogs were walked and socialized on a daily basis. The Beagles were also provided regular health examinations, deworming, and routine vaccinations from certified veterinarians to ensure optimal health.

3.4.2 Diets

The test diets included one control (rice; a grain-containing diet) and five pulse-based diets [all grain-free diets: smooth pea (CDC Inca), wrinkled pea (4,140–4 variety), wrinkled pea (Amigold variety), red lentil (CDC Maxim) and fava bean (CDC Snowdrop)]. All diets were formulated at 20% available carbohydrate using locally obtained flours (flour proximate analyses shown in Supplementary Table S1). A non-digestible Celite marker was also incorporated at 1% for determination of apparent total tract digestibility and measured as acid-insoluble ash in proximate analyses of diet and feces (Sales & Janssens, 2003). Diets were formulated using the software Creative Concept 5 (Creative Formulation Concepts, Pierz, MN, USA) and were structured to meet the nutritional requirements for canine adult maintenance (see Table 3.1 for formulations). These requirements were based on AAFCO and the National Research Council recommendations. Feed ingredients were sourced from local and commercial sources as needed and diets were extruded into a dry kibble format using a laboratory-scale, co-rotating, twin-screw extruder (Baker Perkins Ltd, Peterborough, UK) at the University of Winnipeg (Food Science Laboratory, Winnipeg, MB, Canada). All diets were extruded under the same conditions as described in Supplementary Table S2. Diets were then vacuum coated with fat at the Canadian Feed Research Centre (North Battleford, SK, Canada). Samples of all diets were then sent to analytical laboratories for proximate and amino acid analysis (Central Testing Laboratory Ltd., Winnipeg, MB, Canada) according to AOAC standards (AOAC, 2019). Dry matter was determined by oven-drying the sample and crude protein determined using the Kjeldahl method while non-fiber carbohydrate and fat were determined through acid-hydrolysis solvent extraction. Metabolizable energy (ME) content of diets was determined through calculation (see footnote to Table 3.2 for equation). Fiber analyses were performed according to the AOAC 2011.25 method (Eurofins, Toronto, ON, Canada).

Table 3.1: Formulation of test diets with increasing fiber and amylose content listed from left to right. All diets were formulated to include 20% available carbohydrate.

	Rice Diet	Lentil Diet	Smooth Pea Diet	Fava Bean Diet	Wrinkled Pea Diet (4140-4)	Wrinkled Pea Diet (Amigold)
Flour	23.12	42.19	41.67	46.40	58.65	58.14
Chicken By-Product Meal	37.83	21.49	24.26	17.53	8.85	9.03
Cellulose	15	14	12	14	10	10
Chicken Fat*	10	10	10	10	10	10
Fish Meal	5	5	5	5	5	5
Canola Oil	6.55	5.00	5.00	5.00	5.00	5.00
Celite	1	1	1	1	1	1
Vitamin/Mineral Premix	1	1	1	1	1	1
NaCl	0.3	0.3	0.3	0.3	0.3	0.3
Choline Chloride	0.1	0.1	0.1	0.1	0.1	0.1
Calcium Carbonate	0.05	0.05	0.05	0.05	0.05	0.05
Dicalcium Phosphate	0.05	0.05	0.05	0.05	0.05	0.38

All values are expressed as % inclusion as fed. *Included addition of antioxidant Naturox (Kemin, Des Moines, IO USA)

Table 3.2: Proximate analyses of test diets after extrusion and fat coating. Diets are listed in order of increasing fiber and amylose content from left to right

	Rice Diet	Lentil Diet	Smooth Pea Diet	Fava Bean Diet	Wrinkled Pea Diet (4140-4)	Wrinkled Pea Diet (Amigold)
DM (%)	89.78	90.43	89.90	90.62	89.33	89.69
Crude Protein (%) [a]	33.69	30.96	32.86	30.41	27.64	26.13
Crude Fiber (%) [b]	6.050	5.230	6.460	8.930	8.770	9.620
Fat (%) [c]	19.79	19.99	16.01	18.45	17.81	19.05
Ash (%) [d]	8.960	7.330	8.420	7.170	6.180	6.500
Metabolizable Energy (kcal/kg) [e]	3913	4121	3806	3855	3920	3856

DM = dry matter; All % values are relative to dry matter except excluding Metabolizable Energy (kcal/kg).
[a] Determined using a Nitrogen/Protein Analyzer (CN628, LECO Corporation, St. Joseph, MI, USA), with a conversion factor of 6.25.
[b] Determined by Central Testing Laboratory Ltd. (Winnipeg, Manitoba, Canada), following Crude Fiber Method by Ankom Technology (2017).
[c] Determined by Central Testing Laboratory Ltd. (Winnipeg, Manitoba, Canada), following AOCS Method Am 5-04.
[d] Determined by Central Testing Laboratory Ltd. (Winnipeg, Manitoba, Canada), following AOAC Method 942.05.
[e] Determined using the ME equation for swine: (kcal/kg)=4151-(122*Ash)+(23*Crude Protein)+(38*Fat)-(64*Crude fiber)*(1.003-(0.0021* Crude Protein))

Dogs were fed twice daily, weighed weekly and body condition scored using a 9-point scale (LaFlamme, 1997). During the pre-trial phase of at least a month, each dog was fed a standard commercial diet, and individual food portion/maintenance energy required to maintain ideal weight (body condition score of 4–5 on a 9-point scale) was determined. Once on trial, isocaloric test diet portions determined for each dog in the pre-trial phase was calculated and used throughout the feeding trial without any further adjustment to portion size.

3.4.3 Glycemic Index & Digestibility Testing

To establish both glycemic responses and starch digestibility of diets as well as the effects on circulating amino acid and taurine levels, Beagles were fed each test diet for 7 days. This was done in a randomized, cross-over, blinded, repeated measures design study with a 3-day washout period on the commercial diet between each test diet and another 7-day feeding period followed by a 3-day washout repeated until all diets had been tested in each dog. Apparent total tract digestibility was determined in feces collected on the sixth and seventh day of each feeding period. After collection, feces were kept at−20°C until they were dried at 65°C for 72 h. Fecal samples were then sent to an analytical laboratory to assess nutrient excretion (Central Testing Laboratory Ltd., Winnipeg, MB, Canada). In addition, another portion of dried feces was used to conduct total bile acid analyses using a commercial kit according to manufacturer instructions (Total Bile Acid Assay Kit, Cell Biolabs Inc. San Diego, CA, USA) which uses a colorimetric enzyme driven reaction in which bile acids are incubated in the presence of 3-alpha hydroxysteroid dehydrogenase and thio-NADH. Total fecal output was not measured in this experiment.

Apparent total tract digestibility was calculated using the equation 3.1:

Equation 3.1:

$$Apparent\ Digestibility\ (\%) = \{1 - (\frac{\%\ Nutrient\ in\ Feces}{\%\ Nutrient\ in\ Diet})\ x\ (\frac{\%\ Indicator\ in\ Diet}{\%\ Indicator\ in\ Feces})\} \times 100$$

On day 7 of feeding, dogs were fasted overnight and 8.0 mL of whole blood was collected the next day from the jugular vein. While still fasted, 5.0 mL of whole blood was collected into EDTA tubes and centrifuged at 2,200 RPM to obtain plasma. Samples were stored at−80°C until assayed for plasma cysteine (measured as the total of the cysteine dimer, cystine, plus the deprotonated form of cysteine called half-cystine), methionine, and taurine at a contract analytical laboratory (Amino Acid Laboratory, University of California Davis, Davis, CA, USA). Beagles were then catheterized in the cephalic vein and fasting blood glucose was determined using an Ultra2 glucometer (OneTouch, LifeScan Canada ULC, Malvern, PA, USA). Depending on the diet fed during the week, dogs were either fed glucose (oral glucose tolerance test) providing 1 g/kg body weight of glucose after consumption of the commercial diet or fed a portion of the test diet fed that week to provide 1 g/kg available carbohydrate (1 g/kg divided by % available carbohydrate) according to established protocols in dogs in our group (Adolphe et al., 2012). The amount of available carbohydrate in each diet was determined using a commercially obtained test kit (Available Carbohydrate Assay Kit, Megazyme, Bray, Ireland). Available carbohydrate was defined as total digestible starch (TDS) plus maltodextrins, sucrose, D-glucose, D-fructose, and lactose. The available carbohydrate method measures glucose liberated in vitro after 4 h incubation (AOAC Method 2020.07). Blood glucose levels were monitored using a glucometer over a 5-h time period (time 0, 5, 10, 15, 20, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270, 300) according to methods established in this group (Adolphe et al., 2014).

3.4.4 Sulfur-Containing Amino Acid Plasma Analysis

Plasma sample analyses were conducted at a contract analytical laboratory (Amino Acid Laboratory, University of California Davis, Davis, CA, USA). Modified AOAC Official Method 994.12 alternative III was performed according to GLP (taurine and methionine recovery rates were 97–102%). Variances between duplicates were <5%. Cystine results were corrected by multiplying factor of 2 (recovery rate is about 50%).

3.4.5 Data Handling and Statistics

All data were tested for normality and outliers using the Kolomogorov-Smirnov test, Q-Q plots, and box plots. Depending on the normality of the data either a repeated measure, 1-way ANOVA, or a repeated measure, 1-way ANOVA on ranked data was then conducted followed by post-hoc Tukey's tests if significance was achieved. Differences were considered statistically significant at $p \leq 0.05$. Values obtained during the oral glucose tolerance test were not used for statistical analysis and were provided strictly for reference or in glycemic index calculations. Principal components analysis was also performed on the six dietary treatment groups (34 variables studied). Factors were reduced to two components and variables studied were reduced to 24 where ≥83% of the variance across the data was explained. Analyses were performed using SigmaPlot 12.0 and Systat 12.0 (Systat Software Inc. San Jose, CA, USA).

3.5 Results

3.5.1 Proximate and Amino Acid Analysis of Diets

As expected, crude fiber increased in the diets from the lowest rice control diet to the highest wrinkled pea diets (Table 3.2). Crude fat of the diets ranged from 16.01% to 19.99%, but was unrelated to dietary fiber (Table 3.2). Similarly, metabolizable energy ranged from 3,806 to 4,121 kcal/kg. In the test diets, crude protein (% dry matter) ranged from 26.13% to 33.69% which was well above the AAFCO minimum of 18% dietary protein (AAFCO, 2013). However, dietary protein content slightly decreased with increasing fiber content, as higher levels of pulse ingredients were incorporated (Table 3.2). All test diets met or exceeded the AAFCO requirements of 0.33% methionine and 0.65% cystine+methionine (AAFCO, 2013), except the wrinkled pea CDC 4,140–4 diet which had 0.5% cystine+methionine (Table 3.3). Dietary methionine was highest in the rice diet at 0.84% on a dry matter basis, with all pulse-based diets containing lower methionine to a low of 0.38% for the high fiber CDC 4,140–4 wrinkled pea diet (Table 3.3). Dietary cystine ranged from a high of 1.01% for the rice diet to a low of 0.38% for the CDC 4,140–4 wrinkled pea diet, similar to the methionine results (Table 3.3). The grain-containing rice diet also had the highest level of taurine at 0.14% (Table 3.3). In contrast, the pulse containing diets had lower, varying amounts of taurine which ranged from 0.07% to 0.12% as shown in Table 3.3.

Table 3.3: Cystine, methionine and taurine levels in test diets after extrusion and fat coating. Also shown are the contents of different fiber types, amylose, available carbohydrate and meal size needed to provide 1 g/kg available carbohydrate for each diet. Diets are listed in order of increasing fiber and amylose content from left to right.

	Rice Diet	Lentil Diet	Smooth Pea Diet	Fava Bean Diet	Wrinkled Pea Diet (4140-4)	Wrinkled Pea Diet (Amigold)
Cystine [a]	1.010	0.610	0.170	0.640	0.120	0.490
Methionine [a]	0.840	0.470	0.700	0.480	0.380	0.460
Cystine & Methionine [a]	1.850	1.080	0.870	1.120	0.500	0.950
Taurine [a]	0.140	0.090	0.120	0.090	0.070	0.070
LMWDF [b]	< 0.6	2.2	2.0	1.6	4.8	4.9
Soluble HMWDF [b]	0.9	1.3	1.2	1.7	1.5	1.9
Insoluble HMWDF [b]	15.2	18.1	16.7	20.6	21.3	21.8
TDF [b]	16.1	21.6	19.9	23.9	27.6	28.6
Amylose content [c]	5.1 ± 0.2	7.4 ± 0.2	7.6 ± 0.1	7.7 ± 0.1	13.9 ± 0.1	14.6 ± 0.1
Available carbohydrate (g/100g) [d]	23.9 ± 0.6	24.7 ± 0.3	25.2 ± 0.3	23.9 ± 0.3	22.4 ± 0.6	22.7 ± 0.4
Meal size fed to provide 1 g/kg available carbohydrate (g whole diet/kg dog)	4.2	4.0	4.0	4.2	4.5	4.4

All values are expressed as % dry matter unless stated otherwise. LMWDF = low molecular weight dietary fiber; HMWDF = high molecular weight dietary fiber; TDF = total dietary fiber.
[a] Determined by Central Testing (Winnipeg, MB, Canada) using UPLC and ninhydrin detection.
[b] Determined by Eurofins (Toronto, ON, Canada) using AOAC 2011.25 dietary fibre method.
[c] Determined using an iodine colorimetric method of Chrastil (1987).
[d] Determined using Megazyme Available Carbohydrate Assay Kit following AOAC Method 2020.07.

3.5.2 Fiber, Amylose and Carbohydrate Content of the Diets

Diets were formulated to contain 20% available carbohydrate and results of the available carbohydrate kit for diets showed good agreement with the target (Table 3.3). Available carbohydrate content (defined as the amount of glucose liberated by amyloglucosidase+pancreatin in 4 h in vitro at 37°C) in the formulated test diets ranged from 22.4% to 25.2%, as shown in Table 3.3. This available carbohydrate value was then used to calculate what meal size needed to be fed to provide 1 g available carbohydrate per kilogram body weight of the dogs during glycemic testing (Table 3.3). The same test diets prior to fat coating were tested for in vitro starch digestibility using Englyst methodology in a separate study by this group (Ren et al., 2020). Uncoated wrinkled pea diets after extrusion (both Amigold and 4,140–4) had higher resistant starch content (15.1–19.0% on a dry starch basis) compared to the other pulse-based or rice-based diets (2.7–5.5% on a dry starch basis). Also, both wrinkled pea diets had lower gelatinized starch after extrusion (11.9–12.3% dry matter) compared with those of round pea, lentil, fava bean, and rice diets (17.1–19.6%; 9).

Dietary fiber analyses of the fat coated test diets used in this feeding study demonstrated that they had varying levels of dietary fiber (Table 3.3). Low molecular weight dietary fiber varied from <0.6–4.9% for the rice and the wrinkled pea (Amigold) diets, respectively (Table 3.3). High molecular weight dietary fiber was subdivided into two categories: insoluble high molecular weight dietary fiber and soluble high molecular weight dietary fiber. The soluble high molecular weight dietary fiber varied from 0.9% to 1.9% (rice and Amigold wrinkled pea diets, respectively), while insoluble high molecular weight dietary fiber varied from 15.2% to 21.8% (rice and Amigold wrinkled pea diets, respectively; Table 3.3). Similarly, total dietary fiber ranged from 16.1% to 28.6% (rice and Amigold wrinkled pea diets, respectively; Table 3.3).

Finally, the wrinkled pea (Amigold) pulse-containing diet had the highest amylose content (14.6%) while the rice-containing diet had the lowest level of amylose at 5.1% (Table 3.3).

3.5.3 Digestibility

Apparent total tract digestibility of crude protein varied across the diets (ANOVA, $p < 0.001$). Crude protein digestibility varied from 72.95% to 84.35%, the rice diet had the highest crude protein digestibility, and the wrinkled pea (Amigold) diet had the lowest (Table 3.4). For sulfur-containing amino acids, the apparent total tract digestibility of cystine, methionine, cystine+methionine, and taurine varied among test diets (ANOVA, $p < 0.001$ for all but taurine where $p = 0.034$; Table 3.4). Variability was noted for cystine digestibility which ranged from 6.5% to 90% (Table 3.4). This variability was not due to diarrhea. Although fecal output and score (quality) were not quantitated in this study, qualitatively, no obvious changes were observed among diets. All other macronutrient (fat and starch) digestibility values were also different among diets (Table 3.4).

Table 3.4: Apparent total tract digestibility analyses of protein, fat, starch, cystine, methionine, cystine+methionine and taurine in the 6 different test diets formulated at 20% total digestible starch with variable amounts of amylose, fed for 7 days. Diets are listed in order of increasing fiber and amylose content from left to right.

	Rice	Lentil	Smooth Pea	Fava Bean	Wrinkled Pea (4140-4)	Wrinkled Pea (Amigold)	p-Value
Protein**	84.35 ± 1.3^{b}	81.49 ± 0.69^{b}	80.85 ± 0.66^{b}	$79.21 \pm 0.67^{a,b}$	$78.90 \pm 0.58^{a,b}$	72.95 ± 1.37^{a}	< 0.001
Fat*	98.47 ± 0.18^{c}	$97.52 \pm 0.30^{b,c}$	$96.40 \pm 0.65^{a,b}$	$97.52 \pm 0.27^{b,c}$	$96.85 \pm 0.42^{a,b}$	95.82 ± 0.48^{a}	< 0.001
Starch**	$97.71 \pm 0.41^{b,c}$	97.72 ± 0.76^{c}	$97.77 \pm 0.23^{a,b,c}$	$98.28 \pm 0.20^{b,c}$	$89.68 \pm 2.08^{a,b}$	87.74 ± 2.49^{a}	< 0.001
Cystine*	89.76 ± 0.78^{d}	84.91 ± 1.09^{d}	41.80 ± 2.30^{b}	86.10 ± 0.78^{d}	6.52 ± 4.14^{a}	73.31 ± 3.45^{c}	< 0.001
Methionine**	79.22 ± 1.66^{b}	$76.27 \pm 0.85^{a,b}$	83.29 ± 0.99^{b}	$75.80 \pm 0.82^{a,b}$	64.72 ± 2.20^{a}	53.56 ± 5.24^{a}	< 0.001
Cystine+Methionine**	84.98 ± 1.01^{c}	$80.97 \pm 0.94^{b,c}$	$75.18 \pm 1.08^{a,b}$	$81.85 \pm 0.73^{b,c}$	41.12 ± 6.47^{a}	$63.75 \pm 4.07^{a,b}$	< 0.001
Taurine**	$82.92 \pm 1.83^{a,b}$	80.78 ± 11.58^{b}	$77.44 \pm 3.09^{a,b}$	69.98 ± 2.19^{a}	$77.57 \pm 5.96^{a,b}$	$74.42 \pm 5.11^{a,b}$	0.034

All values are expressed as % of apparent total tract digestibility. Data is shown as Mean ± SEM; n = 8 dogs. Different letters indicate significant differences among diets using Tukey's post-hoc analysis (p < 0.05) after significant p-values reported in 1-Way Repeated Measures ANOVA* and Friedman's One-Way ANOVA on Ranked Data**. Where same letters are indicated in a row, no significant differences among diets were detected.
Protein, and fat, determined by Central Testing (Winnipeg, MB Canada) as described in Table 3.2. Diet and fecal amino acids and starch were determined by Central Testing (Winnipeg, MB, Canada) using UPLC with ninhydrin detection and enzymatically using UV detection, respectively.

