EFFECTS OF PEA STARCH YEAST FERMENTATION ON GLYCEMIC INDEX, PALATABILITY, METABOLIC STATUS AND INTESTINAL HEALTH OF DOGS AND CATS FED A PEA BASED DIET

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ABSTRACT

The premium pet food market has expanded substantially over the past few years, which is related to the growing pet owners concerns with animals welfare and longevity. To address this new market niche, the pet food industry invested in ingredients and formulations that aim to provide health benefits for dogs and cats. Among these new ingredients are peas. Peas are low glycemic index carbohydrates that have become popular in pet food formulation. Despite evidence showing that peas benefit pets metabolic status,the use of peas in cat food formulation still a challenge as cats tend to refuse the taste of pea based diets.

Fermentation is a millenary technique used to improve food flavour and texture. Among the microbes used to ferment food are yeasts. Yeast fermentation yields amino acids that are potent flavour enhancers. Furthermore, yeast fermentation lowers the levels of antinutrients, which may inhibit the bitter taste of peas. In addition, the yeast cell wall contains prebiotics that might benefit intestinal health. The purpose of this thesis was to create a novel pet food using yeast fermented pea starch and to investigate the effects of this diet on palatability, glycemic index and intestinal health of dogs and cats. To research these objectives, four studies were conducted. The first study describes the methodology used to ferment the pea starch and compares pea starch composition before and after being fermented. The second study investigates whether yeast fermentation impacts dogs and cats postprandial glucose response to unfermented and fermented pea starches and diets. The third study investigates whether fermentation enhances the palatability of pea based diets formulated for dogs and cats. The last study investigates whether dogs and cats metabolic status and intestinal health benefits from diets formulated with unfermented and fermented pea starch over a diet formulated with a more traditional carbohydrate source (namely corn).

Besides showing that pea starch can successfully be fermented using a common yeast used in human food processing (*C. utilis*), the results of the first study show that pea starch fermentation slightly increases protein and crude fibre content. The second study reveals that fermentation does not significantly impact dogs and cats glucose response to pea products (starch or diet). The third study results show that dogs and cats have preference for a diet formulated with fermented pea starch over a diet formulated with unfermented pea starch. The results of the last study show that dogs and cats fed peas instead of corn have improved

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metabolic status (regardless of fermentation). Moreover, when compared to a diet formulated with corn, consumption of pea based diets led to a more diverse bacterial intestinal community and increased population of Faecalibacterium, genera often associated with improved intestinal health.

In conclusion, the results of this thesis show that yeast fermentation improves the palatability of pea based diets without compromising the health benefits associated with the consumption of peas in dogs and cats.

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Jasmine for helping me with this project and for keeping me company on my good and bad days. You are the best and deserve to have your names forever printed on this page.

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LIST OF ABBREVIATIONS

NSP: non-starch polysaccharides

SCFA: short-chain fatty acids

RDS: rapidly digestible starch

SDS: slowly digestible starch

RS: resistant starch

FAO: Food and Agricultural Organization of the United Nations

TRCs: taste receptor cells

TPR: transient receptor potential

PKDL: polycystic kidney disease like

DNA: deoxyribonucleic acid

16SrRNA: 16 svedberg ribosomal unit of ribonucleic acid

YPD: yeast peptone dextrose

CRFC: Canadian Feed Research Center

ACU: animal care unit

DM: dry matter

UPS: unfermented pea starch

FPS: fermented pea starch

UPD: unfermented pea diet

FPD: fermented pea diet

ME: metabolizable energy

BW: body weight

RER: resting energy requirements

MER: maintenance energy requirements

WBC: white blood cells

PLR: platelet lymphocyte ratio

NLR: neutrophil lymphocyte ratio

Mfeed: index compound in feed

Mfeces: index compound in feces

BCS: body condition score

NGS: next-generation sequencing

DE: digestible energy

ANOVA: analysis of variance

LSD: least significant difference

ALK: alkaline phosphatase

GGT: gamma-glutamyl transferase

ALT: alanine transaminase

CK: creatinine kinase

A:G ratio: albumin globulin ratio

RBC: red blood cells

GLDH: glutamate dehydrogenase

OTU: operational taxonomic unit

PERMDISP: permutation multivariate analysis of dispersion

PERMANOVA: permutation multivariate analysis of variance

FDR: false discovery ratio

CHAPTER 1

LITERATURE REVIEW

1.1 Carbohydrates

A balanced diet is composed of six major macronutrients: water, carbohydrates, proteins, fats, minerals, and vitamins (Case et al., 2011). Carbohydrates are defined by their chemical composition as natural compounds containing carbon, hydrogen, and oxygen. Carbohydrates may also be classified as sugars or polysaccharides, according to their molecular size (Knudsen, 2013). Sugars are further sub-classified as monosaccharides (e.g., glucose, fructose and galactose) and disaccharides (e.g., sucrose, lactose and maltose). Polysaccharides or complex carbohydrates are polymeric chains of monosaccharides linked by glycosidic bonds that are subclassified into starch and non-starch polysaccharides (NSP) (Knudsen, 2013; Hall and Mertens, 2017).

Non-starch polysaccharides - also known as dietary fiber - can be either soluble or insoluble. Insoluble fibers have laxation properties, while soluble fibers are linked to many other health benefits, such as lower blood cholesterol levels and lower postprandial glucose and insulin response (Cunningham, 2010; Jenkins et al., 1986; Lehmann and Robin, 2007; Nguyen et al.1998). Cellulose, galactomannans, xylans, xyloglucans and lignin are examples of water-insoluble fibers; while pectins, arabinogalactans, arabinoxylans, β- (1,3) (1-4) and β-glucans are examples of soluble fibers (Knudsen, 2015). Despite being classified as soluble or insoluble, all non-starch polysaccharides contain chemical bonds that are not susceptible to digestive enzymatic digestion; therefore, NSP reach the colon where it can either bind to water and increase stool weight or be fermented by intestinal microbes generating short-chain fatty acids (SCFA). Short-chain fatty acids can either be used as an energy source by the host or promote the growth of microbes that inhabits the intestine (Bach Knudsen, 2015; Căpriţă and Căpriţă, 2011; Hall and Mertens, 2017).

Starches are composed of many glucose units linked together. Amylose and amylopectin are the glucose polymers that form starch. Amylose is a long linear unbranched chain of α-1,4-linked D-glucose and amylopectin is a long α-1,6-branched chain of α-1,4-linked glucose units (Knudsen, 2013; Lehmann and Robin, 2007; Thorne et al., 1983). The starch structure usually contains a combination of amylose and amylopectin glucose chains. Because amylose has stronger hydrogen bonds than amylopectin, foods that are rich in amylose are more difficult to digest, which leads to a more gradual postprandial glucose and insulin response (Jenkins et al., 1986; Lehmann and Robin, 2007; Thorne et al., 1983).