3.5.4 Glycemic Response

Blood glucose increased from fasting after feeding glucose or a meal with 1 g available carbohydrate/kg body weight, then returned to baseline within 5 h in dogs (Figure 3.1). From these glycemic response figures, quantitative data and statistical analyses on peak, time to peak, area under the curve, and glycemic index were calculated (Table 3.5). In dogs fed the commercial diet for 7 days, the fasting blood glucose level prior to the oral glucose tolerance test was 3.8 ± 0.2 mmol/L. After 7 days of feeding the test diets, fasting blood glucose had variation with diet in these same dogs (Figure 1; ANOVA, $p < 0.001$; Table 3.5). After ingestion of the glucose standard, glucose levels peaked at 52.5 ± 5.5 min with a blood glucose value of 6.3 ± 0.2 mmol/L (Figure 1, Table 3.5). Time to peak blood glucose was longer after a meal of a whole diet and the peak was lower when compared to the response to the glucose standard (Figure 3.1).

Dogs fed the lentil-based diet and wrinkled pea (4,140–4) diet for 7 days had the lowest fasting blood glucose levels (3.5 ± 0.1 mmol/L) and (3.6 ± 0.1 mmol/L), respectively (Table 3.5). The peak blood glucose was also different among diets (ANOVA, $p = 0.01$), while the time to peak was not different (ANOVA, $p = 0.20$). Dogs fed the rice diet had the highest peak blood glucose at 5.0 ± 0.09 mmol/L, while the lowest peak blood glucose was observed with the lentil diet at 4.4 ± 0.1 mmol/L (Table 3.5). The area under the blood glucose response curve (AUC) was different among diets (ANOVA, $p = 0.02$; Table 3.5), ranging from 810.9 ± 15.8 mmol/L x min for the rice diet to a low of 726.5 ± 21.7 mmol/L x min for the lentil diet (Table 3.5). Glycemic index values of the diets followed the AUC trend, with differences among diets (ANOVA, $p < 0.001$; Table 3.5). The rice diet had the highest glycemic index of 95.7 ± 2.2, while the lowest glycemic index was observed in the lentil diet at 85.8 ± 2.8 (Table 3.5).

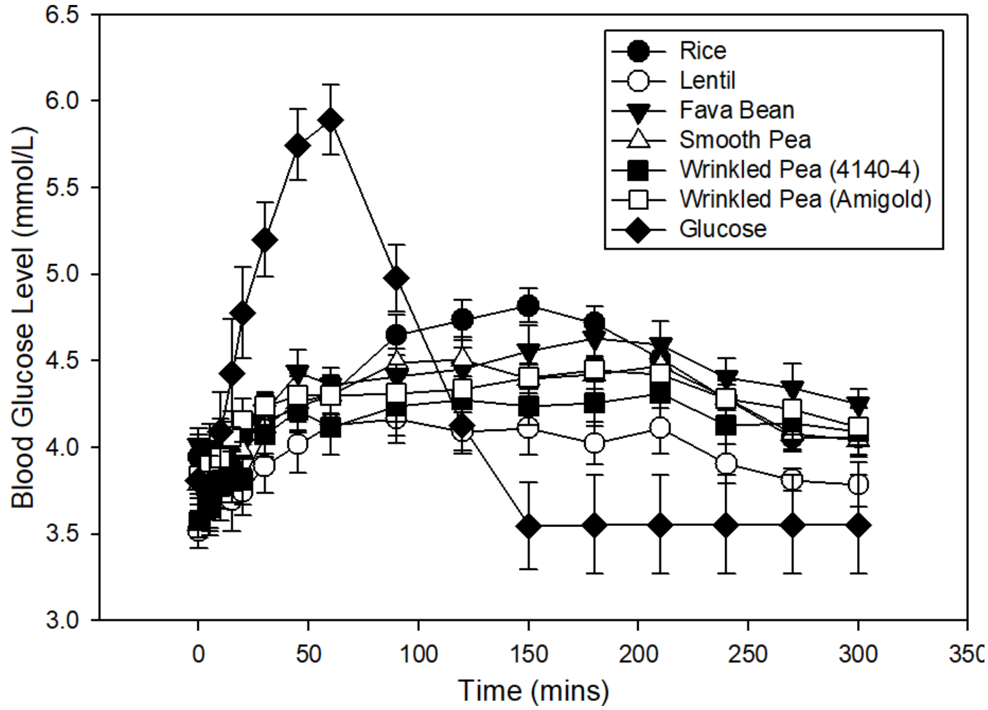

Figure 3.1: Time courses of blood glucose response in fasted dogs after an oral glucose challenge (1 g glucose/kg body weight) after 7 days of feeding a commercial husbandry diet and after consuming a meal of each test diet (1 g available carbohydrate/kg body weight). Dogs were fed each test formulated at 20% total digestible starch with increasing levels of fiber and amylose (lowest to highest from top to bottom in figure legend) for 7 days prior to an overnight fast, followed by the test meal the next day. Data is shown as Mean ± SEM; n = 8 dogs.

Table 3.5: Quantitative measures of glycemic response in fasted beagles after feeding a meal of different test diets. Diets are listed in order of increasing fiber and amylose content from left to right. Data is taken from that shown in Figure 1.

	Glucose	Rice	Lentil	Smooth Pea	Fava Bean	Wrinkled Pea (4140-4)	Wrinkled Pea (Amigold)	p-Value
Fasted Blood Glucose (mmol/L)*	3.8 ± 0.2	3.9 ± 0.1[b]	3.5 ± 0.1[a]	3.8 ± 0.06[a]	4.0 ± 0.1[b]	3.6 ± 0.1[a]	3.8 ± 0.1[b]	< 0.001
Peak (mmol/L)*	6.3 ± 0.2	5.0 ± 0.09[b]	4.4 ± 0.1[a]	4.7 ± 0.1[a,b]	4.8 ± 0.1[a,b]	4.5 ± 0.1[a,b]	4.7 ± 0.1[a,b]	0.01
Time to Peak (mins)**	52.5 ± 5.5	135 ± 12.7	99.4 ± 18.6	91.9 ± 9.2	132.5 ± 20.9	111.6 ± 18.0	130 ± 22.8	0.2
AUC (mmol/L x mins)*	849.3 ± 19.6	810.9 ± 15.8[b]	726.5 ± 21.7[a]	780.2 ± 14.0[a,b]	792.6 ± 12.6[a,b]	749.4 ± 18.8[a,b]	776 ± 14.1[a,b]	0.02
Glycemic Index*		95.7 ± 2.2[c]	85.8 ± 2.8[a]	92.2 ± 2.6[b,c]	93.6 ± 2.2[b,c]	88.3 ± 1.5[a,b]	91.5 ± 1.2[b,c]	< 0.001

Data is shown as Mean ± SEM; n = 8 dogs. Different letters indicate significant differences among diets using Tukey's post-hoc analysis ($p < 0.05$) after significant p-values reported in 1-Way Repeated Measures ANOVA* and Friedman's One-Way ANOVA on Ranked Data**. Where same letters are indicated in a row, no significant differences among diets were detected. Glucose data was used to calculate glycemic index and is shown for reference, but was not used in the statistical analyses of the diets.

3.5.5 Sulfur-Containing Amino Acids in Plasma

After 7 days of feeding each diet, fasting plasma taurine was different in the dogs after feeding different diets (ANOVA, p = 0.021; Table 3.6). Feeding rice and the lentil diets produced the highest plasma taurine levels in the dogs at 99 and 111 nmol/ml (equivalent to μmol/L) while feeding the fava bean diet produced the lowest plasma taurine at 73 nmol/ml (Table 3.6). Despite differences in dietary content of cystine and methionine (Table 3.2), after 7 days of feeding each diet, fasting levels of plasma methionine and half-cystine were not significantly different (Table 3.6). In contrast, plasma cysteine varied greatly among the dogs fed the different diets with the highest levels when the lentil and rice diets were fed.

3.5.6 Bile Acid Assay

Total fecal bile acid content was significantly different among diets, with the rice diet having the highest value and the wrinkled pea 4,140–4 diet having the lowest (Figure 3.2).

Table 3.6: Fasted plasma amino acid levels of taurine, half-cystine, cysteine and methionine observed in fasted dogs after 7 days of feeding each test diet. Diets are listed in order of increasing fiber and amylose content from left to right.

	Rice	Lentil	Smooth Pea	Fava Bean	Wrinkled Pea (4140-4)	Wrinkled Pea (Amigold)	p-Value
Half-cystine*	17.61 ± 0.85	19.16 ± 1.62	17.46 ± 1.14	16.43 ± 1.42	18.34 ± 1.59	17.13 ± 1.12	0.42
Cysteine**	132.5 ± 32.79[a]	2732.75 ± 139.75[b]	807.38 ± 279.88[a,b]	458.63 ± 213.27[a]	158.88 ± 213.27[a]	147.86 ± 28.58[a]	0.002
Methionine*	56.31 ± 3.13	50.18 ± 2.09	53.58 ± 2.08	54.74 ± 2.51	53.35 ± 3.13	52.55 ± 3.54	0.52
Taurine**	99.03 ± 11.90[a,b]	111.14 ± 18.04[b]	82.24 ± 8.67[a,b]	72.68 ± 12.74[a]	86.63 ± 10.95[a,b]	89.74 ± 15.36[a,b]	0.021

All values expressed nmol/ml (equivalent to µmol/L). Amino acids were measured at a contract analytical laboratory (Amino Acid Laboratory, University of California Davis, Davis, CA, USA) using modified AOAC Official Method 994.12 alternative III.
Data is shown as Mean ± SEM; n = 8 dogs. Different letters indicate significant differences among diets using Tukey's post-hoc analysis ($p < 0.05$) after significant p-values reported in 1-Way Repeated Measures ANOVA* and Friedman's One-Way ANOVA on Ranked Data**. Where no letters are indicated in a row, no significant differences among diets were detected.

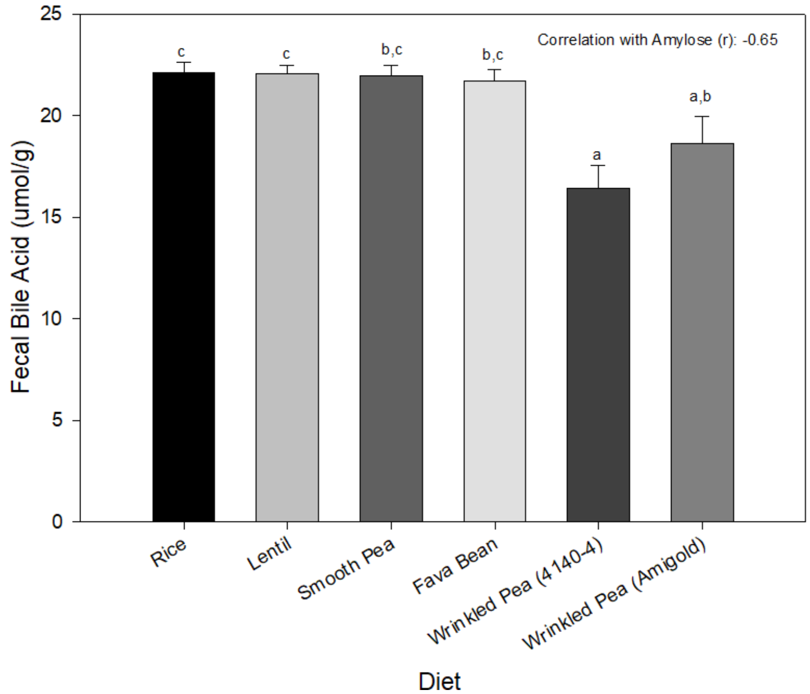

Figure 3.2: Fecal bile acid content from dogs after 7 days of feeding each test diet. Diets are listed in order of increasing fiber and amylose content from left to right. Data is shown as Mean ± SEM; n = 8 dogs. Different letters indicate significant differences using Tukey's post-hoc analysis ($p < 0.05$) after 1-Way Repeated Measures ANOVA.

3.5.7 Principal Components Analysis

In order to assess the relationships among the variables tested, a Principal Components Analysis (PCA) was run initially with all variables measured in all 6 test diets. The top 24 variables that were correlated to the first two components were retained in the final PCA analysis. These 24 variables explained 83.13% of variance in this final analysis (see Table 3.7). A factor loadings plot of these variables (Figure 3.3A) clustered variables with the greatest tendency to be positively related to each other. A plot of weighted factor scores for each of these 24 variables for each diet resulted in a graph where the rice diet was clearly separated from the two wrinkled pea diets, while fava bean, smooth pea, and lentil diets were intermediate (Figure 3.3B). This confirms the order we predicted based on fiber and amylose content. In support of this, the greatest separation among the diets was along the x-axis showing Factor 1 scores

(Figure 3.3B) where high positive scores indicated diets with high dietary content of chicken meal, crude protein, sulfur-containing amino acids (cystine, methionine and taurine) as well as high fecal bile salt content, available carbohydrate and fat digestibility, but low values for dietary content of amylose and all fiber fractions (Figure 3.3B). The diets did not separate as much on the y-axis except for the lentil diet, but the 95% confidence intervals were very large for this diet and it did overlap with all diets on this axis (Figure 3.3B). High positive y-axis values for Factor 2 were associated with high fasting blood glucose, long time to peak blood glucose, high glycemic index and high plasma methionine values, but low plasma cystine, half-cysteine and taurine values.

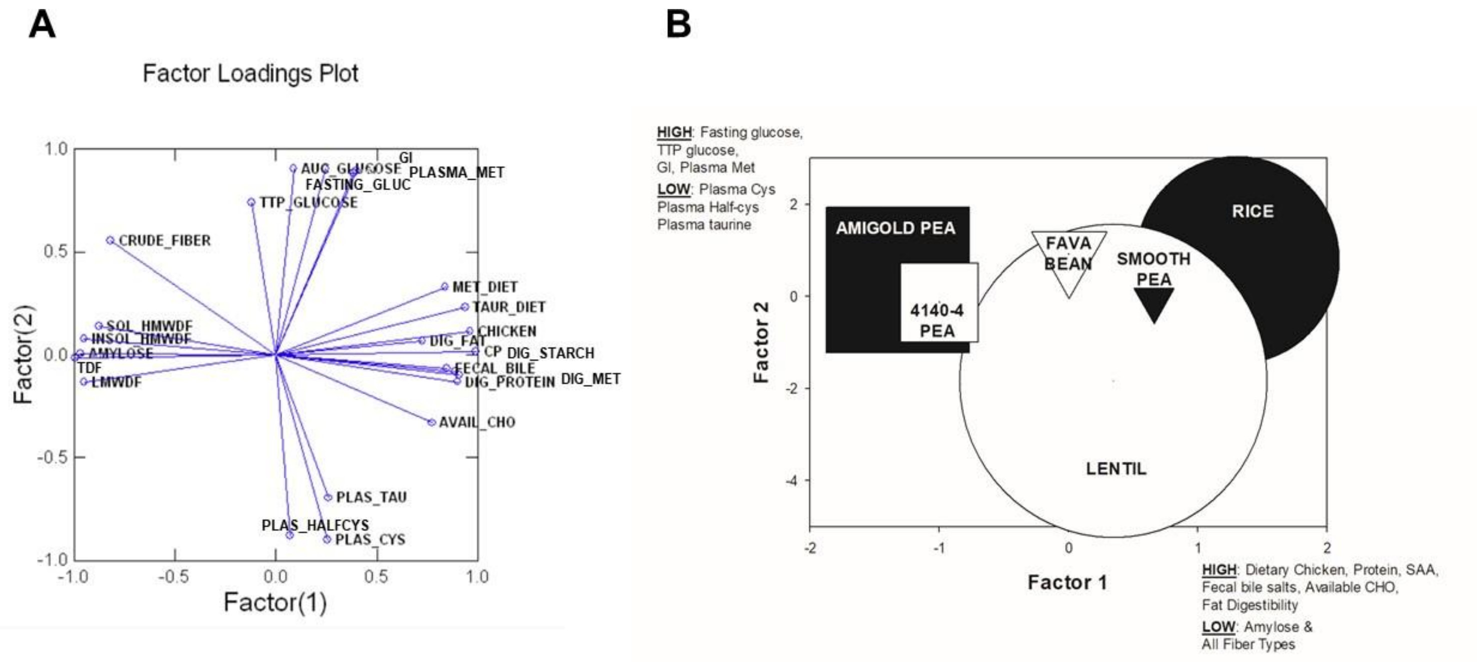

Figure 3.3: Plots from Principal Components Analysis of the top 24 variables that explained the most variability among diets. **A**. Factor loadings plot for factor 1 and factor. **B.** A plot of weighted factor scores for components 1 versus components 2 for each test diet in this study from the Principal Components Analysis. The size of the shape indicates the 95% confidence intervals and distance between shapes indicates greater difference among diets. Higher, positive scores for Factor 1 were associated with high dietary chicken content, dietary crude protein, dietary sulfur-containing amino acid content (SAA; cysteine, methionine and taurine), fecal bile salts, dietary available carbohydrate and fat digestibility, but low amylose and all fiber types. In contrast, a high positive score for Factor 2 was associated with high fasting blood glucose, long time to peak blood glucose, high glycemic index and high plasma methionine, but low plasma cysteine, plasma half-cysteine and plasma taurine. SOL_HMWDF = soluble high molecular weight dietary fiber; INSOL_HMWDF = insoluble high molecular weight dietary fiber; TDF = total dietary fiber; LMWDF = low molecular weight dietary fiber; TTP_GLUCOSE = time to peak glucose; FASTING_GLUC = fasting blood glucose; AUC_GLUCOSE = area under the curve for blood glucose response; GI = glycemic index; PLASMA_MET = plasma methionine; MET_DIET = dietary level of methionine; TAUR_DIET = dietary level of taurine; DIG_FAT = apparent total tract digestibility of fat; DIG_STARCH = apparent total tract digestibility of starch; FECAL BILE = fecal bile salts; DIG_PROTEIN = apparent total tract digestibility of protein; DIG_MET = apparent total tract digestibility of methionine; AVAIL_CHO = available carbohydrate in diet; PLAS_TAU = plasma taurine; PLAS_HALFCYS = plasma half-cysteine; PLAS_CYS = plasma cystine

Table 3.7: Principal Components Analysis of the top 24 variables examined in this study that explained 83% of the variation among diets. The strength of the relationship (r-value) for the two components, referred to as component loadings, are shown for each of these 24 variables.

	Component 1	Component 2
Total Variance Explained (%)	56.03	27.095
Variable	Component Loadings	
Total Dietary Fiber	-0.992	-0.016
Crude Protein	0.990	0.014
Amylose	-0.968	0.007
Chicken By-Product Meal	0.962	0.115
Insoluble High Molecular Weight Dietary Fiber	-0.950	0.076
Low Molecular Weight Dietary Fiber	-0.949	-0.134
Dietary Taurine	0.938	0.231
Methionine Digestibility	0.909	-0.096
Protein Digestibility	0.901	-0.131
Starch Digestibility	0.889	-0.083
Soluble High Molecular Weight Dietary Fiber	-0.876	0.138
Fecal Total Bile Acids	0.847	-0.067
Dietary Methionine	0.839	0.329
Dietary Crude Fiber	-0.818	0.553
Available Carbohydrate	0.773	-0.331
Fat Digestibility	0.726	0.069
Fasting Blood Glucose Levels	0.244	0.904
Area Under the Curve (Glucose)	0.089	0.903
Plasma Cysteine Levels	0.258	-0.898
Glycemic Index	0.399	0.895
Plasma Methionine Levels	0.385	0.883
Plasma Half-Cystine	0.071	-0.880
Blood Glucose, Time to Peak	-0.118	0.740
Plasma Taurine Levels	0.263	-0.696

3.6 Discussion

The most important findings of this study were that the pulse-based, grain-free diets produced a lowered glycemic response, which could be utilized to promote increased satiety and decreased risk of diabetes mellitus. This postprandial glycemic response was negatively associated with fasted plasma taurine and cysteine levels but positively associated with fasted plasma methionine levels in dogs after 7-days of feeding each test diet. Surprisingly, fiber content (all fractions) and dietary amylose content were not strongly related to fasted plasma sulfur-containing amino acid levels. Instead, fiber and amylose were negatively correlated to digestibility of all macronutrients, including sulfur-containing amino acids. Despite increasing levels of dietary fiber as amylose content increased among diets, excretion of fecal bile acids in this study unexpectedly decreased. In addition, on a short-term feeding basis (7 days), grain-free diets did not cause a detrimental impact on the fasting plasma taurine status in dogs, despite decreased taurine digestibility. This study showed promising effects of grain-free, pulse-based diets that could be utilized for the improvement of health in dogs.

3.6.1 Test Diet Properties and Relationship to Fiber Content

All test diets were formulated to be as similar as possible while aiming to achieve ~20% available carbohydrate. This produced desired variations among the diets regarding their dietary fiber and amylose content. Diets with increasing levels of dietary fiber and amylose were among the diets that incorporated the largest amounts of pulse flour which provided plant protein and subsequently needed decreased amounts of animal protein (chicken meal) to be isonitrogenous. In addition, decreasing the amount of animal protein among diets produced decreasing levels of taurine in diets, which is why the rice diet had higher levels of dietary taurine than the two wrinkled pea diets. Other studies confirmed that seafood and poultry products contained high concentrations of taurine, while plant products do not (Spitze et al., 2003). Dietary fiber also did not appear to have any impacts on the other amino acids studied within the different diets such as cystine, methionine, and cysteine + methionine. All pulse diets had lower levels of these amino acids in comparison to the rice diet due to pulses possessing limited content of these amino acids (Singh 2017; Tiwari & Singh, 2012). This could explain why there were variations of dietary cystine, methionine, and cystine+methionine in the test diets.

In this study increasing amylose content among the different diets was associated with increasing levels of total dietary fiber. This was due to pulses with higher amylose levels containing a larger amount of resistant starch, which is the fraction of starch that reaches the colon intact, as it is not digested in the small intestine unless completely cooked (Newberry et al., 2018). As reviewed by Newberry et al., resistant starch such as amylose is also viewed as dietary fiber, partially explaining the link between increased dietary fiber and amylose among diets. Increased amounts of amylose and dietary fiber influence nutrient digestibility and postprandial glucose responses (Carciofi et al., 2008). Thus, based on those diets we produced, our results support our hypothesis that the diets with higher fiber and lower animal-source protein exert negative effects on apparent total tract digestibility of macronutrients and amino acids and produce lower glycemic responses. However, after 7-days of feeding each diet, all fasting plasma levels of cysteine, methionine, and taurine in the dogs were within normal range. Longer term studies are needed to confirm this.

3.6.2 Effect of Different Pulse-Based Diets on Glycemic Response

The diets for this study were chosen to demonstrate how increasing dietary fiber produced positive attributes on glycemic responses but might have negative impacts on other nutrients in dogs. In humans, increased consumption of dietary fiber and amylose decreased postprandial blood glucose (Behall & Hallfrisch, 2002; Behall & Howe, 1995; Behall et al., 1989; Granfeldt et al., 1995). High amylose pulse crops when used as an ingredient in dog diets minimized the risk of obesity and diabetes mellitus (Carciofi et al., 2008) and pulse-based diets have low glycemic properties in dogs (Adolphe et al., 2012; Carciofi et al., 2008; Adolphe et al., 2015; Briens et al., unpublished). However, a study systematically using a gradient of amylose content in dog diets had not yet been performed. The current study demonstrates that while amylose and total dietary fiber content is clearly an important factor in decreasing glycemic response in dogs, the spurious low results with the lentil diet point likely contributed to the lack of correlation of all the glycemic endpoints to dietary fiber and amylose content.

In addition to dietary fiber content determining glycemic response, other factors include digestion rate, amount of diet ingested, processing factors, and dietary composition (Carciofi et al., 2008). In this study, all diets had 22–25% total digestible starch. However, since wrinkled pea flour contains much lower starch levels (~34%), the high amylose wrinkled pea diets contained up to 65% flour which contributed a large amount of fiber compared to the low amylose diets. In human studies, resistant starch is an indication of high levels of amylose and contributes to decreased starch digestibility and increased levels of total dietary fiber (Li et al., 2019). In similar studies, changes in dietary fiber impacts glycemic response by slowing down the rate of passage of feed and the rate of hydrolysis on polysaccharides in starch (Nguyen et al., 1998; Wolever, 1990). Postprandial glucose responses are further impacted by dietary fiber as it is believed to prolong glucose absorption, thus reducing variations in glucose responses (Carciofi et al., 2008). Overall, glycemic response in this study was less impacted by the varying levels of amylose and dietary fiber within the different test diets and instead related to some other unidentified factor that was associated with high plasma taurine and cysteine. Further studies are needed to confirm this finding and explore how this happens.

3.6.3 Effect of Amylose vs. Fiber on Macronutrient and Amino Acid Digestibility

The results of the current study which found decreasing digestibility of all macronutrients (protein, fat, and starch) with increasing dietary amylose and total dietary fiber agrees well with a study conducted by (Beloshapka et al., 2020). Digestibility of crude protein decreased as total dietary fiber consumption increased in dogs (Beloshapka et al., 2020). This could be explained possibly by endogenous factors within the pulse flours having intrinsic interactions to form structures with starch, such as amylose, to limit the digestibility of protein (Rooney & Pflugfelder, 1986). Similar to what is seen with protein digestion, lipids also interact with starches, creating a single-helical structure with amylose molecules and limits the enzymatic digestibility of starch (Debet & Gidley, 2006). These amylose-lipid complexes that are resistant to starch digestion are formed during the exposure to elevated temperatures, which occur during the process of extrusion (Seneviratne & Biliaderis, 1991; Lin et al., 1997). Due to the possibility of these processes, lipid digestibility in this study decreased as amylose levels and total dietary fiber increased. For example, a study in growing pigs demonstrated that increasing levels of fiber decreased the apparent total tract digestibility of both crude protein and fat (Zhang et al., 2013).

In addition to amylose and dietary fiber impacting the digestibility of macronutrients, they also impact the digestibility of amino acids. In this study increasing levels of amylose and dietary fiber were negatively correlated to digestibility of cystine, methionine, and taurine. Diets containing high amounts of dietary fiber not only lead to a greater possibility of sulfur-containing amino acid excretion, but also greater microbial overgrowth and taurine assimilation by the microflora (Mansilla et al., 2019). This would be detected as increased apparent total tract digestibility, so it is inconsistent with our observations. What this study could not determine is whether a particular fiber fraction or amylose content were driving changes in sulfur-containing amino acid digestibility since all fiber fractions and amylose were all equally negatively associated with digestibility.

3.6.4 Effect of Different Pulse-Based Diets on Plasma Levels of Sulfur-Containing Amino Acids

Unlike cats, dogs are able to synthesize taurine from the sulfur-containing amino acids cysteine and methionine (Backus et al., 2006). Thus, taurine is not considered an essential dietary nutrient for dogs, while methionine and methionine+cysteine have dietary minima in dogs (AAFCO, 2013). In this study, fasting plasma taurine levels ranged from 73 to 111 nmol/mL in fasted dogs after 7 days of feeding test diets which falls within previously reported taurine reference ranges of 63–194 nmol/mL (Kaplan et al., 2018; Ontiveros et al., 2020; Tôrres et al., 2003; Delaney et al., 2003). Other studies disagree on whether or not grain-free diets contribute to decreased plasma taurine levels in dogs. Some studies determined that dogs consuming grain-free diets have an increased prevalence of taurine deficiencies (Kaplan et al., 2018; Ontiveros et al., 2020), while others have noted no change or improvements in taurine status in dogs when consuming grain-free diets (Donadelli et al., 2020; Adin et al., 2019). Despite lower dietary taurine content with increasing dietary fiber and amylose in the current study, plasma taurine remained within normal range. This could be due to the short-term nature of the current study (7 days per diet). Another important factor linked to a lack of consistent change in plasma taurine may be that taurine levels in target tissues such as the heart are more relevant and these tissue levels could be depleted before plasma levels of these free amino acids fall (Sanderson et al., 2001). Moreover, the beagle breed is not predisposed to either taurine deficiencies or DCM (Pezzali et al., 2020). Taken together, this study demonstrated that short-term consumption of both grain-containing and grain-free diets had no major effect on plasma taurine levels in dogs.

In this study, fasting plasma levels of half-cystine ranged from 16 to 19 nmol/ml while fasted plasma levels of methionine varied from 50 to 56 nmol/mL. Levels for both of these amino acids are lower than, but close to, the value reported in a dog study with 46 and 57 nmol/mL, respectively (Delaney et al., 2003). Fasted plasma cysteine values had much greater and unexpected variation from 132 to 2,732 nmol/mL among diets in the current study. Cysteine numbers are suspect for two reasons. First, cysteine is unstable after sample collection and rapidly forms disulfide bonds with itself to form the dimer called cystine or with other plasma proteins which are subsequently removed before analysis (personal communication from Amino Acid Laboratory, University of California Davis, Davis, CA, USA). Second, in this experiment, the cysteine data was more likely overestimated due to interference during HPLC analysis from L-alpha-aminoadipic acid that co-eluted with cysteine (Tôrres et al., 2003). Coupled with the fact that dietary cystine levels did not vary that much, it seems likely that dietary fiber has no impact on fasting plasma cysteine, methionine or taurine levels, at least after 7 days of feeding test diets.

3.6.5 Effect of Dietary Fiber and Amylose on Fecal Bile Acids

Contrary to the original hypothesis, fecal excretion of total bile acids decreased as dietary fiber and amylose increased in this study in beagles after 7 days of feeding test diets. The findings of this study disagree with reports that dietary fiber can bind bile acids within the intestinal lumen, leading to increased fecal excretion of bile acids (Donadelli et al., 2020; Garcia-Diez et al., 1996; Stratton-Phelps et al., 2002). However, the results of this study agree with another report that grain-free diets did not lead to an increased excretion of bile acids in dogs (Pezzali et al., 2020). Soluble dietary fiber abundant in pulses was proposed to lower taurine availability in companion animals (Mansilla et al., 2019). One of the major roles of taurine in dogs is conjugation with bile acids to form the predominant bile salt, taurocholate (Sanderson et al., 2001; Mansilla et al., 2019). While soluble fibers bind bile acids to prevent their reabsorption through the entero-hepatic circulation, thereby lowering lipids (a beneficial health effect), this effect could also deplete taurine via taurocholate loss in the feces, leading to taurine wasting (Sanderson et al., 2001). Alternatively, legumes high in dietary fiber can also act as prebiotics for the gut microbiota, changing the microbial population composition and overgrowth, which could enhance taurine degradation in the small intestine before it can be absorbed (Tôrres et al., 2003). Future studies need to explore whether feeding periods >7 days cause increased bile salt loss, whether specific classes of bile acids are affected, or whether the effects on intestinal microbiota lead to taurine depletion in dogs and if so, which dietary fiber components, if any, contribute to this loss.

3.7 Study Strengths and Limitations

A strength of this study was the detailed glycemic response data for each diet. Another strength of this study was the use of multiple pulses to study if dietary fiber and amylose are impacting variables in different pulses or if there is another component that should be studied. One limitation of the study design was the sample size of eight beagles. However, we combated this limitation by using a cross-over, repeated-measures design, allowing all dogs to rotate through all diets and be studied. Another limitation of this study could be insufficient duration of feeding each diet for plasma sulfur containing amino acids to change. Ongoing studies in this group are exploring the effects of longer-term feeding periods but establishing what happens in shorter time frames is also important information. For digestibility measurements, a limitation was the use of the apparent total tract digestibility method, which was necessary since our studies require minimally invasive, non-lethal techniques to allow our dogs to be adopted into homes once retired. True digestibility can only be assessed through either lethal sampling to remove digesta along the length of the intestinal tract or through ileal sampling that requires surgical creation of a permanently disfiguring ileal cannula.

3.8 Conclusions

Pulses are beneficial at producing a low glycemic response in dogs and higher amylose pulses such as the wrinkled pea (4,140–4) have superior low glycemic properties in dogs. However, even pulses such as red lentil with relatively low amylose and dietary fiber could also be processed to produce a low glycemic diet. The trade-off for beneficial low glycemic properties of high dietary fiber and high amylose pulses is decreased macronutrient and amino acid digestibility. However, this study did not find high fiber or amylose to be associated with increased fecal bile acid secretion and instead observed a decrease. In addition, due to the limitations of diet formulation in this study, high-fiber pulse diets contained less animal-source protein and higher plant-based proteins compared with the rice diet, which could be another important factor leading to decreased digestibility of certain nutrients. However, despite decreased nutrient digestibility, plasma levels of sulfur-containing amino acids, including taurine remained within normal range after 7 days of feeding test diets, suggesting at least in the short term, that the benefits may outweigh any negative nutritional effects of high fiber canine diets.

4 CHAPTER 4: Going Against the Grain: Effects of Feeding Grain-Containing versus Pulse-Based Diets on Cardiac Function, Taurine Levels and Digestibility in Domestic Dogs

4.1 Chapter 4 Preface

The following chapter is the second of two studies that were conducted on research Beagles in the Weber Lab. This study used the same research dogs that were previously used in study one. This chapter consists of a long-term feeding study (28 days) that further examined the impacts of feeding pulse-based, grain-free diets in dogs. Pulse-based diets, from the previous 7-day study, containing the highest and lowest amounts of amylose were selected and slightly modified for the following study. The rice diet from the previous 7-day study and a commercial based study were also used as controls. This study has a specific focus on how diets impact cardiovascular health, fasting plasma levels of taurine, and digestibility after 28 days of feeding each diet.

This chapter has been submitted for publication in the journal *PLOS ONE*. The following authors associated with this publication were: C. Quilliam: formulated and created test diets, designed and performed experiments, and analyzed data, and co-wrote the paper. L. Reis: performed canine troponin I assays, assisted in performing experiments, review & editing. Y. Ren: extruded diets, starch analyses, amylose data curation, and review & editing. Y. Ai: Conceptualization, funding acquisition, investigation, project administration, resources, and review & editing. L. Weber: Supervised animal research, experimental design, funding acquisition, investigation, resources, project administration, review & editing, and co-wrote the paper.

4.2 Abstract

In July 2018, the US Food and Drug Administration reported a link between canine dilated cardiomyopathy and grain-free diets, but evidence to support a causative link remains weak. We hypothesized dogs fed pulse-based, grain-free diets for 28 days will show decreased apparent total tract digestibility of macronutrients, increased fecal bile acid excretion, and decreased fasted plasma levels of cystine, cysteine, methionine, and taurine, causing sub-clinical cardiac or blood changes indicative of early DCM. Three diets were formulated using white rice flour (grain), whole lentil, or Amigold wrinkled pea (grain-free) and compared to a commercial, grain-based diet. After 28 days of feeding each diet, the high amylose wrinkled pea diet (Amigold) increased left ventricular size and volume along with increased plasma proBNP, albeit in a sub-clinical manner that was reversible within the next 28-day feeding period. Apparent total tract digestibility of macronutrients and sulfur-containing amino acids, excluding taurine, also decreased with pulse-based compared to grain-based diets, due to higher levels of HMWDF. Fasted plasma taurine levels were unchanged; however, fasted plasma methionine was deficient for all diets. Overall, DCM-like changes with the wrinkled pea diet, but not the lentil diet, show that not all grain-free diets are the same. This complicates the original claim made by the FDA regarding grain-free diets.

4.3 Introduction

Grain-free diets in the pet food industry are diets that do not use traditional grains such as wheat, corn, and rice flours. Instead, these diets use pulses such as peas, chickpeas, and lentils as their carbohydrate source (Prantil et al., 2018). Pulses are feed ingredients that have been used in many dog foods over the past two decades (Mansilla et al., 2019). These pulse-based, grain-free diets are often viewed as premium diets (Pirsich et al., 2017) and currently comprise over 40% of formulated dry dog food diets in the United States (USA) (Plantz, 2017). Pulses have been commonly incorporated into human and pet diets as healthy ingredients as they contain high levels of protein, fiber, vitamins, and minerals (Singh, 2017). Pulse crops are also slowly digested due to their relatively high levels of dietary fiber and amylose, which is ideal for low glycemic responses and increased satiety in dogs (Mudryj et al., 2014; Ren et al., 2021).