In nature, starch is present in plants in the form of starch granules. The grain structure was studied using X-ray diffraction techniques, which allowed the classification of starch according to their physical characteristics into type A (open structure), type B (compact structure) or type C (a combination of A and B). The physical structure of the grain influences starch digestibility, with physically enclosed starches (e.g. coated with protein or lipids) being more resistant to digestion (Bach Knudsen, 2013; Lehmann and Robin, 2007).

Processing methods may change physical starch form and chemical properties, which have an effect on starch digestibility. For instance, starch granules are disrupted when the grain is heated in the presence of water, resulting in a gelatinized starch form that is rapidly digested by pancreatic amylase (Englyst and Englyst, 2005; Yao et al., 2010). Conversely, high amylose starches may instead retrograde and become harder to be digested. Dry heat processing does not lead to gelatinization and, therefore, does not increase food digestibility. Starch digestibility may not be changed by milling, as very small size starch granules might not be changed by this processing technique. As a result, the digestibility of starch flours may be similar to the grains that originated the product, depending on how finely ground the flour is (Englyst and Englyst, 2005; Lehmann and Robin, 2007; Thorne et al., 1983).

Individual variation and interaction among mixed meal ingredients also account for differences in starch digestion. Increased gastrointestinal transit will result in a decreased time of exposure to hydrolytic enzymes and a consequent reduction in starch digestion. Starch digestion tends to increase with mixed meals containing ingredients that reduce transit time (e.g., fat and NSP) . However, other factors such as meal volume, food particle size, viscosity,

and pH may also interfere in the gastric emptying process and play a role in the speed at which starch is digested (Brand-Miller et al., 2009; Englyst and Englyst, 2005; Thorne et al., 1983).

1.1.1   Carbohydrate Metabolism: Dogs & Cats

The fact that cats and dogs are housed with humans made people believe that both species are similar. However, cats and dogs show distinct characteristics in physiology, behavior and nutritional requirements. Although both species belong to the order Carnivore, dogs belong to the superfamily Canoidea, while cats belong to the superfamily Feloidea. Many animals from the Canoidea superfamily, including the dogs, evolved to have an omnivorous diet, while the whole Feloidea superfamily, including the cat, remained as strict carnivores (Caseet al., 2011; MacDonald et al., 1984; de-Oliveira et al., 2008).

Similar to humans, carbohydrate digestion in dogs relies on enzymatic processes. The digestive process starts with the amylase produced by salivary glands. Because amylase cannot survive the stomach's acid environment, most of the starch will be further degraded into the small intestine by the pancreatic juice through further enzymatic reactions. Starch is transformed into glucose by amylase and maltase. Glucose will then be actively absorbed by the enterocyte, going to the portal circulation and finally to the liver or other peripheral tissues. The glucose may be used as an energy source or stored as glycogen. Carbohydrates that are resistant to small intestine digestion will reach the large intestinal and may instead be fermented by the intestinal microbiome to produce SCFAs (Knudsen, 2013; Case et al., 2011; Zoran, 2010).

Cats are obligate carnivores, and like their wild ancestors are meant to be active animals and efficient hunters. However, domesticated cats have a lifestyle and a diet completely different from what they were used to in their natural environment (Zoran and Buffington, 2011). Wild cats consume a heavily meat-based diet that is usually low in carbohydrates and high in protein. Accordingly, cats are evolutionarily designed to digest little carbohydrate, which is reflected by anatomic and metabolic characteristics found in their gastrointestinal tract (Zoran et al., 2002; Verbrugghe et al., 2011). For instance, cats have very little salivary amylase activity, and pancreatic amylase is also reduced in comparison to other species. Moreover, cats

small intestinal brush border enzymes activity (e.g. maltase, sucrase and lactase) is heavily impaired and the transport system for sugars is also compromised. Finally, cats have a shorter small intestine and a shorter colon when compared to other species, which further compromises complex carbohydrate digestion. Despite its short length, a significant number of microbes inhabits the feline gastrointestinal tract (Case et al, 2011; Debra L. Zoran, 2002).

Besides the differences found in enzymatic activity and intestinal anatomy, the liver of cats also shows some peculiar characteristics. In most species, glycolysis relies on two enzymes: hexokinase and glucokinase. A study determined the rate of glucose metabolism by glucokinase, hexokinase and glucose dehydrogenase in different species. Results show that dogs possess high glucokinase activity, while no activity was detected in the liver of cats (Ballard, 1965). Another study investigated the glucokinase activity in the peripheral leukocytes of dogs and cats. The results show high rates of glucokinase in the dog leukocytes contrasting with absent activity in cat leukocytes. Conversely, cat leukocytes show higher rates of hexokinase and pyruvate kinase when compared to dog leukocytes. These findings support the thought that dogs are able to use glucose more efficiently than cats, but it also suggests that cats may be able to use other sugars more efficiently than dogs (Arai et al., 1998).

The gluconeogenesis pathway is used by dogs to generate glucose generally only when glucose levels are low, with minimal gluconeogenic activity when glucose is normal or high. Cats, on the other hand, use gluconeogenesis to meet glucose requirements regardless of being fasted or not (Verbrugghe et al., 2012). Constant gluconeogenesis is a feature common to many carnivorous species that consume low carbohydrate diets and thus must produce glucose endogenously to supply their tissues with energy. Despite differences in carbohydrate handling, digestibility studies show that both species can effectively digest carbohydrates (Laflamme, 2008; de-Oliveira et al., 2008). However, the physiological mechanisms involved in carbohydrate digestion by the cat are still under investigation (Laflamme, 2008; de-Oliveira et al., 2008; Verbrugghe and Hesta, 2017).

1.1.2   In Vitro and In Vivo Techniques to Assess Carbohydrate Metabolism

In vitro and *in vivo* techniques were developed to determine the speed of digestion of different starch sources. The Englyst method is an *in vitro* technique that measures how readily starches are digested. By mimicking the enzymatic digestion in the gastrointestinal tract, carbohydrates are classified into rapidly-digestible starch (RDS), slowly-digestible starch (SDS) and resistant starch (RS) (Englyst and Englyst, 2005). RDS is defined as the portion of starch that is converted into glucose within 20 minutes, while SDS refers to the starch that is converted into glucose between 20 and 110 minutes. The remaining portion is named as resistant starch and is thought to be the portion that cannot be digested by enzymes produced by the gastrointestinal tract of the host. However, microbes harboured in the host large intestine may instead be able to digest the resistant starch that escapes digestion in the upper intestinal tract (Tas and El, 1999; Englyst and Englyst, 2005; Englyst et al., 2018; Walter et al., 2005). Similar to soluble non-starch polysaccharides, resistant starch has been deemed a functional food ingredient with potential health benefits. For instance, the consumption of resistant starch has been shown to lower blood levels of cholesterol and glucose. Moreover, a diet rich in resistant starch benefits intestinal health by increasing growth and diversity of intestinal microbes (Cerbo et al., 2014; Lehmann and Robin, 2007; Niba, 2002)

In 1929, McCance and Lawrence introduced the concept of available carbohydrate, arguing that, because carbohydrates are metabolized differently, they should be classified differently as available or unavailable carbohydrate. Available carbohydrates are digested and used by the host (starch and sugars), while unavailable carbohydrates cannot. By developing a methodology to determine available carbohydrates content in foods, McCance and Lawrence intended to use this knowledge to advise better food choices for diabetic patients (Cummings and Stephen, 2007; McCance and Lawrence, 1929).