Though pulses are high in plant proteins, they lack taurine, which is only obtained through animal protein sources. Recently, there has been a growing concern that DCM has a dietary cause in dogs, mainly attributed to insufficient dietary taurine (Backus et al., 2006; Kaplan et al., 2018). These factors combined to form the unproven hypothesis that pulse-based/grain-free diets are linked to DCM due to insufficient taurine in these diets. Additionally, pulses contain a limited amount of the amino acids cysteine and methionine (Singh, 2017). Cysteine and methionine are both used to synthesize taurine via the transsulfuration pathway in the dog. Thus, the limited amount of these amino acids in pulse-based diets can further contribute to the taurine deficiency which in turn may link to DCM in dogs.

In July of 2018, the US Food and Drug Administration (FDA) reported that there was a link between canine DCM and grain-free diets containing peas, lentils, other legumes, and potatoes, as the main carbohydrate source (U.S. FDA, 2019). Following this initial report, there was a decrease in sales of grain-free dog food and reduced use of pulses in pet food (Phillips-Donaldson, 2019). Confusion regarding the safety of feeding grain-free diets to dogs among veterinarians and pet owners was further exacerbated in the Fall of 2020 when the FDA released another report regarding grain-free diets. This report acknowledged that dietary causes of DCM in dogs are more complex than originally proposed and likely multi-factorial, not due to just a single ingredient such as pulses (U.S. FDA, 2020). Overall, specific connections between grain-free diets and DCM in dogs has yet to be established and more research is required (U.S. FDA, 2020).

DCM in dogs is a myocardial disorder that is accompanied by left ventricular systolic dysfunction (ejection fraction < 40% accompanied by increased end diastolic and systolic volumes), reduced left ventricular wall thickness, and ventricular chamber dilation (Cunningham & Pierce, 2018). The diagnosis of DCM in dogs has been associated with low whole blood levels of taurine (Mansilla et al., 2019). Taurine holds high importance for myocardial function but also plays a role in the conjugation of bile acids, such as taurocholic acid (Sanderson, 2006). Taurine depletion has been associated with increases in dietary fiber (Mansilla et al., 2019; Sanderson et al., 2001), but is also associated with low dietary protein through enterohepatic losses (Sanderson, 2006). In dogs, DCM associated with low blood taurine levels has been reported to be reversible with taurine supplementation, especially when DCM remains subclinical (Mansilla et al., 2019). During sub-clinical stages of DCM, dogs remain asymptomatic, but, begin having echocardiographic changes demonstrating structural irregularities such as increased ventricular chamber volume and size during diastole (Cunningham & Pierce, 2018). Elevated levels of the blood markers NT-ProBNP and cardiac troponin I are additional screening measures that can be performed to detect sub-clinical DCM as they increase with cardiac changes associated with

DCM (Cunningham & Pierce, 2018; Oyama et al., 2007). Larger breeds such as Dobermans and Boxers are commonly diagnosed with DCM and are predisposed to this condition (Mansilla et al., 2019). Despite this, there has been an increased observance of both large and medium breeds experiencing DCM (Mansilla et al., 2019).

The following study aims to further evaluate the safety of grain-free diets in dogs with a specific focus on apparent total tract digestibility of macronutrients, bile acid excretion, plasma amino acid levels (cystine, cysteine, methionine, and taurine), blood markers of overall health, and cardiac function. Specifically, this study investigated whether dietary fiber or amylose content of grain-free diets were potential links to DCM. This research was performed as a further investigation to a study previously conducted in our lab looking at the effects of feeding pulse-based, grain-free diets for 7 days (Quilliam et al., 2021). From this 7-day study that examined 6 diets, the pulse diets with the highest and lowest fiber/amylose content (Amigold wrinkled pea and lentil, respectively) were compared to a rice-based diet, all formulated at 15% enzymatic starch. For this study, it was hypothesized that in dogs, feeding pulse-based, grain-free diets for a longer period of 28 days will cause a decrease in apparent total tract digestibility of macronutrients, an increase in the excretion of fecal bile acids, and a decrease in fasted plasma levels of cystine, cysteine, methionine, and taurine, resulting in sub-clinical cardiac and blood changes consistent with early signs of DCM.

4.4 Materials and Methods

All procedures and handling of the dogs were conducted following protocols approved by the University of Saskatchewan's Animal Research Ethics Board according to guidelines that were established by the Canadian Council on Animal Care (Animal Utilization Protocol #20190055).

4.4.1 Animals

Adult Beagle dogs (n=8; 4 spayed females, 4 neutered males, 9.4 ± 0.44 kg 3-5 years old) were obtained from certified scientific breeders (Marshal Bioresources, North Rose, NY, USA, and King Fisher International, Stouffville, ON, Canada). The research Beagles were housed at the Animal Care Unit (ACU) in the Western College of Veterinary Medicine at the University of Saskatchewan (Saskatoon, SK, Canada). The Beagles were group-housed during the day in a large enclosure, allowing for daily socialization, but were individually kenneled during feedings and overnight. The dogs were walked and socialized on a daily basis. The Beagles were also provided regular health examinations, deworming, and routine vaccinations from certified veterinarians to ensure optimal health.

4.4.2 Diets

Diets formulated for this study included one control (rice; a grain-containing diet) and two pulse-based diets (red lentil (CDC Maxim) and wrinkled pea (Amigold variety); both grain-free diets). All diets for this study were formulated to contain 15% enzymatic starch (total enzymatic starch determined with an R-BIOPHARM Enzymatic BioAnalysis) using flours that were locally obtained (flour proximate analyses shown in Supplemental Table S1). A non-digestible Celite marker was also incorporated into all diets at 1% to determine the apparent total tract digestibility of each diet. This marker was measured as acid-insoluble ash in the proximate analyses of both diet and feces (Sales & Janssens, 2003). Research diets were formulated using the software Creative Concept 5 (Creative Formulation Concepts, Pierz, MN, USA). All diets were formulated to meet the nutritional requirements for canine adult maintenance (See Supplemental Table S4 for diet formulations) based on recommendations made by AAFCO. However, some diets following extrusion failed to meet these recommendations regarding methionine and cysteine+methionine. Feed ingredients for all diets were from both local and commercial sources as required and extruded into a dry kibble format using a laboratory-scale, co-rotating, twin-screw extruder (TwinLab-F 20/40, C. W. Brabender Instruments Inc., South Hackensack, NJ, USA) at the University of Saskatchewan (Food Science Laboratory, Saskatoon, SK, Canada). Extrusion conditions for all diets remained constant and are described in the Supplemental Table S3. After extrusion, diets were air dried for 48 hours and then vacuum coated with oil and chicken fat at the Canadian Feed Research Centre (North Battleford, SK, Canada). Once all diets were complete individual samples were sent to multiple analytical laboratories for analyses. Proximate and amino acid analyses (Central Testing Laboratory Ltd., Winnipeg, MB, Canada) were performed according to the AOAC standards (AOAC, 2019). Dry matter of all samples was determined by oven-drying the sample, while crude protein was determined using the Kjeldahl method. In addition, non-fiber carbohydrate and fat were determined through acid-hydrolysis solvent extraction. All diet samples were also subjected to fiber analyses to determine the soluble, insoluble, and total high molecular weight dietary fiber (HMWDF) content (Eurofins, Toronto, ON, Canada) according to the AOAC 991.43 methods (AOAC, 2019).

4.4.3 Feeding of Diets

Research Beagles were fed twice daily, weighed weekly, and assessed for body condition using a 9-point scale (LaFlamme, 1997). During the pre-trial phase, the dogs were fed a standard commercialized, grain-containing diet (See Table S5 for diet details), which was used to establish baseline values before starting the research study. During this period each dog was fed individual feed portions that were calculated to maintain ideal body weight and body condition (score of 4-5 on a 9-point scale). Once the feeding trial started, isocaloric measurements of each test diet were calculated and measured for each dog, as determined in the pre-trial phase, and used throughout the entire feeding trial without any further adjustment of portion size. All diet portions were eaten fully by each dog.

The dogs were fed each test diet for 28 days continuously. Diet types were blinded to the researchers and all dogs were fed the same diet within the same time periods. The rice diet was fed twice in two separate 28-day feeding periods. Thus, the rice diet was used as a control as well as a washout between feeding the lentil and wrinkled pea (Amigold) diets to ensure that results were reliable and repeatable. Results from the two rice feeding periods did not statistically differ from each other, thus the values from the two feeding periods were averaged and the mean value used in all graphs and statistical analyses. The feeding order that was performed was commercial-rice–lentil–rice –wrinkled pea (Amigold). This was conducted to ensure none of the dogs were fed the pulse-based diets twice in a row, ensuring more reliable results following each pulse-based diet feeding period. Similarly, to the pre-trial phase, all dogs were fed individually measured portions of test diet twice daily, weighed weekly, and assessed weekly for body condition using a 9-point scale.

4.4.4 Digestibility Testing

Apparent total tract digestibility was determined using feces that were collected on days 27 and 28 of each feeding period. Total fecal output was not studied in this experiment. Once fecal samples were collected, they were placed in a -20˚C until they were dried at 65˚C for 72 hours. Once all samples were dried, they were sent to an analytical lab to assess nutrient excretion (Central Testing Laboratory Ltd., Winnipeg, MB, Canada). Apparent total tract digestibility was calculated using the formula:

Apparent Digestibility (%) = {1-((% Nutrient in Feces)/(% Nutrient in Diet)) x ((% Indicator in Diet)/(% Indicator in Feces))}×100

In addition, total bile acid analyses were conducted on the feces using a commercial kit as per manufacturer instruction (Total Bile Acid Assay Kit, Cell Biolabs Inc. San Diego, CA, USA). This assay kit used a colorimetric enzyme driven reaction while bile acids are incubated with the presence of 3-alpha hydroxysteroid dehydrogenase and thio-NADH.

4.4.5 Whole Blood and Plasma Analyses

Leading into day 28 of feeding each test diet, dogs were fasted overnight and 8.0 mL of whole blood was collected the following morning from the jugular vein. 3.0 mL of fasted whole blood was collected and sent to an external laboratory for the performance of a complete blood count (CBC) and Chemistry Panel (Prairie Diagnostic Ser-vices, Saskatoon, SK, Canada), while 5.0 mL of whole blood was collected into an EDTA tube and centrifuged at 2200 RPM to collect plasma. Once plasma was obtained from fasted dogs, it was stored at -80˚C until the end of the research project. Once all plasma samples were collected for all diets, they were sent to a contract analytical laboratory to assay for cysteine, cystine, methionine, and taurine (The Metabolomics Innovation Centre, Edmonton, AB, Canada). Quantitation of these amino acids was determined using UPLC-MRM MS methodology. Plasma collected from fasted dogs was also used to determine canine NT-ProBNP and canine cardiac troponin I (hs-cTnI, High Sensitivity Cardiac Troponin I) using commercially available Elisa kits as per instructed (Nordic Biosite, Täby, Sweden).

4.4.6 Cardiac Assessment

Prior to performing this experiment, researchers were instructed in echocardiography techniques by an experienced echocardiographer and logged a minimum of 200 hours of practice. Prior to experiments, repeated measurements were taken of the same dogs to ensure technique was robust enough to produce repeatable cardiac results. On day 28 of feeding each test diet, a cardiac assessment was performed on each dog. Each dog was conscious and not sedated during all cardiac procedures. The cardiac assessment was performed and analyzed by one researcher. To begin the cardiac assessment, multiple blood pressure measurements were taken using a high-definition canine/feline oscillometer (VET HDO High Definition Oscillometer, Babenhausen, Germany). An average of three readings per dog were taken and used to determine the systolic and diastolic pressures. After blood pressure measurements were taken, dogs were shaved creating windows for echocardiography and flow-mediated dilation. Echocardiography and flow-mediated dilation was performed using a Sonosite Edge II ultrasound (Fujifilm Sonosite Canada, Markham, ON, Canada). A P10x transducer (8-4 Hz) was used for echocardiography views, while an L38xi (10-5 Hz) transducer was used for vascular imaging. All values from the echocardiography were normalized to the body weight of each dog according to methodology found within literature and previously performed by our lab (Reis et al., 2021; Visser et al., 2019; Cornell et al., 2004). During the echocardiography, multiple views were performed such as a 2/4 chamber apical view, a 2-chamber view, and a short axis view according to protocols validated and performed by our lab among others (Reis et al., 2021; Otto et al., 2019; Adolphe et al., 2015; Adolphe et al., 2014; Al-Dissi & Weber, 2011; Thomas et al., 1993). Echocardiography was performed to assess the dogs cardiac function using multiple endpoints. Ejection fraction (EF) measures the amount of blood that is pumped by the left ventricle and should remain above 40% in healthy animals (Cunningham & Pierce,2018). Cardiac output (CO), is the volume of blood that the heart pumps per minute and in healthy dogs should range from 118-194 mL/min/Kg (O'Rourke & Bishop, 1971). Stroke volume (SV) is the amount of blood the left ventricle pumps during systolic contraction and ranges from 1.8-2.1 mL/Kg (O'Rourke & Bishop, 1971). Heart rate (HR) measures the heart beats per minute and in conscious healthy dogs generally ranges from 60-120 BPM. Left ventricular end systolic volume (LVESV) and left ventricular end diastolic volume (LVEDV) are studied to determine if there is dilation or volume overload of the left ventricle (Cunningham & Pierce, 2018). Left ventricular internal diameter systolic (LVIDs) and left ventricular internal diameter diastolic (LVIDd) are key indications to determine chamber dilation of the left ventricle (Cunningham & Pierce, 2018). Finally, the E:A ratio is an indication of left ventricular diastolic function. This ratio is representative of the passive ventricular filling during early-diastole to the active ventricular filling that occurs during end-diastole. Lowered E:A ratios are an indication of ventricular stiffness and diastolic dysfunction (Bonagura & Fuentes, 2015).

Vascular health assessment was performed using the brachial artery of the dog forelimb and the ability to dilate in response to a shear stress from a sudden cuff release is indicative of healthy endothelium in arteries. The diameter of the brachial artery was captured during baseline and a blood pressure cuff was then placed on the dog forelimb, distal to the brachial artery. The blood pressure cuff was then inflated for a minute above normal systolic blood pressure (220 mmHg). After a minute the cuff was deflated and another image of the brachial artery was captured at the time of peak dilation (30 s post cuff release) (Adolphe et al., 2012; Raitakari & Celermajer, 2000). Captured images obtained pre and post cuff inflation were then analyzed using the software Adobe Premiere Elements 2.0 (Adobe Systems Incorporated, San Jose, CA, USA) and Image-Pro 10 (Media Cybernetics Incorporated, Rockville, MD, USA). Flow-mediated dilation was then calculated using the following equation:

Flow Mediated Dilation (%) = 100% x [(maximum diameter post-cuff re-lease)-(baseline diameter)]/(baseline diameter)

4.4.7 Data Handling and Statistics

All data were tested for normality and outliers using the Kolomogorov-Smirnov test, Q-Q plots, and box plots. All data from both rice feeding periods were subjected to paired t-tests to determine if they were statistically different ($p < 0.05$). It was determined that the data was not significantly different ($p > 0.05$) between the two rice feeding periods and all rice data for each dog was averaged and used for further statistical analyses. Depending on the normality of the data either a repeated measures, one-way ANOVA, or a repeated measures one-way ANOVA on ranked data was then performed followed by post-hoc Tukey's tests where significance was achieved. Differences were considered to be statistically significant at $p \leq 0.05$. Principal components analysis was also performed on the four dietary treatment groups initially with all 78 variables where measurements were made in all diets. Factors were reduced to two components and variables studied were reduced to 14 variables which explained 89% of the variance. Analyses were performed using SigmaPlot 12.0 and Systat 12.0 (Systat Software Inc. San Jose, CA, USA).

4.5 Results

4.5.1 Proximate, Fiber and Amylose Analyses of Diets

All 3 formulated diets and the commercial diet contained similar levels of crude protein (% dry matter) which ranged from 30.92% - 33.47% (Table 4.1). This surpassed the minimum requirement set by AAFCO of 18% dietary protein for adult dogs (AAFCO, 2013). Slight decreases in crude protein in the grain-free diets (lentil and wrinkled pea) compared to the rice diet are likely due to higher levels of pulse protein being incorporated, leading to a lower need for animal protein to achieve the desired protein content (Supplemental Table S4). Among the formulated test diets (rice, lentil, wrinkled pea) crude fiber varied from 7.39% - 7.47% (Table 4.1), while the commercial diet contained 0.86% crude fiber (Table 4.1). The formulated research diets were seen to have higher levels of crude fiber due to the addition of powdered cellulose to produce balanced diets. Cellulose was included in diets to maintain 15% enzymatic starch across all test diets (Supplemental Table S4). Crude fat of all diets ranged from 15.12% - 20.26% (Table 4.1) and was unrelated to the carbohydrate ingredient used. Non-fiber carbohydrate of all diets ranged from 26.80% - 45.05% (Table 4.1). The rice diet had the lowest level of non-fiber carbohydrate. This again was likely due to less rice flour being used compared to the grain-free diets flour source and the addition of the powdered cellulose in the diet (Supplemental Table S4). Similarly, the total digestible nutrients of the rice diet were the lowest compared to all diets at 74.30% (Table 4.1), likely for reasons mentioned previously. When comparing the total digestible nutrients of the grain-free test diets (lentil and wrinkled pea) to the commercial grain-containing diet, however, there appears to be slightly less total digestible nutrient in the grain-free diets (lentil and wrinkled pea) than the commercial grain-containing diet (Table 4.1). This is likely due to the amylose content and fiber fractions observed within the grain-free diets (Table 4.1).

Soluble high molecular weight dietary fiber (HMWDF) across diets ranged from 0.50% - 0.80% (Table 4.1). Insoluble HMWDF varied from 5.00% - 16.50% (Table 4.1) and was higher in both grain-free diets (lentil and wrinkled pea). Total HMWDF ranged from 5.50% - 17.20% (Table 4.1) and was again greater in both the lentil and wrinkled pea (Amigold) diets. All formulated diets contained approximately 15% enzymatic starch, while the commercial diet had 24.53% enzymatic starch. Amylose content (% dry matter) ranged from 4.57% - 12.81% (Table 4.1) and was highest in the grain-free wrinkled pea (Amigold) diet.

Table 4.1: Proximate analyses, fiber and amylose content of the commercial grain-containing diet and different formulated test diets after extrusion and fat coating. Diets ordered from left to right in order of increasing total HMWDF fiber content.

	Commercial Diet	Rice Diet	Lentil Diet	Wrinkled Pea Diet (Amigold)
DM	93.65	90.89	90.63	87.76
Crude Protein [a]	30.92	33.47	31.81	30.96
Crude Fiber [b]	0.86	7.47	7.39	7.40
Fat [c]	15.12	20.26	18.49	18.12
Ash [d]	7.11	0.96	7.51	7.28
Non-Fiber Carbohydrate [e]	45.05	26.80	33.89	35.36
Total Digestible Nutrients [f]	83.09	74.30	76.19	76.44
Metabolizable Energy [g]	4234.68	3801.34	3928.36	3930.04
Soluble HMWDF [h]	0.50	0.80	0.60	0.70
Insoluble HMWDF [h]	5.00	14.80	16.10	16.50
Total HMWDF [h]	5.50	15.60	16.70	17.20
Enzymatic Starch [i]	24.53	15.75	15.28	15.92
Amylose content [j]	8.09	4.57	6.68	12.81

DM = dry matter; All % values are relative to dry matter except excluding Metabolizable Energy (kcal/kg).

[a] Determined by Central Testing Laboratory Ltd. (Winnipeg, MB, Canada), following AOAC Method 990.03(M)

[b] Determined by Central Testing Laboratory Ltd. (Winnipeg, MB, Canada), following Crude Fiber Method by Ankom Technology (2017), based on AOCS Ba 6a-05.

[c] Determined by Central Testing Laboratory Ltd. (Winnipeg, MB, Canada), following AOCS Method AM 5-04.

[d] Determined by Central Testing Laboratory Ltd. (Winnipeg, MB, Canada), following AOAC Method 942.05.

[e] Determined using the equation for Non-Fiber Carbohydrates (NFC, Dry Matter): NFC (%) = 100-[(%Dry Matter/100)+%Protein+%Fat+%Ash+%Crude Fiber]

[f] Determined by Central Testing Laboratory Ltd. (Winnipeg, MB, Canada) using the equation for Total Digestible Nutrients (TDN, Dry Matter): TDN (%) = 100-[(%Dry matter/100)+%Ash+%Crude Fiber]-8

[g] Determined by Central Testing Laboratory Ltd. (Winnipeg, MB, Canada) using the ME equation for swine: (kcal/kg) = 4151-(122*Ash)+(23*Crude Protein)+(38*Fat)-(64*Crude fiber)*(1.003-(0.0021* Crude Protein)) by Central Testing Laboratory Ltd. (Winnipeg, MB, Canada)

[h] Determined by Eurofins (Toronto, ON, Canada) using AOAC 991.43 dietary fibre method.

[i] Determined by Central Testing Laboratory Ltd. (Winnipeg, MB, Canada), R-Biopharm Test kit (UV Method)

[j] Determined using an iodine colorimetric method of Chrastil (1987).

HMWDF = high molecular weight dietary fiber.

4.5.2 Amino Acid Content of Diets

All test diets were formulated to meet or exceed the AAFCO requirements of 0.33% methionine and 0.65% cystine+methionine (AAFCO, 2013). However, analysis of the final extruded and fat-coated product showed some diets failed to meet this recommendation and were slightly below what was required. The wrinkled pea (Amigold) diet had slightly lowered methionine levels at 0.32%, while both the lentil and wrinkled pea (Amigold) diets had lowered cystine+methionine levels of 0.56% and 0.58% respectively (Table 4.2). Dietary methionine was highest in the rice diet at 0.59% on a dry matter basis, with all pulse-based, grain-free diets containing lower but adequate methionine levels (Table 4.2). Taurine levels varied from 0.08% - 0.10%, with no major differences being observed between grain-containing and grain-free diets as shown in Table 4.2.

Table 4.2: Content of cystine, methionine, cystine+methionine and taurine in the 3 different test diets formulated at 15% enzymatic starch with variable amounts of HMWDF, fed for 28 days. Diets ordered from left to right in order of increasing total HMWDF fiber content.

	Commercial Diet	Rice Diet	Lentil Diet	Wrinkled Pea Diet (Amigold)
Cystine [a]	0.32	0.23	0.20	0.26
Methionine [a]	0.43	0.59	0.36	0.32
Cystine+Methionine [a]	0.75	0.82	0.56	0.58
Taurine [a]	0.08	0.10	0.08	0.09

All values are expressed as % dry matter unless stated otherwise.
[a] Determined by Central Testing (Winnipeg, MB, Canada) using UPLC and ninhydrin detection.

4.5.3 Apparent Total Tract Digestibility

Apparent total tract digestibility of crude protein varied across the diets (ANOVA, $p = 0.008$). Crude protein digestibility varied from 77.96% - 86.17% being highest in the rice diet and lowest in the grain-free diets (Table 4.3). Fat digestibility ranged from 94.29% - 98.43% (ANOVA, $p = 0.018$) with fat digestibility being lowest in the wrinkled pea (Amigold) diet as seen in Table 4.3. Digestibility of starch varied across the diets (ANOVA, $p < 0.001$), with the wrinkled pea (Amigold) diet having the lowest starch digestibility of 92.73% (Table 4.3). Non-fiber carbohydrate digestibility differed across the diets (ANOVA, $p = 0.018$) ranging from 54.62% - 66.76%, and was lowest in the lentil diet as shown in Table 4.3. The total digestible nutrients varied from 73.85% - 82.40% (Table 4.3), being lowest in the lentil diet and highest in the rice diet (ANOVA, $p < 0.001$).

Digestibility of cystine decreased across diets (ANOVA, $p = 0.003$) ranging from 69.09% - 81.83% with the lentil diet having the lowest level of digestibility (Table 4.3). Both the lentil and the wrinkled pea (Amigold) diets were seen to have lowered methionine digestibility when compared to the rice diet (ANOVA, $p < 0.001$), shown in Table 4.3. Similarly, both grain-free diets also had lowered cystine+methionine digestibility when compared to the rice diet (ANOVA, $p < 0.001$), shown in Table 4.3. No significant differences were observed in the apparent total tract digestibility of taurine between grain-containing and grain-free diets after 28 days of feeding.

Table 4.3: Apparent total tract digestibility analyses of different test diets formulated at 15% enzymatic starch with variable amounts of HMWDF, fed for 28 days. Diets ordered from left to right in order of increasing total HMWDF fiber content.

	Rice Diet	Lentil Diet	Wrinkled Pea Diet (Amigold)	p-Value
Crude Protein **	86.17 ± 0.45^{a}	77.96 ± 0.62^{b}	79.39 ± 2.36^{b}	0.008
Fat **	98.43 ± 0.41^{a}	$96.52 \pm 0.87^{a,b}$	94.29 ± 2.21^{b}	0.018
Starch *	99.26 ± 0.48^{a}	99.74 ± 0.19^{a}	92.73 ± 1.57^{b}	<0.001
Non-Fiber Carbohydrate *	$62.20 \pm 1.79^{a,b}$	54.62 ± 1.59^{b}	66.76 ± 3.56^{a}	0.018
Total Digestible Nutrients **	82.40 ± 0.81^{a}	73.85 ± 0.76^{b}	$78.62 \pm 2.12^{a,b}$	<0.001
Cystine *	81.83 ± 0.88^{a}	69.09 ± 2.73^{b}	78.69 ± 2.39^{a}	0.003
Methionine *	91.70 ± 0.65^{a}	82.71 ± 1.55^{b}	79.60 ± 1.99^{b}	<0.001
Cystine+Methionine *	88.93 ± 0.70^{a}	77.85 ± 1.65^{b}	79.19 ± 2.10^{b}	<0.001
Taurine **	100.00 ± 0.0	99.38 ± 0.62	98.88 ± 0.74	0.79

All values are expressed as % of apparent total tract digestibility.
Data is shown as Mean ± SEM; n = 8 dogs. Different letters indicate significant differences among diets using Tukey's post-hoc analysis ($p < 0.05$) after significant p-values reported in one-way repeated measures ANOVA* and Friedman's one-way ANOVA on ranked data**.
Protein, fat, non-fiber carbohydrate, and total digestible nutrient determined by Central Testing (Winnipeg, MB Canada) as described in Table 4.1. Diet and fecal amino acids and starch were determined by Central Testing (Winnipeg, MB, Canada) using UPLC with ninhydrin detection and enzymatically using UV detection, respectively.
Commercial diet was not included for TTAD due to not having a digestible marker in it.

4.5.4 Bile Acid Analysis

Fecal bile acid content significantly differed among the different diets, with the commercial grain-containing diet having the highest excretion and the wrinkled pea (Amigold) diet having the lowest (Figure 4.1).

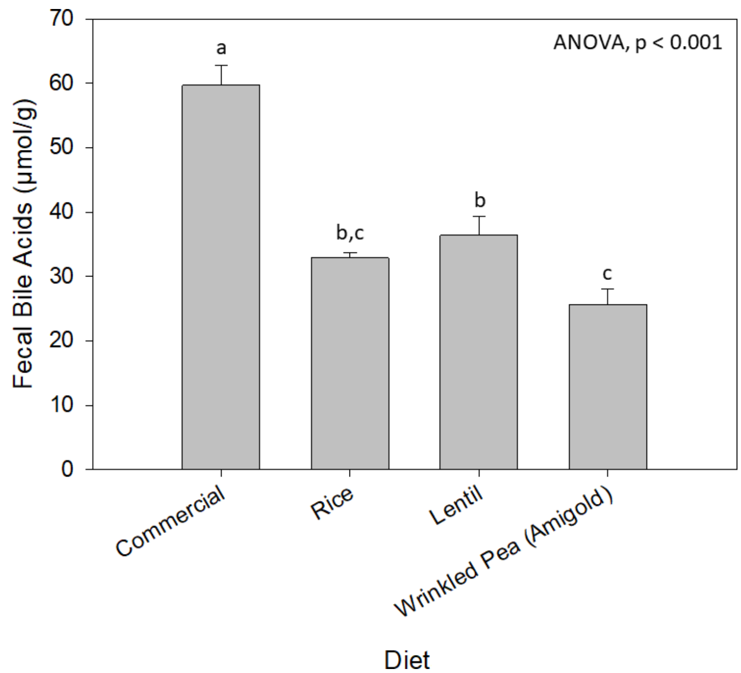

Figure 4.1: Fecal bile acid content (µmol/g) from dogs after 28 days of feeding each test diet. Diets ordered from left to right in order of increasing total HMWDF fiber content. Data is shown as Mean ± SEM; n = 8 dogs. Different letters indicate significant differences using Tukey's post-hoc analysis ($p < 0.05$) after one-way repeated measures ANOVA.

4.5.5 Complete Blood Count and Blood Chemistry

The CBC in dogs had no significant differences among diets for both white blood cells (WBC) and platelets (Table 4.4). However, red blood cell (RBC) values varied across the diets from 6.49 x1012/L – 6.97 x1012/L, with dogs consuming the rice diet having the lowest RBC count and dogs on the commercial diet having the highest RBC count (ANOVA, $p = 0.022$), shown in Table 4.4. Despite the differences, all values for the dogs on all diets in the CBC fall within normal limits as seen in Table 4.4.

Blood parameters of hepatic function from the blood chemistry performed after feeding each diet for 28 days are shown in Table 4.5. Slight elevations in serum cholesterol were observed in dogs on the commercial diet, when compared to the other diets (ANOVA, $p < 0.001$), but still fell within normal limits for a healthy adult dog (Table 4.5). Total bilirubin for dogs on the lentil diet fell below the reference range for adult dogs at 0.96 μmol/L but was not significantly different from when dogs consumed the other diets (Table 4.5). Direct bilirubin had significant differences in dogs among the diets, with the dogs consuming the commercial diet having the lowest direct bilirubin of 0.44 μmol/L, but still fell within normal limits (Table 4.5). Indirect bilirubin levels in dogs after eating all diets fell within normal limits, and ranged from 0.39 – 0.90 μmol/L, with consumption of the wrinkled pea (Amigold) diet having the lowest value and the commercial diet having the highest (ANOVA, $p = 0.001$) as shown in Table 4.5. Similarly, alkaline phosphate (ALP) in the dogs ranged from 32.38 – 49.25 U/L and was highest when the commercial diet was eaten and lowest when the wrinkled pea (Amigold) diet was consumed (ANOVA, $p < 0.001$). Creatine kinase (CK) levels fell within normal limits for the dogs on all diets but the lentil diet was significantly different from all the other diets at 227.75 U/L (ANOVA, $p < 0.001$). Total protein for all dogs on all diets also fell slightly below normal limits and was lowest on the wrinkled pea (Amigold) diet at 51.38 g/L (ANOVA, $p = 0.011$), shown in Table 4.5. Globulin levels for dogs on all diets also fell below normal limits and was lowest when the rice diet was consumed (ANOVA, $p < 0.001$).

All blood electrolyte values for the dogs fell within normal limits, except for their calcium levels when they consumed the commercial diet (Table 4.6), which was above normal limits at 3.39 mmol/L (ANOVA, $p = 0.002$). Chloride values were also seen to be higher in dogs after the commercial diet feeding period (ANOVA, $p = 0.012$). As shown in Table 4.6, bicarbonate, phosphorus, and magnesium levels in dogs all significantly differed among diets, being highest after consuming the lentil diet for 28 days and lowest after consuming the rice diet. NA:K values ranged from 31.75 – 34.31 and was highest in dogs after consuming the rice diet (ANOVA, $p = 0.006$, Table 4.6). Potassium was highest in dogs following the wrinkled pea (Amigold) feeding period and was lowest in dogs after eating the rice diet (ANOVA, $p = 0.024$), shown in Table 4.6.

Blood parameters of kidney function, digestive enzymes and fasting blood glucose are shown in Table 4.7. All values fell within normal limits. Urea ranged from 5.48 – 7.59 mmol/L in dogs and was highest after consuming the rice diet and lowest after eating the wrinkled pea (Amigold) diet (ANOVA, $p <0.001$). Amylase levels in dogs also varied significantly and was seen to be the highest after consuming the commercial diet for 28 days, as shown in Table 4.7. Fasting blood glucose levels of the dogs also significantly differed and was lowest after dogs were fed the lentil diet for 28 days at 3.69 mmol/L.

Table 4.4: Complete blood count in dogs fed different grain-containing and grain-free diets for 28 days. Diets ordered from left to right in order of increasing total HMWDF fiber content.

	Commercial Diet	Rice	Lentil	Wrinkled Pea (Amigold)	p-Value	Reference Range
WBC (Ref. Int. x 10^9/L)*	6.44 ± 0.45	5.67 ± 0.37	6.20 ± 0.39	5.89 ± 0.38	0.064	4.9 – 15.4
Platelets (Ref. Int. x 10^9/L)**	267.00 ± 33.04	257.69 ± 30.17	263.13 ± 31.87	281.50 ± 34.04	0.27	117 – 418
RBC (x 10^{12}/L)*	6.97 ± 0.24^a	6.49 ± 0.12^b	6.64 ± 0.12^{a,b}	6.51 ± 0.12^b	0.022	5.80 – 8.50

Data is shown as Mean ± SEM; n = 8 dogs. Different letters indicate significant differences among diets using Tukey's post-hoc analysis ($p < 0.05$) after significant p-values reported in one-way repeated measures ANOVA* and Friedman's one-way ANOVA on ranked data**.

Reference range was not used in the statistics and is provided to determine if values fall above, below, or within normal limits.

White blood cells (WBC), red blood cells (RBC).

Table 4.5: Blood parameters of hepatic function in dogs fed different grain-containing and grain-free diets for 28 days. Diets ordered from left to right in order of increasing total HMWDF fiber content.

	Commercial Diet	Rice Diet	Lentil Diet	Wrinkled Pea Diet (Amigold)	p-Value	Reference Range
Cholesterol (mmol/L)**	5.37 ± 0.31[a]	4.51 ± 0.25[b]	4.15 ± 0.28[b]	4.57 ± 0.30[a,b]	<0.001	2.70 – 5.94
Total Bilirubin (μmol/L)*	1.34 ± 0.15	1.17 ± 0.090	0.96 ± 0.11	1.03 ± 0.086	0.060	1.0 – 4.0
Direct Bilirubin (μmol/L)*	0.44 ± 0.032[a]	0.65 ± 0.045[b]	0.55 ± 0.068[a,b]	0.64 ± 0.053[a,b]	0.035	0.0 – 2.0
Indirect Bilirubin (μmol/L)*	0.90 ± 0.15[b]	0.52 ± 0.059[a]	0.41 ± 0.29[a]	0.39 ± 0.085[a]	0.001	0.0 – 2.5
ALP (U/L)*	49.25 ± 7.27[a]	33.94 ± 4.47[b]	36.13 ± 5.29[b]	32.38 ± 4.90[b]	<0.001	9.0 – 90
GGT (UL)*	1.75 ± 0.41[a,b]	2.00 ± 0.25[a]	0.38 ± 0.38[b]	1.13 ± 0.48[a,b]	0.026	0.0 – 8.0
ALT (U/L)**	22.25 ± 0.96[b,c]	20.63 ± 0.87[a,b]	24.13 ± 1.03[c]	20.63 ± 0.87[a,b]	<0.001	19.0 – 59.0
GLDH (U/L)*	3.63 ± 0.18	3.63 ± 0.32	3.13 ± 0.35	3.25 ± 0.45	0.52	0.0 – 7.0
CK (U/L)*	136.80 ± 10.44[a]	126.06 ± 5.18[a]	227.75 ± 17.20[b]	119.88 ± 8.24[a]	<0.001	51.0 – 418.0
Total Protein (g/L)**	53.13 ± 1.37[a,b]	51.56 ± 1.02[a]	53.13 ± 1.19[b]	51.38 ± 1.41[a, b]	0.011	55.0 – 71.0
Albumin (g/L)**	33.38 ±1.24	33.50 ± 0.87	34.25 ± 0.10	32.88 ± 1.21	0.052	32.0 – 42.0
Globulin (g/L)*	19.75 ± 0.65[a]	18.06 ± 0.38[b]	18.88 ± 0.69[a,b]	18.50 ± 0.63[b]	<0.001	20.0 – 34.0
A:G	1.71 ± 0.089[b]	1.86 ± 0.057[a]	1.83 ± 0.089[a]	1.79 ± 0.089[a,b]	0.006	1.06 – 1.82

Data is shown as Mean ± SEM; n = 8 dogs. Different letters indicate significant differences among diets using Tukey's post-hoc analysis ($p < 0.05$) after significant p-values reported in one-way repeated measures ANOVA* and Friedman's one-way ANOVA on ranked data**.

Reference range was not used in the statistics and is provided to determine if values fall above, below, or within normal limits.

Alkaline phosphate (ALP), gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT), glutamate dehydrogenase (GLDH), creatinine kinase (CK).

Table 4.6: Blood electrolytes in dogs fed different grain-containing and grain-free diets for 28 days. Diets ordered from left to right in order of increasing total HMWDF fiber content.