The interest in studying carbohydrate bioavailability emerged from the fact that carbohydrate content of foods is labelled using a deductive methodology. According to this methodology, carbohydrate content is not determined directly in standard food industry labelling, but instead is determined indirectly by subtracting from 100%, the sum of other food macronutrient components that are determined directly (ash, protein, moisture, fat and crude fiber). Despite being widely used, the determination of carbohydrates by deduction has some

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limitations (McCance and Lawrence, 1929). First, the value obtained by the difference is simply identified as "carbohydrates," which includes all types of carbohydrate plus other substances such as organic acids and lignin. Second, any analytical error that possibly occurs in any of the previous techniques used to determine the other four food components compromises the value obtained and identified as carbohydrates (Cummings and Stephen, 2007; Southgate, 1969; Mccance and Lawrence, 1929). Finally, the total carbohydrate food content fails to inform differences in types of carbohydrates, which may prevent consumers from making healthier food choices (Cummings and Stephen, 2007).

Despite being useful tools to differentiate the type of carbohydrates present in foods, *in vitro* techniques fail to consider carbohydrate physiological effects (Jenkins et al., 1981). In contrast, the glycemic index methodology accounts not just for the carbohydrate content of foods, but it also considers the *in vivo* physiological process involved in carbohydrate digestion (Brouns et al., 2005; Jenkins et al., 1981; Wolever et al., 1991). The glycemic index measures the quality of carbohydrates according to their post prandial peak glucose and duration of glycemic responses (Brouns et al., 2005; Jenkins et al., 1981; Louie et al., 2016). The glycemic index concept was first introduced to enable reliable nutritional guidance for human diabetic patients (Jenkins et al., 1981; Wolever et al., 1991). Since the glycemic index concept was first introduced, the methodology has evolved and is becoming more inclusive. Some countries integrated this concept into their dietary guidelines and human food labelling. Thus, the glycemic index has been used not just for research purposes, but also by health professionals and food companies (Brand-Miller et al., 2009; Louie et al., 2016).

To determine the glycemic index value, the post prandial glycemic response in fasted individuals to the test food is compared to the glycemic response to a standardized reference food (white bread or pure glucose). The glucose response to the reference food is considered to be the highest response possible (100%). The fasted test subjects consume a fixed amount of available carbohydrate of either the test food or the reference food; then, blood is collected at pre-determined time points to create a curve of time *versus* blood glucose concentration. The glucose area under the curve is calculated using the trapezoidal rule, and the glycemic response to each food is expressed as a percent of the mean response to the reference food. The

calculated value for each test subject is averaged to obtain the glycemic index value of the food (Brouns et al., 2005; Wolever et al., 1991).

Many factors can affect the test results and the outcome of glycemic index testing. Therefore, it is important to standardize the technique to obtain comparable values for the glycemic index. The number of subjects enrolled in the test, the number of replications for the reference and test foods, the physiological state of the subject, the choice of the reference food, the consumption time of the meals and the amount of available carbohydrate offered are among the factors that must be addressed (Brouns et al., 2005).

A large number of studies support the health benefits associated with consumption of low glycemic index foods in humans (Brand-Miller et al., 2009; Dahl et al., 2012; Rizkalla et al., 2002; Venn and Green, 2007). Observational studies, clinical trials and meta-analyses studies in humans suggest a link between glycemic nature of carbohydrates, obesity and other related metabolic diseases (Livesey, 2005; Rizkalla et al., 2002; Siegel et al., 2011; Venn and Green, 2007).

Obesity is considered the most common nutritional disease in dogs, and it is estimated that at least 33% of the pets that are taken to vet clinics are obese (German, 2006; Zoran, 2010). As observed in humans, obesity also plays a key role in the development of diabetes and insulin resistance in cats (Osto et al., 2013; Verbrugghe et al., 2012). In fact, the prevalence of diabetes in cats has drastically increased since obesity became a common disease in pets (German, 2006; Osto et al., 2013; Zoran, 2010). Although carbohydrates are not considered essential for cats and dogs, these nutrients have a physiologic impact on pet nutrition. The most important role of carbohydrates is as a cheap energy source, consequently sparing the need for higher-priced protein in the diet (Thompson, 2008). The energy supplied by carbohydrates can be utilized in the body directly as glucose or stored as glycogen. However, the amount of carbohydrate that can be stored as glycogen is limited. Once the body reaches this limit, the excess carbohydrate consumed in the diet will be metabolized to body fat. Therefore, consuming high amounts of carbohydrates can lead to obesity (Case et al., 2011).

Dry pet foods may contain 30 to 60% carbohydrates in their formulation (de-Oliveira et al., 2008). Common sources of digestible carbohydrates used in the industry are whole grain corn, brewer's rice, barley, and whole-grain wheat. Various grain flours, brown rice, oats,

sorghum, and potatoes can also be used (Thompson, 2008). However, all of these carbohydrate sources are considered high to moderate glycemic index foods in humans, with values in pets being unknown. Considering the abundance of carbohydrates used in pet food formulation and the increasing concerns about obesity and other metabolic diseases in dogs and cats, the replacement of traditional high glycemic index food ingredients with lower glycemic index ingredients might benefit the general health of dogs and cats, improving quality of life and increasing life span.

1.2   Health Benefits Associated with Pea Consumption

According to the Food and Agricultural Organization of the United Nations (FAO), pulses are defined as "a type of leguminous crop that is harvested solely for the dry seed" (e.g., dried beans, lentils and dry peas) (FAO, 1994). When compared to cereal grains, pulses have higher protein content (17 to 30% or approximately twice the protein content found in most cereal grains), higher rates of amylose (5-10% more than cereals) and higher levels of antinutrients (Mudryj et al., 2014; Parca et al., 2018; Thorne et al., 1983). In contrast, cereal grains have a higher concentration of methionine and tryptophan, while pulses are rich in lysine (Dahl et al., 2012).

Despite high carbohydrate content (50 to 65% of the seed), pulses have low energy density. Furthermore, pulses are rich in fiber and slowly digestible carbohydrates, which places them low on the glycemic index food scale in humans. The slow post prandial response observed after consumptions of pulses also correlate to its physical structure. Pulses contain type C starch, which means that the starch in the middle of the seed is surrounded by proteins that protect against amylase digestion (Bach Knudsen, 2013; Dahl et al., 2012; Thorne et al., 1983). Pulses also provide other important nutrients such as selenium, thiamin, niacin, folate, riboflavin and pyridoxine. Although pulses may also contain a high concentration of calcium, zinc and iron, the bioavailability of these minerals is compromised by the presence of antinutrients, especially phytate (Thompson, 1993; Parca et al., 2018; Thorne et al., 1983).

Peas, the naturally dried seeds of *Pisum sativum L.*, are classified as pulses and are widely produced in Canada (Dahl et al., 2012). On average, approximately 46% of the pea seed

is composed of starch, while fiber comprises at least 20% of peas. Like other starch obtained from pulses, pea starch has high amylose, protein and antinutrient content as well as being considered a slowly digestible starch in humans (Dahl et al., 2012; Yao et al., 2010). Consumption of peas is associated with many health benefits in humans and animals (Mudryj et al., 2014).

The Canadian Cancer Society and the United States Food and Drug Administration recommend regular consumption of peas and other pulses to reduce the risk of cancer in humans. High fiber consumption reduces the risk of colon cancer in humans (Mudryj et al., 2014; O'Keefe, 2008; Ou et al., 2013). Dietary zinc and selenium protect cells against oxidative stress. Moreover, pea compounds such as saponins, protease inhibitors, phytic acid and tannins, all considered antinutritional factors in production animals, may instead provide benefit through anticarcinogenic and antioxidant properties for species where healthy longevity is more of a concern (Bultman, 2017; Dahl et al., 2012; Thompson, 1993).

Because peas contain fiber, resistant starch and prebiotic oligosaccharides, regular consumption of peas might also improve intestinal health (Dahl et al., 2012; O'Keefe, 2008). Furthermore, peas are rich in lysine, which makes pea proteins prone to spontaneous glycation during storage and cooking. Glycated food proteins that escape enzymatic digestion in the upper gastrointestinal tract reach the colon and are metabolized by intestinal microbes, generating SCFA and other metabolites that lead to a shift in the microbial composition and enhanced intestinal health (Topping and Clifton, 2001). A study using human gastrointestinal tract simulators investigated the effects of glycosylated pea proteins on the intestinal microbiome. The results show an increase in intestinal Bacteroides, Lactobacillus and Bifidobacterium, as well as an increase in the production of SCFA and other beneficial bacterial metabolites (Dominika et al., 2011).

Peas contain plant sterols, as well as mono- and polyunsaturated fat, all of which increase blood levels of high-density lipoprotein while reducing low-density lipoprotein and total blood cholesterol in humans (Abeysekara et al., 2012). Isoflavones present in pulses have antihypertensive and anti-atherosclerotic activity in humans and rodent models of human disease. Also, high consumption of fiber lowers blood pressure and reduces inflammation in humans and models of human disease. Taken together, these findings justify the inverse

correlation between regular consumption of peas and the risk of developing cardiovascular diseases (Dahl et al., 2012; Kerver et al., 2003)

The high fiber content associated to the high levels of resistant starch found in peas improves glucose tolerance and insulin sensitivity, thereby reducing the risk of type 2 diabetes in rodent models of human disease (Dahl et al., 2012; Jenkins et al., 1981, 1986; Mudryj et al., 2014; Rizkalla et al., 2002). Due to the low glycemic index, peas are also recommended to prevent human obesity. However, few studies directly evaluating the effects of pea consumption on weight management are available in either humans or animals (Dahl et al., 2012; Siegel et al., 2011).

Although antinutritional factors impair protein digestion and mineral absorption, some antinutrients found in peas also show potential health benefits (Thompson, 1993). Peas contain enzyme inhibitors (trypsin and chymotrypsin), oxalates, phytates, oligosaccharides, phenolic compounds, tannins, and lectins (Çabuk et al., 2018; Champ, 2002). Enzyme inhibitors, phytic acid, lectins and phenolic compounds, have been shown to reduce postprandial glycemic responses to starchy foods as well as lowering blood concentrations of cholesterol and triglycerides in humans and rodent models of human disease (Campos-Vega et al., 2010; Thompson, 1993; Millar et al., 2019). Also, evidence supports the hypothesis that phytic acid and phenolic compounds reduce the risk of developing cancer (Campos-Vega et al., 2010; Parca et al., 2018), while oligosaccharides have prebiotic effects (Dahl et al., 2012; O'Keefe, 2008). However, once again, these health benefits are all either in humans or rodent models of human disease. Little is known about whether comparable benefits exist in dogs or cats.

1.2.1 The Use of Peas in Pet Food Formulation

The pet food industry follows human food trends. Over many years, the pet food industry has introduced ingredients that were initially implicated in human health to promote novel dog and cat food formulations (de Godoy et al., 2013). For example, the so-called "grain-free movement" became very popular, mirroring the gluten-free movement in human food. Once grains were removed from pet foods, an alternative ingredient that was increasingly used in new pet food formulas were all pulses, but most commonly peas (Skerrit Jen, 2018). Few

studies evaluating the health benefits of peas in dog and cat nutrition are available. One previous study from our laboratory showed that dog postprandial blood glucose response after eating pea flour was lower than after consuming barley or rice flours. However, no statistically significant difference was observed after the ingredients were processed and included in a whole diet (Adolphe et al., 2015). Another study evaluated the long-term effects of feeding a diet formulated with 30% rice or pea flour in obese dogs. Although no difference was observed in body weight, fat distribution, adipokines and cardiovascular variables, the dogs that received the pea-based diet showed lower postprandial insulin response after a glucose challenge, which supports the hypothesis that pea-based diets benefit dog metabolic status (Adolphe et al., 2014).