	Commercial Diet	Rice	Lentil	Wrinkled Pea (Amigold)	p-Value	Reference Range
Sodium (mmol/L)*	148.0 ± 0.63	147.4 ± 0.50	147.6 ± 0.57	146.6 ± 0.75	0.27	140.0 – 153.0
Potassium (mmol/L)*	4.58 ± 0.092[a,b]	4.33 ± 0.071[b]	4.61 ± 0.081[a,b]	4.63 ± 0.080[a]	0.024	3.80 – 5.60
Na:K*	32.38 ± 0.60[a,b]	34.31 ± 0.55[a]	32.00 ± 0.54[b]	31.75 ± 0.56[b]	0.006	28.0 – 38.0
Chloride (mmol/L)*	115.13 ± 0.72[a,b]	113.50 ± 0.53[a,b]	113.13 ± 0.79[b]	112.75 ± 0.90[b]	0.012	105.0 – 120.0
Bicarbonate (mmol/L)*	20.00 ± 0.87[a]	19.81 ± 0.34[b]	22.13 ± 0.40[a]	21.13 ± 0.64[a,b]	0.026	15.0 – 25.0
Anion Gap (mmol/L)*	17.50 ± 0.42	18.50 ± 0.48	17.25 ± 0.31	17.50 ± 0.50	0.055	12.0 – 26.0
Calcium (mmol/L)**	3.39 ± 0.042[a]	2.45 ± 0.023[b]	2.47 ± 0.024[b]	2.44 ± 0.029[b]	0.002	1.91 – 3.03
Phosphorus(mmol/L)*	1.26 ± 0.041[a,b]	1.19 ± 0.043[a]	1.40 ± 0.041[b]	1.25 ± 0.053[a]	0.004	0.63 – 2.41
Magnesium (mmol/L)*	0.83 ± 0.011[b,c]	0.79 ± 0.013[a,b]	0.85 ± 0.012[c]	0.82 ± 0.016[a,b,c]	0.005	0.70 – 1.16

Data is shown as Mean ± SEM; n = 8 dogs. Different letters indicate significant differences among diets using Tukey's post-hoc analysis ($p < 0.05$) after significant p-values reported in one-way repeated measures ANOVA* and Friedman's one-way ANOVA on ranked data**.

Reference range was not used in the statistics and is provided to determine if values fall above, below, or within normal limits.

Table 4.7: Blood parameters of kidney function, digestive enzymes and fasting blood glucose in dogs fed different grain-containing and grain-free diets for 28 days. Diets ordered from left to right in order of increasing total HMWDF fiber content.

	Commercial Diet	Rice	Lentil	Wrinkled Pea (Amigold)	p-Value	Reference Range
Urea (mmol/L)**	$5.76 \pm 0.16^{b,c}$	7.59 ± 0.278^{a}	$7.03 \pm 0.45^{a,b}$	5.48 ± 0.28^{c}	<0.001	3.5 – 11.4
Creatinine (μmol/L)**	62.63 ± 3.15	70.63 ± 6.13	64.88 ± 5.94	60.75 ± 3.40	0.70	41.0 – 121.0
Amylase (U/L)*	531.38 ± 56.76^{c}	$457.81 \pm 46.88^{a,b}$	$482.13 \pm 41.11^{b,c}$	$468.75 \pm 40.26^{a,b}$	0.011	343.0 – 1375
Lipase (U/L)*	55.13 ± 8.18	47.38 ± 4.80	51.00 ± 6.93	55.13 ± 5.16	0.19	25.0 – 353.0
Fasting Glucose (mmol/L)*	4.58 ± 0.16^{a}	4.82 ± 0.13^{a}	3.69 ± 0.15^{b}	4.70 ± 0.16^{a}	<0.001	3.1- 6.3

Data is shown as Mean ± SEM; n = 8 dogs. Different letters indicate significant differences among diets using Tukey's post-hoc analysis ($p < 0.05$) after significant p-values reported in one-way repeated measures ANOVA* and Friedman's one-way ANOVA on ranked data**.

Reference range was not used in the statistics and is provided to determine if values fall above, below, or within normal limits.

4.5.6 Sulfur-Containing Amino Acids in Plasma

After 28 days of feeding each diet, fasting plasma levels of taurine observed in the dogs was not significantly different among diets (Table 4.8). Plasma levels of cysteine did vary in dogs with the highest values being reported after consuming the wrinkled pea (Amigold) diet (0.96 nmol/mL) and lowest after consuming the commercial diet (0.66 nmol/mL, Table 4.8). After consuming the lentil diet, fasting plasma levels of cystine in the dogs were at their highest (18.50 nmol/mL), while the fasting plasma levels of cystine were at their lowest following the consumption of the commercial diet (11.88 nmol/mL, Table 4.8). Plasma levels of methionine in dogs appeared to show an inverse relationship to plasma cystine. Fasting plasma methionine levels significantly differed in the dogs and was highest after consuming the commercial diet (53.90 nmol/mL) and lowest when the lentil diet was fed (45.05 nmol/mL, Table 4.8).

Table 4.8: Fasted plasma amino acid levels of taurine, cystine, cysteine, and methionine observed in dogs fed different grain-containing and grain-free diets for 28 days. Diets ordered from left to right in order of increasing total HMWDF fiber content.

	Commercial Diet	Rice Diet	Lentil Diet	Wrinkled Pea Diet (Amigold)	p-Value
Cysteine **	0.66 ± 0.029^{b}	0.91 ± 0.057^{a}	0.86 ± 0.052^{a}	0.96 ± 0.083^{a}	0.002
Cystine *	11.88 ± 1.01^{b}	17.03 ± 1.28^{a}	18.50 ± 2.07^{a}	15.11 ± 1.32^{a,b}	0.004
Methionine *	53.90 ± 2.62^{a}	47.71 ± 2.20^{b}	45.05 ± 1.53^{b}	49.12 ± 1.66^{a,b}	0.002
Taurine *	81.21 ± 9.56	87.88 ± 8.79	88.56 ± 13.23	83.62 ± 13.77	0.86

All values are expressed as nmol/mL.
Data is shown as Mean ± SEM; n = 8 dogs. Different letters indicate significant differences among diets using Tukey's post-hoc analysis (p < 0.05) after significant p-values reported in one-way repeated measures ANOVA* and Friedman's one-way ANOVA on ranked data**.

4.5.7 NT-ProBNP and Cardiac Troponin I in Plasma

Fasting plasma levels of canine NT-ProBNP were significantly different in dogs after consuming each of the different diets for 28 days. Levels were highest after dogs consumed the wrinkled pea (Amigold) diet and lowest after consuming the lentil diet (Figure 4.2). Despite this variation, reported mean NT-ProBNP values remained within normal limits. Fasting plasma levels of canine cardiac troponin I also significantly differed in the dogs after consuming each diet for 28 days. Highest levels of cardiac troponin I were in dogs after consuming the commercial diet, while the lowest levels were recorded in dogs after consuming the lentil diet (Figure 4.2). Reported mean canine cardiac troponin I values were within normal limits.

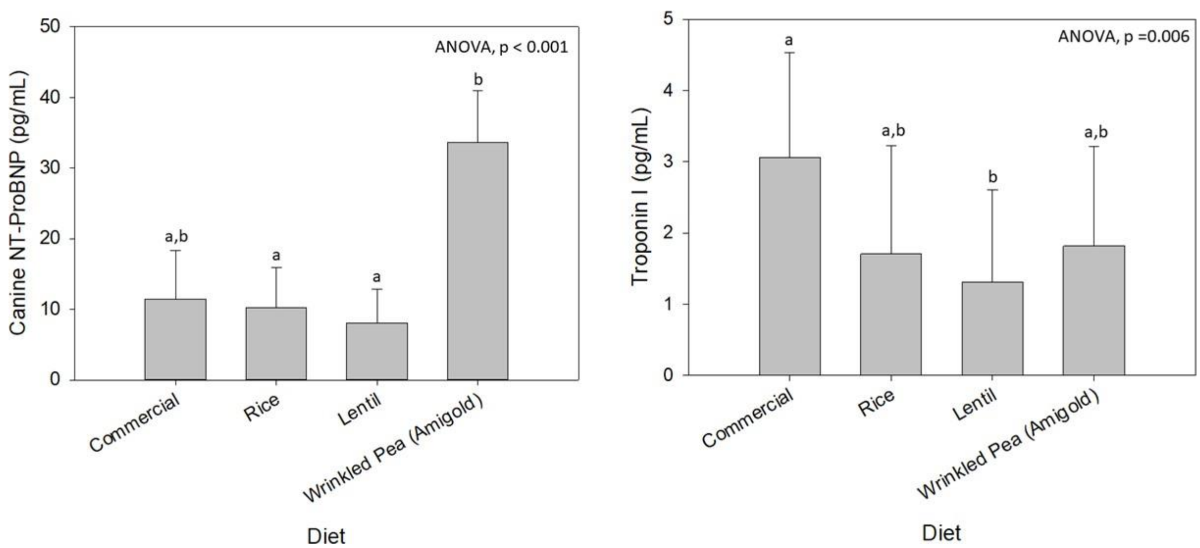

Figure 4.2: Plasma levels of canine NT-Pro BNP (left graph) and cardiac-specific Troponin I (right graph) circulating in dogs after 28 days of feeding each test diet. Diets ordered from left to right in order of increasing total HMWDF fiber content. Data is shown as Mean ± SEM; n = 8 dogs. Different letters indicate significant differences using Tukey's post-hoc analysis ($p < 0.05$) after conducting a Friedman's one-way ANOVA on ranked data

4.5.8 Blood Pressure, Echocardiography, and Flow-Mediated Dilation

As shown in Table 4.9, systolic pressure and pulse pressure in dogs were not significantly different in dogs after eating grain-containing and grain-free diets for 28 days. Significant differences were seen in diastolic pressure with the wrinkled pea (Amigold) diet having the lowest, and the lentil diet having the highest pressures (Table 4.9).

Echocardiography and flow-mediated dilation are displayed in Table 4.10. Ejection fraction and heart rate were significantly lower in dogs after the wrinkled pea (Amigold) diet feeding period (Table 4.10) but was within normal limits of a healthy adult dog. Stroke volume, left ventricular end systolic volume, left ventricular end diastolic volume, left ventricular internal diameter (LVID) at systole, and LVID diastolic measurements were also significantly increased in dogs after consuming the wrinkled pea diet for 28 days compared to the lentil diet (Table 4.10). The E:A ratio significantly differed in dogs when fed the commercial diet and wrinkled diet, being at its lowest during the wrinkled pea feeding period (Table 4.10). Maximum blood velocity (E-wave), which reflects passive ventricular filling, was highest in dogs after consuming the rice diet and lowest after consuming the commercial diet (ANOVA, $p < 0.001$, Table 4.10). No significant differences in flow-mediated dilation were observed in dogs after consuming all diets.

Table 4.9: Systolic, diastolic and pulse pressure measurements observed in dogs fed different grain-containing and grain-free diets for 28 days. Diets are ordered from left to right in order of increasing total HMWDF fiber content.

	Commercial Diet	Rice Diet	Lentil Diet	Wrinkled Pea Diet (Amigold)	p-Value
Systolic *	137 ± 6	136 ± 3	138 ± 6	137 ± 6	0.99
Diastolic **	74 ± 5^{a}	78 ± 3^{a}	86 ± 5^{a}	72 ± 6^{a}	0.047
Pulse Pressure *	63 ± 5	58 ± 3	52 ± 5	65 ± 3	0.24

All values are expressed as mmHg.
Data is shown as Mean ± SEM; n = 8 dogs. Different letters indicate significant differences among diets using Tukey's post-hoc analysis ($p < 0.05$) after significant p-values reported in one-way repeated measures ANOVA* and Friedman's one-way ANOVA on ranked data**.

Table 4.10: Echocardiography and flow-mediated dilation in dogs fed different grain-containing and grain-free diets for 28 days Diets ordered from left to right in order of increasing total HMWDF fiber content.

	Commercial Diet	Rice Diet	Lentil Diet	Wrinkled Pea Diet (Amigold)	p-Value
Ejection Fraction (%)*	68.31 ± 2.71[a]	61.69 ± 2.05[a,b]	65.44 ± 3.21[a,b]	60.13 ± 2.91[b]	0.021
Cardiac Output (mL/min/Kg)*	172.60 ± 30.24	144.28 ± 14.96	157.21 ± 17.49	113.94 ± 11.04	0.082
Stroke Volume (mL/Kg)*	1.63 ± 0.17[a]	1.04 ± 0.098[b]	1.48 ± 0.13[a]	1.65 ± 0.17[a]	< 0.001
Heart Rate (bpm)*	105 ± 12[a,b]	93 ± 5[a,b]	107 ± 8[a]	73 ± 7[b]	0.026
Left Ventricular End Systolic Volume (mL/Kg)*	0.74 ± 0.081[b]	0.97 ± 0.067[a,b]	0.77 ± 0.079[b]	1.13 ± 0.14[a]	0.002
Left Ventricular End Diastolic Volume (mL/Kg)*	2.37 ± 0.20[a,b]	2.53 ± 0.19[a,b]	2.25 ± 0.13[b]	2.79 ± 0.28[a]	0.043
LVID Systolic (cm/Kg$^{1/3}$)*	0.51 ± 0.040[b]	0.57 ± 0.037[a,b]	0.52 ± 0.038[b]	0.60 ± 0.05[a]	0.002
LVID Diastolic (cm/Kg$^{1/3}$)*	0.82 ± 0.066[a,b]	0.84 ± 0.063[a,b]	0.81 ± 0.061[b]	0.88 ± 0.071[a]	0.036
E:A**	0.17 ± 0.01[a]	0.20 ± 0.05[a,b]	0.15 ± 0.01[a,b]	0.12 ± 0.01[b]	0.031
V Max (E-Wave, cm/s/ Kg$^{1/3}$)*	20.38 ± 2.12[b]	24.85 ± 2.90[a]	24.16 ± 3.28[a]	24.00 ± 2.13[a]	<0.001
Flow Mediated Dilation (%)*	12.87 ± 2.91	18.78 ± 3.55	12.91 ± 4.46	19.08 ± 4.88	0.47

Data is shown as Mean ± SEM; n = 8 dogs. Different letters indicate significant differences among diets using Tukey's post-hoc analysis ($p < 0.05$) after significant p-values reported in one-way repeated measures ANOVA* and Friedman's one-way ANOVA on ranked data**.
Left Ventricular Internal Diameter (LVID), maximum velocity (V Max)

4.5.9 Principal Component Analysis

In order to assess the relationships among the variables tested, a Principal Components Analysis (PCA) was run initially with all 78 variables measured in all 4 test diets in the current study. Digestibility had to be excluded because this could not be determined in the commercial diet. The top 14 variables that were correlated to the first two components were retained in the final PCA analysis. These 14 variables explained 89% of variance in this final analysis (see Table 4.11). It is important to note that only variables that naturally correlated to the top two factors were kept in the final analyses. No variable was forced into the top factors. Surprisingly, dietary amylose content correlated to a separate component than soluble and insoluble HMWDF. Also noteworthy, the cardiac function and health parameters consistent with early signs of DCM (higher left ventricular end-diastolic volume and diameter as well as high plasma NT-ProBNP) were positively correlated to Component 2 and high levels of dietary amylose. In contrast, both soluble and insoluble HMWDF, as well as high plasma taurine and high passive ventricular filling velocity, were positively correlated to Component 1, while plasma methionine, plasma cardiac troponin, and dietary cysteine were negatively correlated to this first Component. A plot of weighted factor scores for each of these 14 variables for each diet resulted in a graph where the commercial diet was clearly separated from the lab-formulated diets along the first component x-axis (Figure 4.3). In contrast, there were very large 95% confidence intervals in the commercial diet that made it overlap with all lab-formulated diets along the y-axis for Component 2 and the DCM-related variables (Figure 4.3). However, the lab-formulated diets did separate along the y-axis. Specifically, the wrinkled pea (Amigold) diet showed high Component 2 scores with DCM-related variables, while the lentil diet showed the lowest tendency and the rice diet was intermediate (Figure 4.3).

Table 4.11: Principal components analysis of data from this study that explains 89% of the variability. The original set of 78 variables where values were available for all test diets (digestibility not included since no values for commercial diet available) was reduced to the top 14 variables that explained the greatest amount of variability in the first two factors in the final PCA. The strength of the relationship (r-value) for the two components, referred to as component loadings, are shown for each of these 14 variables.

	Factor 1	**Factor 2**
Total Variance Explained (%)	52.17	37.06
Variable	Component Loadings	
Plasma cardiac troponin I	-0.983	-0.006
Plasma cystine	0.975	-0.215
Cystine in diet	-0.974	0.209
Plasma methionine	-0.969	0.177
Passive ventricular filling velocity (E-wave)	0.964	0.250
Plasma taurine	0.932	-0.327
Insoluble HMWDF in diet	0.925	0.309
Soluble HMWDF in diet	0.702	0.529
Left ventricular end diastolic volume	0.047	0.999
Left ventricular end diastolic diameter	0.090	0.991
Plasma NT-ProBNP	-0.054	0.903
Flow-mediated dilation	0.385	0.861
Fasting blood glucose	-0.336	0.726
Amylose content in diet	-0.280	0.676

HMWDF = high molecular weight dietary fiber; NT-proBNP = N-terminal pro-B –type natriuretic peptide.