Another previous study from our laboratory (unpublished) comparing dog and cat postprandial glycemic response to a variety of starches concluded that pulse starches lead to low post prandial glycemic and insulinemic response on both species (Briens, 2018). The same authors evaluated the chronic effects of different diets on the cardiovascular and metabolic health of dogs and cats. When compared to a diet formulated with 30% modified corn starch, dogs fed pulse-containing diets showed increased insulin sensitivity and improved weight control. In cats, insulin sensitivity decreased after being fed diets containing lentils and modified corn starch but was not affected by diets containing peas and faba beans, which suggests that some pulses may be healthier carbohydrate sources in cat food formulation than others (Briens, 2018). However, studies evaluating the effects of chronic consumption of pea-based diets are made difficult in cats by the highly astringent, bitter taste (Bradshaw et al., 1996; Briens, 2018). One factor that likely explains cat refusal to eat peas is the high content of antinutrients. A study reveláled that human consumers refuse to eat peas due to the bitter taste created by the same antinutrients (Drewnowski and Gomez-Carneros, 2000). Although some differences exist, dogs, cats, and humans share the receptors and genes required for bitter taste perception and therefore it would be reasonable to assume that peas bitter taste is perceived similarly by all the three species (Behrens and Meyerhof, 2006; Lei et al., 2015; Li and Zhang, 2014). However, the refusal to eat pea-based diets was observed in cats, not beagle dogs (Briens, 2018), which may be related to the fact that dogs are less selective eaters than cats (Bradshaw, 2006; Bradshaw et al., 1996). Another possible explanation relates to differences in amino acid preference between dogs and cats. Both species show a preference

for "sweet" amino acids (L-proline, L-cysteine, L-ornithine, L-lysine, L-histidine and L-alanine), but cats tend to refuse the taste of "bitter" amino acids with hydrophobic side-chains (L-tryptophan, L-isoleucine, L-leucine, L-arginine and L-phenylalanine). In dogs, these same amino acids that are inhibitory in cats are considered either neutral or stimulatory (Bradshaw, 1991). Although the amino acid content can vary, peas usually contain high concentrations of lysine, leucine, arginine, phenylalanine, aspartic acid and glutamic acid (Boye et al., 2010; Casey and Short, 1981; Iqbal et al., 2006) and some of these amino acids (leucine, arginine and phenylalanine) may inhibit cats appetite, but at the same time would not compromise food intake in dogs.

1.3   Pet Food Palatability

In 2018 the global pet food market was worth $98.3 billion USD. Further projections show that by 2024, it will reach a value of $128.4 billion USD (Research and Market, 2019). This increase in the pet food market value might be explained by the growing number of pet owners, but it also correlates to a new and high profitable niche of consumers demanding high-quality pet food (Surie, 2014).

Originally, dogs and cats were domesticated to play a functional role (e.g., hunting, herding, control rodent population), in exchange for shelter and food. However, in the past few decades, the owner-pet relationship has evolved to a different type of mutually beneficial relationship where pets offer emotional support and are considered as family members by their owners, often referred to as "pet parents" (Petfood Industry, 2016). The "pet parenting" trend is shaping the pet food market and manufacturers are now interested in releasing new products formulated not just to meet the minimum dog and cat nutrient requirements, but also to provide health benefits that might increase pet longevity (Di Cerbo et al., 2017; Petfood Industry, 2016; Surie, 2014). A previous study showed that over the past 15 years, the premium pet food market increased 170% in comparison to low and medium price pet food in the USA (Surie, 2014).

Pet owners consider the quality of ingredients and diet formulation the two most important factors determining pet food purchase. However, re-purchasing of a diet is mainly based on the pet owner's perception of the diet (Petfood Industry, 2016; Surie, 2014). Most pet

owners perceive food quality through the animal's feeding behavior and consider that meal time must be a pleasant experience for their pets. Research also shows that pet parents have a preference for highly palatable pet foods and that palatability is perceived as how promptly the animal accepts the diet (Aldrich and Koppel, 2015; Becques and Niceron, 2014; Tobie et al., 2015).

Palatability is defined as "the physical and chemical properties of the diet which are associated with promoting or suppressing feeding behavior during the pre-absorptive or immediate post-absorptive period" (National Research Council, 2006). Due to the economic importance of the topic, the pet food industry considers palatability a crucial feature in the development and refinement of pet diets (Aldrich and Koppel, 2015). Furthermore, a recent study shows that 72% of dog and 67% of cat owners consider that high-quality diets are crucial to maintaining health status. Moreover, over 75% of pet owners believe that special food formulations benefit dogs and cats with a particular health condition (Nutraceuticals World, 2019). The expanding demand for high quality and healthy food options has increased the pet food industry's interest in new ingredients that can both promote health and add flavour to pet food formulation (Petfood Industry, 2018). This thesis will examine whether fermentation of peas with *C. utilis* is capable of both of these improvements.

1.3.1   Methods to Assess Palatability in Dogs and Cats

Palatability may be determined through consumption and non-consumption tests. Non-consumption tests rely on physiological responses to a meal (e.g., Pavlovian response in dogs) or on instruments used to measure food preference using a conditioning task. In consumption tests, palatability is determined by the difference between the amount of food offered and the amount of food consumed (Aldrich and Koppel, 2015). Non-consumption palatability tests are designed to avoid allowing the dogs and cats to eat the test food, although consumption of a minimal amount of food might be allowed in some cases. Conversely, animals used in palatability consumption tests might be offered a variety of test foods, sometimes in amounts that exceed their daily energy requirements (Aldrich and Koppel, 2015; Tobie et al., 2015).

Both approaches have advantages and disadvantages. Palatability test results obtained from non-consumption tests are less affected by satiation or previous food experiences than results obtained from consumption tests. Moreover, different from consumption tests, non-consumption tests do not require overfeeding the test subjects, and therefore the animals performing the test are less prone to develop obesity and other correlated conditions (Tobie et al., 2015). However, to be able to perform the tests and show food preference, the animals used in non-consumption tests need intensive training, which increases both the costs of the test and the preparation time of the test subjects. Furthermore, the peculiar behavior of cats increases the challenges of animal training, and therefore, palatability non-consumption tests might not suitable for cats (Becques and Niceron, 2014; Tobie et al., 2015).

Regarding the animals enrolled in palatability testing, palatability tests may be classified as performed by expert panels or by in-home panels. Expert panels are composed of animals housed in a research facility or pet centers, while in-home panels are composed of owner housed pets (Aldrich and Koppel, 2015). Research indicates that kennel dogs are more susceptible to neophobia and/or neophilia than owner housed dogs. However, due to the lack of environmental control and potential bias introduced by pet owners, expert panels are considered more reliable than in-home panels. To reduce the impact of neophilia and neophobia on the results, tests using expert panels should be repeated for at least 5 consecutive days (Griffin et al., 1984).

Although in-home panels are not the best choice to assess intrinsic palatability, it might be a useful tool to evaluate the pet owner's perception of pet diets. A new technique developed to link the owner's perception of a pet diet showed similar results to a traditional palatability consumption test, which reinforces the idea that pet owners are a reliable source of information to evaluate pet food palatability (Becques et al., 2014a).

A variety of no consummatory tests are available to assess food preference in dogs. Most techniques involve training dogs to determine food preference based on animal behavior. In some studies, the dogs are allowed to see, smell, or even consume a restricted amount of food. Rashotte and Smith developed a methodology where dogs were trained to show preference through the use of an apparatus that, once activated, allows access to the test food (Rashotte and Smith, 1984). In 2016, Thompson et al. compared the time that dogs spend

investigating foods to the rate of food extracted from puzzle feeders. The authors found that the time spent investigating the diet was proportional to the rate of diet extraction from the puzzle feeders. In other words, the food that had a greater extraction rate was the same that the dogs spent more time sniffing in the first part of the trial (Thompson et al., 2016). A similar approach was used in a study using rubber toys designed to hide food and treats. The dogs were expected to extract the food from the toys, and the order of extraction was used to rank the foods to determine food preference (Li et al., 2018). All these studies support the idea that trained dogs can express food preference and that nonconsumption approaches are reliable to determine pet food palatability (Basque et al., 2019; Thompson et al., 2016). However, because specific training is needed, that takes a long while to validate and because these tests are not reliable in cats, this thesis will use consumption tests instead.

Palatability consumption tests using expert panels is the methodology commonly used by the pet food industry. Two types of consumption tests are currently available: one-bowl and two-bowl tests (Aldrich and Koppel, 2015). In the one-bowl test, the animals are offered one diet only at a time, and food acceptability is measured by the amount of food consumed by subtracting the amount of food offered from the amount of food consumed by the animals. A diet is considered acceptable when the number of calories eaten is sufficient to maintain the animal weight and support animal performance (Tobie et al., 2015). Although this methodology reproduces the way dogs are generally fed at home, it does not provide information about food preference. In the two-bowl test, the animals are presented two diets simultaneously, and preference is determined by the difference in food consumption between diets (Aldrich and Koppel, 2015). The fact that the animals prefer one of the test diets over another infers that the "winner diet" has a better flavour than the "defeated diet." However, the results of a two-bowl test are applicable only to the particular diets being compared, as the animal choice may change their choice if a different diet competes against the "winner" (Aldrich and Koppel, 2015; Griffin et al., 1984; Tobie et al., 2015).

The time allowed to complete palatability consumption tests may vary significantly depending on the species of interest. Usually, the food is made available for a limited period for dogs (15-30 minutes), while cats may be allowed a very long period to complete the test. The difference in the time allowed to complete consumption palatability tests is justified by

species differences in feeding behavior (Bradshaw, 2006; Zaghini and Biagi, 2005). Before domestication, dogs used to be collective hunters that would share the carcass. Thus, dogs have evolved to eat large amounts of food during a short period because this had evolutionary advantages. Conversely, cats have evolved as solitary hunters that use their olfactory senses to select the food carefully, avoiding anything that seems dangerous or unfamiliar. As a result, evolutionary pressure selected fast eater dogs and selective/slower eater cats (Becques et al., 2014b; Bradshaw, 2006).

The animals participating in palatability trials must be carefully selected and screened for diseases. Because all health conditions interfere in appetite, animal health status needs to be assured by performing a physical exam and complete blood work. Aggressive animals should be excluded, and all the animals should be castrated/spayed to avoid interferences of hormones on food intake (Aldrich and Koppel, 2015). Moreover, an obvious choice test should be previously conducted to certify the animal's ability to make choices. In an obvious choice test, known tasteful food is compared to a less palatable food option. All the animals included in palatability trials should consistently choose the most palatable diet (Tobie et al., 2015). Additional experimental bias is reduced by the use of clean bowls made of a non-porous material and by randomization of the position of the bowls in the two-bowl test. Furthermore, the test should be repeated at the same time of the day, preferentially by the same individual (Aldrich and Koppel, 2015; Tobie et al., 2015).

A study combining the two-bowl consumption test and an olfactory discrimination task using a dual-port- olfactometer compared the food preference results of a two-bowl test using a taster dog panel to the results of a two-bowl test conducted with an olfactory expert panel of dogs. After two diets with equal preference were selected by the taster dog panel, the selected diets were offered to the olfactory expert dog panel. The taster dog panel and the olfactory expert dog panel showed similar results in the two-bowl test regarding the first choice of the diet and food intake ratio, which reinforces the credibility of the two-bowl test as a method to determine food preference in dogs (Basque et al., 2019).

As mentioned before, cats are difficult to be trained, and very few studies exploring palatability assessment through cats behavior are available (Tobie et al., 2015). Researchers found a relationship between cat body language and food intake ratio, indicating that some

postures and behavior are associated with pleasant or unpleasant food flavours. For instance, "licking lips" is associated with high palatable foods while "flicking the tail," "grooming the body," and "flicking ears backward" are associated with food rejection (Becques et al., 2014b; Savolainen et al., 2016). This thesis will attempt to use the amount of food consumed in one-bowl and two-bowl tests in cats to assess palatability.

1.4   Physiology of Taste

The animal gustatory system relies on an intricate series of events and physiological mechanisms that result in taste receptor activation in the gastrointestinal system and taste perception in the brain (Simon et al., 2016; Uneyama and Takeuchi, 2010). The gustatory system of mammals evolved to help in food quality evaluation and to prevent the ingestion of potentially harmful compounds (Rozengurt, 2006) . Evidence also suggests that the gustatory system plays a role in feeding behavior and stimulates the choice of nutritious food options. (Ekstrand et al., 2017 ; Simon et al., 2016; Firestein et al., 2012). The gustatory system recognizes five basic tastes: sweet, umami, salt, sour and bitter. Usually, carbohydrates generate the sweet taste, while umami taste is elicited by amino acids (especially glutamate and ribonucleotides; Conigrave and Brown, 2006; San Gabriel and Uneyama, 2013; Zhang et al., 2003). Sodium ions, essential for electrolyte homeostasis, produce the salt taste. Unpleasant sour and bitter tastes are linked to harmful substances and are usually elicited by spoiled food or toxic plants, respectively (Behrens and Meyerhof, 2011; Uneyama and Takeuchi, 2010).

Taste buds can be found in the tongue, soft palate epiglottis and esophagus and are able to detect the five basic tastes. Taste buds are composed of a group of different taste receptor cells (TRCs) (Uneyama and Takeuchi, 2010). In the oral cavity, taste receptor cells have a small opening (taste pore) on their apical side. The taste pore microvilli have receptors that can sense the presence of tastants. The basolateral side of the cells is protected by tight junctions, and small groups of TRCs communicate through gap junctions, which allows the taste sensing to be transmitted to adjacent groups of TRCs (Simon et al., 2016). The facial nerve is responsible for the innervation of TRCs in the tongue and palate. TRCs present in the epiglottis and esophagus are innervated by the vagus nerve. Both the facial and the vagus nerve respond

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to tastants and contribute to brainstem-based arch reflexes involved in the ingestion and rejection of foods (Adler et al., 2000; Simon et al., 2016; Stuart J Firestein et al., 1999).

The different cells present in the taste buds are classified according to their function and morphology. Type I and type IV have supporting functions. The undifferentiated type I cell, found in the basal part of the taste buds, is also responsible for the replacement of old cells. Synapses allow communication between Type III cells and afferent nerve fibers. Type II cells can be subclassified into different subtypes. Either, sweet, umami or bitter taste are detected by a particular type II cell subtype, which is determined by the taste receptor gene that is expressed on the cell (Adler et al., 2000; Behrens and Meyerhof, 2011; Iwatsuki and Uneyama, 2012).