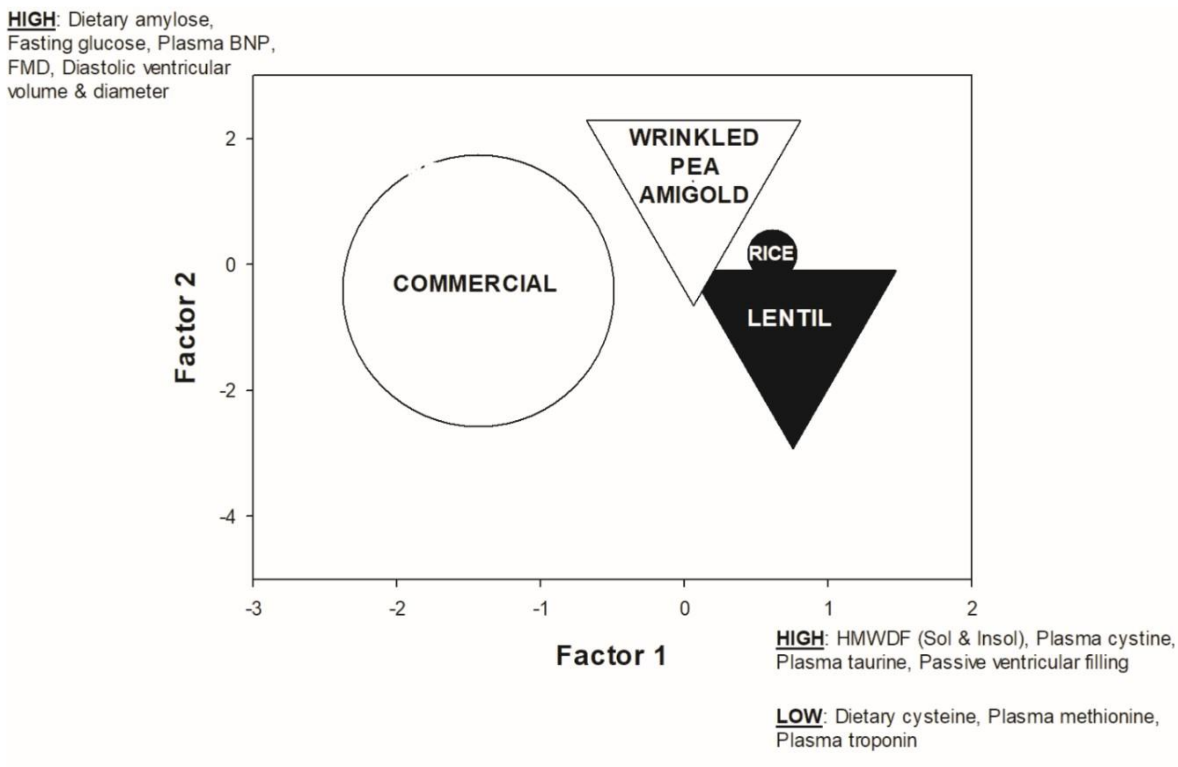

Figure 4.3: A plot of weighted factor scores for component 1 versus component 2 for each test diet in this study from the Principal Components Analysis. The size of the shape indicates the 95% confidence intervals and distance between shapes indicates greater difference among diets. Higher, positive scores for Factor 1 were associated with high soluble and insoluble HMWDF (high molecular weight dietary fiber), high plasma cystine, high plasma taurine and high passive ventricular filling, but low dietary cysteine, low plasma methionine and low plasma cardiac-specific troponin I. In contrast, a high positive score for Factor 2 was associated with high dietary amylose content, high fasting blood glucose, high plasma NT-ProBNP (N-terminal pro B-type natriuretic peptide), high flow-mediated arterial dilation (FMD) as well as high diastolic ventricular volume and diameter.

4.6 Discussion

Important findings of this study were that different pulse-based, grain-free diets caused divergent cardiac changes in dogs. Specifically, the high amylose wrinkled pea diet caused changes that would suggest early, subclinical stages of DCM after 28 days of feeding, while the lentil diet had the least tendency and the grain-based rice diet was intermediate. There were no detrimental changes in basic blood chemistry levels in the dogs associated with consuming the grain-free diets, with the exception of total bilirubin when consuming the lentil diet. Apparent total tract digestibility of macronutrients and sulfur-containing amino acids was decreased when grain-free diets were eaten, except for taurine which remained similar among diets. Also, fasted plasma levels of taurine in dogs were not impacted by grain-free diets. Methionine levels, however, were decreased below mean plasma methionine values, when all diets were consumed, regardless of meeting AAFCO requirements or not. This could pose an issue in the long-term, especially within the grain-free diets as they contained lowered levels of dietary methionine. Overall, this study suggests a correlation between dietary amylose, not soluble or insoluble HMWDF, in diets that may be linked to DCM in dogs and urgently needs further exploration.

4.6.1 Properties of Diets

The test diets created for this study were formulated to be similar while aiming to achieve approximately 15% enzymatic starch. This in turn resulted in variations in insoluble HMWDF, total HMWDF, and amylose content. Amylose content was highest in the wrinkled pea grain-free diet, while both grain-free diets incorporated large amounts of pulse-based flours to achieve formulation goals. In this study, pulse flours provided the grain-free lentil and wrinkled pea (Amigold) diets with large amounts of plant proteins which decreased the requirements for the use of animal proteins (chicken meal). It is known that plant products do not contain taurine, but animal proteins such as seafood and poultry products contain high concentrations of taurine (Spitze et al., 2003). Despite reduced levels of animal protein in the grain-free diets, no major differences were observed among diets regarding their taurine levels, due to the appropriate inclusion of both chicken meal and fish meal. Dietary levels of both methionine and cystine+methionine in both grain-free diets however were lower than both grain-containing diets. Limited levels of these amino acids in the grain-free diets were likely due to pulses being limited in these amino acids (Singh, 2017; Tiwari & Singh, 2012).

The amylose content was highest in the wrinkled pea diet compared to the grain-containing diets. When compared to grains, pulse starch sources have higher amylose and resistant starch contents, which generally undergoes fermentation within the large intestine (Hoover et al., 2010). As reported by Newberry et al (2018) resistant starches, such as amylose contribute to increases in dietary fiber (Newberry et al., 2018), which is why the pulse diets in this study have higher levels of insoluble HMWDF and total HMWDF. However, dietary cellulose is also a contributing ingredient that influences the dietary fiber content of a diet (Morse, 1978). Since all diets were formulated to have 15% enzymatic starch, powdered cellulose was used to achieve isonitrogenous and isocaloric lab formulated diets despite large differences in flour inclusion. Due to higher cellulose inclusion, the rice diet had similar levels of total HMWDF as the lentil and wrinkled pea (Amigold) diets. This cellulose inclusion likely led to the rice diet mapping intermediate between the two grain-free, pulse-based diets tested in the PCA analysis.

4.6.2 Effect of Grain-Containing vs Grain-Free Diets on NT-ProBNP, and Canine Troponin I, and Cardiovascular Health

In dogs, DCM is commonly seen within larger breeds, while Beagles are considered less susceptible (Noszczyk-Nowak, 2011). Confirmation of a DCM diagnosis is performed doing echocardiography, however, there are multiple cardiac indicators in dogs such as blood levels of NT-ProBNP and cardiac troponin I that can be used for early detection of DCM (Noszczyk-Nowak, 2011). NT-ProBNP is a volume dependent, vasodilatory peptide that is released by myocardial tissue when the myocardial wall undergoes stress from chronic excess blood volume (Oyama et al., 2007). In contrast, cardiac troponin I is released when myocytes are injured and necrotic. Troponin is a part of the filamentous structure of the cardiac sarcomere that should not appear in the blood unless myocytes have been damaged (Oyama et al., 2007). In this study, plasma levels of NT-ProBNP ranged from 8.09 – 33.6 pg/mL, with the grain-free wrinkled pea diet having the highest levels, but even this fell well within normal limits (Kellihan et al., 2009). However, it should be noted that the increase in NT-ProBNP is consistent with the increased left ventricular chamber volume and diameter that you would expect in early, sub-clinical DCM. It is important to note that these adverse cardiac and NT-ProBNP changes reversed when dogs were fed the next test diet

Similar to a study performed by Reis et al (2021), this study demonstrated that feeding diets comprised of pulses for 28 days in Beagles did not cause advanced signs of DCM (Reis et al., 2021). In dogs with clinical DCM, it is common for them to be tachycardic (Noszczyk-Nowak, 2011), which was not observed in the dogs after consuming all diets. Heart rate was lowest in dogs after the wrinkled pea (Amigold) feeding period and likely contributed to the lower cardiac output (cardiac output = stroke volume x heart rate) (Zhang et al., 2015). After feeding the wrinkled pea (Amigold) diet, ejection fraction in dogs was slightly reduced but remained above 60%, while left ventricular internal diameter (LVID) during systole and diastole were both increased. Results of the PCA determined that these slight DCM-like changes were correlated with high levels of dietary amylose rather than HMWDF. HMWDF however, was correlated with high passive ventricular filling as well as low cardiac troponin I and plasma methionine levels. While these observations of cardiac changes in dogs consuming the wrinkled pea (Amigold) diet are all consistent with early development of DCM, full clinical heart failure is defined as ventricular ejection fraction falling below 40%, which was not observed (Petrič & Tomsič, 2008). The wrinkled pea (Amigold) diet did cause a slight reduction in the E:A ratio, which could be an indication of a trend towards diastolic dysfunction (Bonagura & Fuentes, 2015). These changes in cardiac function, though sub-clinical, are extremely important to make note of as these slight changes were seen only after 28-days of feeding and have the potential to be more detrimental with longer feeding periods.

4.6.3 Effect of Grain-Containing vs Grain-Free Diets on Plasma Levels of Sulfur-Containing Amino Acids

Taurine is not considered to be essential in dogs as they are able to synthesize taurine from the sulfur-containing amino acids cysteine and methionine (Backus et al., 2006). In this study, no major changes in taurine status were observed in the dogs after consuming each diet for 28 days. It has yet to be determined whether or not grain-free diets contribute to decreases in plasma taurine levels in dogs. Some studies have shown an increase in taurine deficiencies in dogs while consuming grain-free diets (Kaplan et al., 2018), while others have shown that grain-free diets do not cause deficiencies in taurine status of dogs (Donadelli et al.,2020; Adin et al., 2019). In this study, plasma levels of taurine in dogs ranged from 81.21– 88.56 nmol/mL, and all values fell within the normal limits of 63 - 194 nmol/mL determined by other studies (Kaplan et al., 2018; Tôrres et al., 2003, Delaney et al., 2003). Overall, this study demonstrates that feeding pulse-based, grain-free diets for 28-day periods is not detrimental on fasted plasma taurine levels in dogs. Moreover, the fact that sub-clinical trends consistent with early DCM were observed in this study, refutes the hypothesis that taurine deficiency is the causative link to DCM, at least in Beagles.

Although no major changes were observed in plasma taurine status, plasma levels of cystine and cysteine slightly varied. Free forms of cystine and cysteine were measured in this study. Despite pulses being known to have limiting amounts of cysteine (Singh, 2017), the pulse-based, grain-free diets in this study did not appear to cause major decreases in plasma levels of these amino acids when compared to grain-based diets. In fact, the pulse-based grain-free diets resulted in higher plasma levels cystine and cysteine when compared to a commercial grain-based diet in this study. An explanation for this could be attributed to the utilization of plasma methionine. Methionine is a precursor for cysteine which subsequently is used to produce taurine in dogs (Harrison et al., 2020). In this study, results of the PCA determined a correlation between decreasing plasma levels of methionine with increases in HMWDF. In the dogs, plasma levels of methionine ranged from 45.05 - 53.90 nmol/mL. Regardless of whether a diet met AAFCO methionine requirements or not, all plasma methionine values observed after consuming test diets fell below the reported reference range by Delaney et al (2003) which was 57.0 nmol/mL (Delaney et al., 2003). Methionine levels and availability in dogs is largely influenced by taurine and cysteine status (Harrison et al., 2020). Methionine status could be an issue in grain-free diets as they are comprised mainly of plant proteins instead of animal proteins, meaning taurine availability is often reduced (Spitze et al., 2003). However, in this study taurine levels were not negatively impacted. Performing studies in a diverse dog breed population should be considered to better determine the impacts of grain-free diets on taurine status and sulfur-containing amino acids in dogs, as Beagles are not pre-disposed to either taurine deficiencies or DCM (Pezzali et al., 2020; Ko et al., 2007).

4.6.4 Effect of Dietary Fiber and Amylose on Fecal Bile Acids

Excretion of total fecal bile acids in this study decreased as total HMWDF increased after feeding each diet for 28 days. For example, the grain-free, wrinkled pea (Amigold) diet, with the highest level of both amylose and total HMWDF had the lowest excretion of total fecal bile acids in dogs. This was opposite of what was hypothesized, but it does agree with a 7-day feeding study performed previously by our lab (Quilliam et al., 2021). A large proportion of studies confirms and supports that dietary fiber is able to bind bile acids in the intestinal lumen, which in turn would lead to an increased excretion of total fecal bile acids (Donadelli et al., 2020; Garcia-Diez et al., 1996; Stratton-Phelps et al., 2002). The results of this study do agree though with the results of a study performed by Pezzali et al (2020) which demonstrates that grain-free diets did not cause increases in the excretion of fecal bile acids in dogs (Pezzali et al., 2020). In addition to this, a recent meta-analysis demonstrated that high levels of carbohydrates are associated with decreased excretions of fecal bile acids (Pezzali et al., 2021).

One major concern addressed by the FDA, but has yet to be confirmed, is that grain-free diets are low or lacking in taurine (U.S. FDA, 2019). Taurocholate is a predominant bile salt that is conjugated from taurine and cholic acid (Mansilla et al., 2019; Sanderson et al., 2001). Soluble dietary fiber had been suggested to lower taurine availability in dogs, as they bind bile acids and prevent their reabsorption into the enterohepatic circulation. This could deplete taurine, due to taurocholate loss in feces (Sanderson et al., 2001). However, this was not observed in this study, as both the rice diet and the wrinkled pea (Amigold) diets had the highest levels of soluble HMWDF but the lowest excretion of fecal bile acids, which includes taurocholate. This hints to a possible microbiome effect that could be causing taurine degradation, but cannot be confirmed by this study. Some grain-free diets are high in dietary fiber that can act as a prebiotic for the microbiota. This prebiotic fiber effect changes the microbial population and composition, resulting in overgrowth that increases to taurine degradation (Tôrres et al., 2003). Overall, more studies should be performed to further investigate a possible connection between the microbiome and dietary taurine availability.

4.6.5 Apparent Total Tract Digestibility of Macronutrients and Amino Acids

Consistent with a 7-day study conducted in our lab with similar diets, the results of this 28-day study found that grain-free, pulse-based diets decrease the digestibility of macronutrients (Quilliam et al., 2021). An explanation for this could be due to the increased amounts of dietary amylose and fiber found in the diets that is known to influence nutrient digestibility (Carciofi et al., 2008). Similarly to other studies, crude protein digestibility decreased as total dietary fiber increased (Beloshapka et al., 2020). One reason for this could be due to the rice diet having higher amounts of animal proteins, while the pulse-based diets contained less animal protein and higher amounts of plant protein. Another possible explanation for limited protein digestibility is due to the intrinsic interactions of amylose (Rooney & Pflugfelder, 1986). Digestibility of starch in this study was also decreased in the grain-free diets, especially after feeding the wrinkled pea (Amigold) diet. This could be mainly due to the higher gelatinization temperature of wrinkled pea starch, which led to lower gelatinization level of the starch compared with those of rice and lentil diets. Besides, the interactions between starch and lipids, the process of extrusion can further reduce starch digestibility of the kibble (Ren et al., 2021; Seneviratne & Biliaderis, 1991;

Lin et al., 1997). In addition to this, lipid digestibility was also reduced as amylose and dietary fiber increased among the grain-free diets.

Digestibility of cystine, methionine, and cystine+methionine in this study were also decreased in the grain-free diets when compared to the grain-containing rice diet. It is known that sulfur-containing amino acid excretion can increase with increasing levels of dietary fiber (Mansilla et al., 2019). However, this study was unable to determine which fiber fraction or if amylose was causing digestibility decreases in the different diets. In addition to this, no major decreases in the digestibility of taurine were observed, with all values close to 100% in dogs among all diets after 28 days of feeding. An explanation for this could be that any unabsorbed taurine in the gastrointestinal tract is likely to be fermented and degraded by microbial populations, whose numbers are increased after feeding diets with high amounts of fermentable fiber (Mansilla et al., 2019). Further studies should be performed to continue to study the impacts of dietary fiber and amylose in grain-containing and grain-free diets on the apparent total tract digestibility in dogs. Microbiome studies should also be conducted to determine microbial changes that occur during the feeding of grain-containing and grain-free diets.

4.6.6 Effect of Diet on Blood Chemistry and Complete Cell Counts

Based on the results of this study there are no major findings demonstrating compromised overall health in dogs consuming both grain-containing and grain-free diets. Biologically, results of the CBC did not pose any concern for animal health. Following the commercial diet feeding period, cholesterol levels in the dogs were elevated compared to the other diets but was within normal limits. In humans, cholesterol is reduced by dietary fiber, specifically various soluble fibers (Brown et al., 1999). Supporting this, in the commercial diet, crude fiber and total HMWDF levels were less than the test diets. Although the mechanism of fiber lowering blood cholesterol is not fully known, it is suggested that it can bind cholesterol, ultimately leading to a fecal clearance (Brown et al., 1999), which could explain why consuming high fiber diets had lower cholesterol levels compared to the low fiber commercial diet. Fasting blood glucose levels were also higher in diets with increased levels of amylose, which is suspected to be due to a gluconeogenic effect. Total bilirubin and indirect bilirubin were also seen to decrease in the grain-free diets, the cause for this however remains unknown.

4.7 Study Strengths & Limitations

A strength of this study was the use of multiple grain-containing and grain-free diets and how they impact cardiovascular function in dogs after feeding each diet for a longer term (28 days) than a previous study from this group. Moreover, a strength is the use of two separate rice feeding periods that produced statistically similar results, lending confidence to the repeatability of all results. Another strength included examining the impact grain-free diets have on fasted plasma levels of sulfur-containing amino acids and taurine. In addition, this study contains blood chemistries and CBC analyses in dogs after feeding grain-containing and grain-free diets, which has sparsely been reported in current literature. A major limitation was the formulation of the test diets to include 15% enzymatic starch and 58% inclusion of wrinkled pea flour to achieve this. This extremely high inclusion of pulse flour is responsible for the low animal protein and low methionine levels in the diets. While these test diets are not representative of commercial diets, we did include a popular commercial diet as a control and the high pulse/low methionine levels were likely needed to push the Beagle physiology to show early DCM-like changes. Beagles are not predisposed to cardiovascular disease or taurine deficiencies, but if we managed to observe DCM-like changes in this resistant breed, then it seems likely that more susceptible breeds would have suffered greater adverse cardiac changes. Further studies should be performed in dog breeds that are predisposed to DCM to better determine if grain-free diets are safe for all dogs. Another limitation was that this study was conducted on a sample size of eight beagles, due to budget and housing constraints. Larger scale studies using client-owned dogs from multiple breeds will strengthen conclusions in future. Another limitation is use of apparent total tract digestibility instead of ileal cannulation to determine true digestibility. Apparent total tract digestibility is minimally invasive, needed to satisfy ethical review and allow research dogs to be adopted into homes when retired from nutrition studies. Another limitation of this study was having only 4 diets for the PCA analysis which limits the ability to detect relationships among the many variables. However, despite these limitations, some clear relationships were detected that merit exploration and can be used to guide further experiments, especially regarding amylose and different fiber fractions.

4.8 Conclusions

In this study, not all grain-free diets caused changes associated with early signs of DCM in research Beagles after 28 days of feeding each diet. Specifically, the high amylose wrinkled pea diet (Amigold) did increase left ventricular size and volume along with increased plasma proBNP, albeit in a sub-clinical manner that was readily reversed within the next 28-day, crossover feeding period. Moreover, this change was not associated with changes in fasted plasma taurine levels. The lentil diet, another grain-free diet tested, had the least tendency toward DCM-associated changes, while the rice diet was intermediate. The commercial diet, a grain-containing diet, showed great variability in these DCM-related parameters and overlapped with all 3 test diets. Whether longer feeding periods than 28 days with the wrinkled pea diet would have produced overt DCM is unclear. In addition to this, grain-free diets did not have any major detrimental impacts on overall health that was observed through blood chemistries and CBCs. While plasma taurine was unchanged with all diets, plasma methionine was reduced below mean expected values after feeding all diets studied and should be further monitored on a long-term basis. The grain-free diets were seen to have higher levels of total HMWDF, which in turn decreased macronutrient and amino acid digestibility, except for taurine. Diets with a higher content of dietary fiber produced decreases fecal bile salt excretion after 28 days, similar to what we observed previously after 7 days. Due to the limitations of having all diets formulated to 15% enzymatic starch, high-fiber pulse diets contained less animal-sourced protein and higher plant-based protein, while the high-fiber rice diet had more animal-sourced protein. This could be an important factor leading to the decreases in nutrient digestibility in the grain-free diets. Overall, the potential link between DCM-like changes and the wrinkled pea diet, but not lentil diet, show that not all grain-free diets can be treated the same. This complicates the original claim made by the FDA regarding grain-free diets. While high dietary amylose correlated in PCA to the early DCM-like changes, this study design cannot establish cause and effect. Moreover, additional unknown factors that changed in parallel to amylose content in the test diets may be more important in this potential link to DCM in dogs. Further studies urgently need to be performed to better explore these links.

5 CHAPTER 5: OVERALL STUDY FINDINGS

5.1 Results of Study 1 and Study 2

The objective of the two studies conducted was to examine the 7-day and 28-day effects of feeding pulse-based, grain-free diets on the health of domestic dogs. The results of these studies had a number of interesting observations that are summarized in Figure 5.1.

Figure 5.1: Flow-chart of summarized results from the 7-day and 28-day feeding experiments to compare and contrast grain-containing and grain-free diets.

After 7-days of feeding it was evident that the grain-free diets were capable of lowering the glycemic response in dogs. This lowered response was attributed to the increased levels of dietary fiber and amylose within the pulse-based diets. Increased fiber and amylose levels within the diet was also associated with decreasing levels of apparent total tract digestibility of macronutrients in both the 7-day and 28-day studies. Dietary fiber however does not appear to be a contributing factor to increased fecal bile acids excretion, as fecal bile acid excretion decreased as dietary fiber increased in the diets in these two studies. It was recently reported in a meta-analysis that increasing levels of carbohydrate can decrease fecal bile acid excretion (Pezzali et al., 2021), but further studies should be performed to confirm this. Both studies in this thesis demonstrated that pulse-based diets can cause decreased fasted plasma levels of methionine in the dogs. However, the 28-day study showed that this is not only limited to the pulse-based diets as both grain-containing and grain-free diets caused decreased levels of fasted plasma methionine in the dogs. This could pose an issue for taurine synthesis in the long-term, however despite this, for both studies, the fasted plasma taurine levels of the dogs remained within normal limits after consuming both grain-containing and grain-free diets. In the 7-day study, there was an observed trend in decreasing plasma levels of taurine in the dogs as dietary fiber and amylose increased across the pulse-based diets. However, after 28-days of feeding each diet to the dogs, there was no concern for plasma taurine levels after consuming grain-free and grain-containing diets. It is important to note that beagles are not predisposed to taurine deficiency or DCM (Pezzali et al., 2020). With that being said, after 28-days of feeding the wrinkled pea (Amigold) diet to the dogs, there were sub-clinical changes in cardiac function that would be a trending towards the indication of DCM. Decreases in ejection fraction, cardiac output, and E:A as well as increased left ventricular chamber volume and left ventricular internal diameter were observed.

In addition to this, slight elevations of the cardiac market NT-ProBnP were also recorded. Despite trends, it is important to note that all cardiac functions and blood markers remained within normal limits, and were reversible by diet replacement. It is also critical to understand that although these changes were observed within the wrinkled pea (Amigold) diet, they were not observed within the lentil diet, another grain-free diet. After conducting a PCA it appears that there is an association with DCM-like changes in cardiac function and increased levels of amylose. This suggests that grain-free diets cannot all be categorized as the same, let alone have the same detrimental impact on cardiac health in dogs.

Based on the results found from both studies the overall hypothesis that pulse-based, grain-free diets would produce a low glycemic response in dogs due to decreased rates of digestion and lowered bioavailability of nutrients for absorption, which would lower plasma concentrations of cystine, cysteine, methionine, and taurine, resulting in early indications of DCM was partially rejected. Though decreases in glycemic response, nutrient digestibility, and plasma methionine were observed in dogs after consuming the pulse-based, grain-free diets there was no indication that plasma levels of taurine were compromised beyond normal limits. Not all grain-free diets appeared to cause any indication of DCM either. The wrinkled pea (Amigold) diet caused some changes in cardiac function that would indicate sub-clinical DCM, however, the lentil diet caused none whatsoever. Therefore, not all diets have the same capabilities of causing DCM. It is possible that the DCM-like changes associated with the wrinkled pea (Amigold) diets are attributed to amylose content of the diet, however further studies examining this and any other underlying causes should be performed as this thesis was unable to determine a cause-and-effect. Overall, this thesis demonstrates that the controversial discussions surrounding grain-free diets are more complicated than originally expected and that this is likely more than just a taurine deficiency issue. Thus, complicating the original statement released by the FDA surrounding grain-free diets. Overall, grain-free diets do have their benefits, and not all grain-free diets are as detrimental as many have been previously led to believe.

5.2 Study Strengths and Limitations

A strength of the 7-day feeding study is the detailed glycemic response data of each diet fed to the dogs. Another strength for both the 7-day and 28-day feeding studies was the use of multiple pulse crops to study if dietary fiber and amylose are impacting variables associated with pulses or if there are other components to be studied. In addition, the 28-day feeding study was able to examine grain-containing and grain-free diets to determine if they have any impacts on cardiovascular function in the dogs. Both studies were also able to focus on the 7-day and 28-day impacts of feeding pulse-based grain-free diets on plasma levels of sulfur-containing amino acids and taurine, which is currently a controversial topic.

A limitation in both studies was that research was conducted using Beagles, which is a breed that is not predisposed to cardiovascular illness or taurine deficiencies (Pezzali et al., 2020). Due to this, major findings regarding taurine status and cardiovascular health should only be applied to other breeds that are also not predisposed, while further studies on susceptible breeds should be performed. Another limitation for both studies was the use of the apparent total tract digestibility method. True digestibility would have been a better method to use, however, this can only be performed through lethal sampling or by placing ileal cannulas in the dogs, which was not possible as our lab performs only non-lethal and minimally invasive techniques. This is done to allow the adoption of dogs following the studies.

The use of a small sample size (n=8) was another limitation for both studies conducted, and in the future should be combated by conducting studies with larger sample sizes. In the 7-

day feeding study, this was combated by using a randomized, cross-over, repeated-measures, blinded study design, while in the 28-day feeding period we were only able to perform a repeated-measures, cross-over, blinded study. Lastly, it is important to note that many pet owners feed their dogs the same food for long periods of time ranging from months to years, making it difficult to truly emulate the true feeding nature of pet dogs in research dogs.

In order to accommodate formulating all diets in study one to have 20% available carbohydrate and study two to have 15% enzymatic starch, a large amount of pulse-flour had to be used. This was limiting as it caused some pulse diets to have up to 58% pulse flour added to the formulation. As a result, the pulse diets were extreme and not something you would typically see in a commercial diet. Furthermore, NRC nutritional values were used for ingredients such as fish meal and chicken meal instead of directly measuring key nutrient levels in the ingredients used. Variation from NRC values in the feed ingredients due to variable quality resulted in some diets falling below AAFCO requirements for some amino acids. Despite this, only minor, reversible changes in cardiac function and biomarkers were observed. This suggests that it was necessary to use extreme diets in order to observe the cardiac changes and that more commercially representative diets would have resulted in a false conclusion of no effect of grain-free diets.

Finally, diets from study one were extruded using a different extruder than diets made for study two. With that, different extruder parameters were used to make the batches of kibbles in the 7-day study when compared to the 28-day study (see supplemental Tables 2 & 3). These changes in the extrusion process could have possibly impacted kibble structure and degree of cooking, ultimately impacting the digestibility due to starch retrogradation and protein denaturing. This makes it more difficult to compare the results from study one to study two.

However, notable trends and changes in biological responses should not be ignored and further examined.

5.3 Future Studies

Studies further examining the roles of dietary fiber in pulse-based diets on bile acid excretion, nutrient digestibility, and cardiovascular health should be conducted. Dietary fiber binds bile acids and prevents their reabsorption resulting in the excretion of bile acids (Ellegård & Andersson, 2007). Despite this, the opposite was observed in both studies and bile acid excretion decreased as dietary fiber and amylose increased. Decreases in bile acid excretion in these high fiber diets could also possibly be attributed to fiber fractions and fermentation by the microbiome in the gut which is why it is also important to study the microbiome in future studies and its impact on bile acid excretion.

Microbiome studies should also be conducted to further examine if the microbiome impacts taurine availability/digestibility. It is known that many gut microbiota catabolize taurine for energy as well as for biosynthesis, which increases the degradation and decreases the availability of taurine for the host (Ridlon et al., 2016). Diet has been known to impact microbial populations (Wernimont et al., 2020), so it is crucial to study the microbiome and how it changes over time as dogs consume different diets to determine if the microbiome impacts taurine availability/digestibility and in dogs.

Future studies examining pulse-based, grain-free diets should also be conducted in a larger sample size using breeds of dogs that are predisposed to DCM and taurine deficiencies to better examine the safety of these diets on those breeds. Breeds such as Newfoundland, Irish Wolfhounds, Doberman Pinschers, Scottish Terriers, Dalmatians, Boxers, Golden Retrievers, and Cocker Spaniels are susceptible to the development of DCM (Chetboul, 2015; Kittleson et al., 1997; Fascetti et al, 2003; Kaplan et al, 2018), so it may be beneficial to conduct studies in these breeds to determine if diet can exacerbate this pre-disposition. Longer study durations for breeds both pre-disposed and non-predisposed may also be advantageous to better determine if pulse-based, grain-free diets pose any major risks regarding taurine status and cardiovascular health.

Lastly, future studies examining the impacts of increased amylose content versus other fiber fractions in dog diets on the development of DCM should be performed. In study two of this thesis, DCM-like changes were associated with increased amylose, however, this study was unable to establish a concrete cause and effect due to a PCA approach. Further studies analyzing this and determining any other unknown dietary components that are associated with or cause DCM-like changes should be performed.

5.4 Overall Conclusion

Findings of these studies are beneficial in providing insight towards the grain-free controversial debate. After 7-day and 28-day feeding studies, it is apparent that pulse-based, grain-free diets reduce nutrient digestibility due to higher levels of plant matter increasing insoluble HMWDF. This however does appear to negatively impact overall animal health. In fact, pulse-based, grain-free diets have a beneficial effect on glycemic response, which lowers the risk of diabetes mellitus and obesity but also increases animal satiety. Surprisingly, both studies demonstrated decreased excretions of fecal bile acids as amylose content and insoluble HMWDF increased, which is opposite of what was originally hypothesized. Further studies looking into microbiome effects should be considered.

In addition to this, the pulse-based, grain-free diets fed in both studies did not appear to cause detrimental changes in plasma taurine levels of the dogs. In the 28-day study though, there were deficient levels in the dog's plasma methionine levels after consuming all diets, both grain-containing and grain-free, which could possibly lead to a taurine deficiency if prolonged. The 28-day feeding study also found cardiac changes in the dogs associated with increased amylose content of the diet. After consuming the wrinkled pea (Amigold) diet, there were slight indications of sub-clinical DCM in the dogs. The lentil diet however did not show indication of sub-clinical DCM, demonstrating that not all grain-free diets can be treated or viewed the same. Thus, complicating original claims made by the FDA regarding grain-free diets. Altogether, these studies demonstrated that grain-free diets have their benefits, but should not all be viewed as one in the same when it comes to their detriments. Differences in diets based on fiber and amylose content, let alone the use of different pulse ingredients in the diet further supports that there are many different types of grain-free diets. In addition to this, if amylose content is truly associated with diet induced DCM in dogs, this could be an issue that could occur in both grain-containing and grain-free diets. In conclusion, grain-free diets can not be viewed as one in the same, as a large proportion of grain-free diets may not be as detrimental as many have been led to believe.

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7 Supplemental Data

Table S1: Chemical compositions of flour samples from the 7-day feeding study in Chapter 3. [a, b]

Flour	Starch content (%, dry flour basis) [c]	Amylose content (%, dry flour basis) [d]	Amylose content (%, dry starch basis) [e]	Protein content (%, dry flour basis) [f]	Crude fiber content (%, dry flour basis) [g]	Fat content (%, dry flour basis) [h]	Ash content (%, dry flour basis) [i]	Dry matter (%)
Rice	86.5 ± 0.8 d	21.9 ± 0.7 c	25.3 ± 0.9 a	7.3 ± 0.1 a	0.02 ± 0.01 a	0.74 ± 0.12 a	0.48 ± 0.03 a	88.1 ± 0.0
Round pea (CDC Inca)	48.0 ± 0.4 c	19 ± 0.2 b	39.5 ± 0.4 b	23.9 ± 0.3 b	5.64 ± 0.18 c	1.23 ± 0.27 b	2.35 ± 0.18 b	92.6 ± 0.3
Lentil (CDC Maxim)	47.4 ± 0.6 c	17.4 ± 0.7 ab	36.8 ± 1.4 b	27.6 ± 0.1 d	3.55 ± 0.10 b	0.39 ± 0.09 a	2.41 ± 0.02 b	93.6 ± 0.1
Fava bean (CDC Snowdrop)	43.1 ± 1.0 b	16.4 ± 0.4 a	38.0 ± 0.9 b	30.3 ± 0.1 f	7.98 ± 0.21 e	0.69 ± 0.14 a	3.02 ± 0.16 c	93.2 ± 0.1
Wrinkled pea (4140-4)	34.1 ± 0.3 a	24.1 ± 1 d	70.6 ± 3 c	28.7 ± 0.1 e	7.79 ± 0.29 e	1.82 ± 0.09 c	3.11 ± 0.02 c	92.7 ± 0.0
Wrinkled pea (Amigold)	34.4 ± 0.6 a	27.1 ± 0.4 e	78.8 ± 1.1 d	26.4 ± 0.3 c	7.00 ± 0.31 d	1.90 ± 0.11 c	3.40 ± 0.03 d	92.8 ± 0.0

[a] Values are presented as average ± standard deviation of triplicate measurements.
[b] Values followed by the same letter in the same column are not significantly different at $p < 0.05$.
[c] Determined using Megazyme Total Starch Assay Kit following AACC Method 76-13.01.
[d] Determined using an iodine colorimetric method of Chrastil (1987).
[e] Amylose content (%, dry starch basis) = [Amylose content (%), dry flour basis] / [Total starch content (%), dry flour basis] × 100.
[f] Determined using a Nitrogen/Protein Analyzer (CN628, LECO Corporation, St. Joseph, MI, USA), with a conversion factor of 6.25.
[g] Determined by Central Testing Laboratory Ltd. (Winnipeg, Manitoba, Canada), following Crude Fiber Method by Ankom Technology (2017).
[h] Determined by Central Testing Laboratory Ltd. (Winnipeg, Manitoba, Canada), following AOCS Method Am 5-04.
[i] Determined by Central Testing Laboratory Ltd. (Winnipeg, Manitoba, Canada), following AOAC Method 942.05.

Table S2: Extruder parameters used to create test diets for the 7-day feeding study in Chapter 3.

Parameters	Condition
Feed Moisture (%)	30
Solid Feed Rate (kg/h)	3.2
Screw Speed (rpm)	200
Temperature (Section 1, ˚C)	40
Temperature (Section 2, ˚C)	60
Temperature (Section 3, ˚C)	80
Temperature (Section 4, ˚C)	100
Temperature (Section 5, ˚C)	120
Die Diameter (mm)	5.5

Table S3: Extruder parameters used to create test diets for the 28-day feeding study in Chapter 4.

Parameters	Condition
Feed Moisture (%)	25
Solid Feed Rate (kg/h)	10
Screw Speed (rpm)	300
Temperature (Section 1, ˚C)	30
Temperature (Section 2, ˚C)	60
Temperature (Section 3, ˚C)	90
Temperature (Section 4, ˚C)	120
Temperature (Section 5, ˚C)	120
Temperature (Section 6, ˚C)	120
Die Diameter (mm)	4

Table S4: Formulation of rice, lentil and wrinkled pea (Amigold) diets from the 28-day feeding study in Chapter 4. Diets ordered from left to right in order of increasing total HMWDF fiber content.

	Rice Diet	Lentil Diet	Wrinkled Pea Diet (Amigold)
Flour	23.12	42.19	58.14
Chicken By-Product Meal	39.28	23.14	14.36
Cellulose	15	12.17	5
Chicken Fat	10	10	10
Fish Meal	5	5	5
Canola Oil	5	5	5
Celite	1	1	1
Vitamin/Mineral Premix	1	1	1
NaCl	0.3	0.3	0.3
Choline Chloride	0.1	0.1	0.1
Calcium Carbonate	0.05	0.05	0.05
Dicalcium Phosphate	0.05	0.05	0.05

All values are expressed as % inclusion as fed. *Included addition of antioxidant Naturox (Kemin, Des Moines, IO USA)

Table S5: Ingredient list of commercial diet fed in the pre-trial phase of the 28-day feeding study in Chapter 4.

	Ingredients:
Commercial Diet	Chicken, Brewers Rice, Whole Grain Wheat, Poultry By-Product Meal (Natural Source of Glucosamine), Corn Gluten Meal, Whole Grain Corn, Animal Fat (Preserved with Mixed-Tocopherols, Form of Vitamin E), Corn Germ Meal, Fish Meal (Natural Source of Glucosamine), Animal Digest, Dried Egg Product, Salt, Potassium Chloride, Calcium phosphate, Calcium Carbonate, Vitamin E Supplement, Choline Chloride, Zinc Sulfate, Ferrous Sulfate, L-Ascorbyl-2-Polyphosphate (Source of Vitamin C), L-Lysine Monohydrochloride, Manganese Sulfate, Niacin, Vitamin A Supplement, Calcium Pantothenate, Thiamine Mononitrate, Copper Sulfate, Riboflavin Supplement, Vitamin B-12 Supplement, Pyridoxine Hydrochloride, Garlic Oil, Folic Acid, Vitamin D-3 Supplement, Calcium Iodate, Biotin, Menadione Sodium Bisulfite Complex (Source of Vitamin K Activity, Sodium Selenite