A superfamily of proteins known as G-protein coupled receptors is recognized as the receptors for sweet, umami, and bitter taste. Two families of genes are responsible for the coding of G protein-couple receptors: the Tas1r gene family (Type 1 taste receptor-T1Rs) and the Tas2r gene family (Type 2 taste receptor- T2Rs). Type 1 taste receptor is subdivided into TAS1R1, TAS1R2, and TAS1R3 (Behrens and Meyerhof, 2011; Simon et al., 2016; Zhang et al., 2003).

The interaction between two different protein subunits creates a homodimeric (formed by the same two subunits) or heterodimeric (contain two different subunits) functional protein. Both sweet and umami tastants are perceived by heterodimeric G-protein coupled receptors; sweet tastants are detected by T1R2/ T1R3, and umami taste is detected by T1R1/T1R3. The transduction of bitter tastants requires homodimeric T2Rs (Behrens and Meyerhof, 2011; Zhang et al., 2003). A protein called gustducin is expressed with T2Rs in TRCs, which suggests that gustducin is also necessary for bitter taste transduction (Adler et al., 2000).

The mechanisms for sour and salty tastant transduction are still under investigation, and the exact cell type involved in the pathway of salt tastant transduction has not yet been elucidated. However, it has been established that salt taste is mediated by the epithelial sodium channel superfamilies (Simon et al., 2016; Uneyama and Takeuchi, 2010). A diverse group of cation channels called the transient receptor potential (TRP) superfamily seems to be involved in the pathway for sour taste transduction. A recent study concluded that the lack of one of the members of the TRP superfamily (the like polycystic kidney disease ion channel PKD2L1)

make animals totally unresponsive to sour-tasting stimuli, which suggests that PKD2L1 ion-channel is responsible for sour taste transduction (Montell, 2005; Rozengurt, 2006; Simon et al., 2016).

New evidence suggests that TRCs also express a fatty acid receptor/ transporter, which raises the question of whether fatty should be considered the sixth taste. Two receptor candidates have been proposed: CD36 and G protein-coupled receptor 120. A study using CD36 knocked out mice concluded that in the absence of this protein, mice lose their preference for long-chain fatty acids, which supports the involvement of CD36 in fatty acids transduction mechanisms. However, more studies are needed to elucidate the role of TRCs in fat perception (Keast and Costanzo, 2015; Simon et al., 2016).

1.4.1 Differences in Taste Perception of Dogs and Cats

Although bitter, sweet, umami, salt and sour taste are equally important to all species, studies comparing the gustatory system of different species found some particularities that might be explained by differences in dietary habits (Behrens and Meyerhof, 2011; Li and Zhang, 2014). In comparison to the number of sweet and umami receptors, all species have a higher number of bitter taste receptors. It is hypothesized that due to the higher possibility of ingestion of toxic plant compounds, herbivorous species evolved to possess more bitter taste receptors than carnivorous species (Li and Zhang, 2014). Indeed, compared to the number of bitter functional receptors found in other mammals, the numbers of bitter receptors found in dogs and cats are considered low (Chandrashekar et al., 2006; Lei et al., 2015). Dogs have only 12 functional bitter receptors, while rats have 37. The same is valid for the number of genes involved in bitter taste perception; while most mammals have 34-42 Tas2r genes and pseudogenes, dogs have just 21 (Go, 2006; Ling-Ling HU and Peng SHI, 2013).

If we consider that bitter receptors are not essential for carnivorous species and that diet acts as an evolutionary factor determining bitter taste number and function, it would be reasonable to speculate that the numbers and functionality of bitter receptors in strict carnivores species, such as the cat, would be compromised. However, research expressing cat TAS2R receptors in cell-based assays found a reasonable number of functional bitter taste receptors

in this species (Lei et al., 2015). According to this study, cats have a minimum of seven bitter functional receptors, a number that is similar to the numbers of intact bitter receptors found in other members of the order Carnivora. The results found in this study do not support the hypothesis that diet has an evolutionary impact on the number or functionality of bitter receptors of the species tested (Lei et al., 2015). One of the possible explanations for the expression of bitter taste in strictly carnivores is that bitter tastes are not exclusively located in the gastrointestinal tract and are important in defence mechanisms outside the gut. For instance, there is evidence that bitter receptors located in the respiratory tract of rats participate in innate protection against bacterial infections. Another reasonable explanation is that prey meat may also contain bitter and harmful components that need to be avoided (e.g., bile acids) (Lei et al., 2015; Tizzano et al., 2010).

The numbers of sweet and umami taste receptors of vertebrates are relatively constant. T1R1, T1R2, and T1R3 are the only three receptors linked to sweet and umami taste perception in mammals, except for the cat (Chandrashekar et al., 2006; Masatoshi Nei et al., 2009). When cat taste nerve fibers were evaluated to determine the cats response to salty, sour, bitter umami, and sweet tastants, no neural response to sugars were detected (Boudreau and Alev, 1973). Further research explained the reason behind the cat's inability to taste sugars. Both genes involved in sweet taste perception in mammals (T1R2/ Tas1r2 and T1R3/ Tas1r3) were screened in the cat. Tas1r2 and Tas1r3 were analyzed to determine gene sequence, functionality, and expression in taste buds. Tas1r3 sequencing revealed similarities to functional Tas1r3 genes of other species. The gene is also expressed in taste buds and elicits a response in the taste tissue of cats, as observed in other species that are able to taste sweets. Nevertheless, Tas1r2 sequencing revealed a disruption in its coding sequence as well as a lack of expression of the gene in taste buds. The authors concluded then that the Tas1r2 gene of cats is an unexpressed pseudogene and, as both taste receptors (T1R2 and T1R3) are required to build the functional heterodimeric G-protein-coupled receptor necessary for perception of sweet taste, cats are unable to taste sweets (Xia L et al., 2005). Comparison between the Tas1r3 and Tas1r2 of dogs and cats revealed that dogs have intact and functional Tas1r3 and Tas1r2 genes, which supports the preference for sweet tastants observed in dogs (Thombre, 2004; Xia Li et al., 2006).

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As observed in humans, dogs and cats are strongly attracted by umami flavours (Kenzo Kurihara and Makoto Kashiwayanag, 2000). T1R1 and T1R3 are the two most important taste receptors involved in umami taste perception. However, the taste-metabotropic glutamate 4 (mGluR4) and one more mGlu receptor that acts in the brain (mGluR1) also play a role in the umami sense of taste, which is described as a meat-like or savoury taste (Zhang et al., 2017). Umami taste is enhanced by the addition of salts in both humans and dogs. Sodium (especially NaCl), potassium, and calcium salts seem to improve the umami response to most amino acids in both species (Kumazawa, 1990; Kumazawa et al., 1991). In spite of the finding that umami taste is improved in dogs by the addition of salts, appetite for salts has not been demonstrated in a carnivore. A study shows that dogs have a little appetite for salts under unique experimental conditions (Fitzsimons and Moore- Gillon, 1980; Shiguang Yu et al., 1997). Another study found that adult cats do not show a preference for salt solution or water. However, a slight preference was found when salt concentration was equal to 0.1 M (Thombre, 2004). Yet, a study shows that kittens do not have an appetite for sodium even when sodium is depleted from the diet (Shiguang Yu et al., 1997).

The recent discovery that the polycystic- kidney-disease-like ion channel is involved in sour taste transduction may elucidate the mechanisms behind sour taste perception and allow comparison between species (Huang et al., 2006). To date, the literature lacks information on dog and cat sour taste perception.

1.5   The Effects of Yeast Fermentation on Peas Taste and Glycemic Index

Pulses are a valuable source of nutrients in many cultures and different processing techniques have been used to enhance the taste and sensory characteristics of pulse based foods. These techniques are more commonly baking, cooking and soaking, but also includes fermentation. Besides increasing shelf-life and improving flavour, pulse fermentation adds functional properties to the final product (Adebo et al., 2017; Frias et al., 2017). Bacteria (Bacillus), fungus and yeasts are used to ferment pulses. These microorganisms convert the substrate into new compounds leading to a reduction in pH as well as changes in carbohydrates, proteins, lipids, and antinutritional factors present in pulses. Depending on the microorganism

and substrate used, fermentation may also result in secondary compounds (e.g., alcohols, ketones, organic acids, aldehydes, yeast extract) that creates a fermented pulse product with unique aroma and flavour (Adebo et al., 2017; Reddy and Pierson, 1994).

Enzyme inhibitors, oxalates, phytates, oligosaccharides, phenolic compounds, and lectins are the major class of antinutrients found in peas (Çabuk et al., 2018; Champ, 2002). In addition to the production of substances that adds aroma and flavour to the food, fermentation may also reduce the concentration of some antinutrients found in peas. (Çabuk et al., 2018; Dvořák et al., 2011; Khattab and Arntfield, 2009). These antinutrients are responsible for the bitter taste of peas and may also reduce nutrient bioavailability by reducing protein digestion and mineral absorption. On the other hand, some of these antinutrients also provide a variety of health benefits, including lower postprandial glycemic response (Thompson, 1993; Parca et al., 2018). Despite being fermented, pulse-based foods still possess significant levels of phenolic compounds, lectins, polysaccharides and phytates when compared to other conventional sources of carbohydrates (Adebo et al., 2017). Therefore, yeast fermentation has the potential to enhance the taste and nutrient bioavailability of peas without totally erasing potential health benefits related to antinutrient consumption (Reddy and Pierson, 1994).

Yeasts are also used in pet food to increase food palatability. Enzymatic extraction of yeast intracellular components generates yeast extract, a fine power with meaty flavour rich in proteins, peptides, nucleotides, and amino acids (Swanson and Fahey, 2003; Oliveira et al., 2016; Shurson, 2018). Studies show an increase in food intake of dogs and cats fed diets with the inclusion of yeast extract at different rates (Swanson and Fahey, 2003; Oliveira et al., 2016). However, some studies report a reduction in food intake in cats when yeast extract was added at inclusion rates higher than 2% (Aquino et al., 2010; M. Lima et al., 2015; Ogoshi et al., 2014). All these studies were conducted using *Saccharomyces cerevisiae* in the form of yeast extract or yeast cell wall. To our best knowledge, no information is available about the use of *Candida utilis* as a food enhancer for pet food. Moreover, no study has been conducted using this yeast to ferment pet feed ingredients.

Candida utilis has the advantage of not producing alcohol (Kieliszek et al., 2017). Since ethanol can be a feed detractant with dogs and cats, the use of *C. utilis* should enable yeast fermentation as a processing technique to be used for starchy ingredients to be included in pet

food formulation (Swanson and Fahey, 2003; Postma et al., 1989). Furthermore, *C. utilis* can assimilate carbon and nitrogen sources such as nitrite, nitrate, and urea. These features facilitate the biomass production of *C. utilis* in a variety of substrates (Boze et al., 1992; Buerth et al., 2016) . *C. utilis* biomass is a rich source of amino acids (including lysine), nucleotides, β-glucans, glucomannans, and mannoproteins. In humans, *C. utilis* is used as a food additive or meat substitute in vegetarian and processed foods (Bekatorou et al., 2006). The meaty flavour is attributed to the high concentration of glutamic acid, an amino acid that triggers the umami taste of foods and is considered a potent flavour in humans (Bekatorou et al., 2006; Boze et al., 1992).

Dogs and cats are strongly attracted by umami food ingredients, and it has been proved that they can perceive umami stimuli (Kurihara and Kashiwayanag, 2000; Kumazawa et al., 1991). Therefore, the inclusion of the *C.utilis* and other yeasts that contains a high concentration of glutamic acid should enhance palatability in these species. However, some controversy exists regarding the concentration of glutamic acid that elicits a positive hedonic response in humans, dogs, and cats. Some researches argue that high concentration of monosodium glutamate and other umami substance may negatively affect palatability, which could explain the reduced food intake observed in studies testing diets with high inclusion of yeast extract (M. Lima et al., 2015; Ogoshi et al., 2014; Yamaguchi and Ninomiya, 2000; Zhang et al., 2017).

Studies evaluating the effects of pulse fermentation on the glycemic index are scarce. Considering that antinutritional factors play a role in lowering the postprandial glucose response (Champ, 2002; Rizkalla et al., 2002), fermented foods may have a higher glycemic response than unfermented foods. On the other hand, the yeast uses food carbohydrates to produce secondary metabolites (Adebo et al., 2017; Shurson, 2018), which may lead to a reduction in the glycemic index of fermented foods. The current thesis used *Candida utilis* to ferment pea starch. To the best of our knowledge, no previous study has evaluated the effects of *C.utilis* yeast fermentation on pea starch glycemic index in humans or animals.

1.6   Intestinal Health

Despite being often cited in the scientific literature and widely used by the food industry, the terms "intestinal health" or "gut health" lack a specific definition. The World Health Organization defines "health" as "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity" (Bickenbach, 2015; Bischoff, 2011). Therefore, intestinal heath does not refer only to the absence of intestinal disease but instead might be defined as the state in which the intestinal tract can optimally perform all its physiological functions. Considering that both upper and lower gastrointestinal tract is needed for optimal intestinal performance, the term 'intestinal health' should be used in reference not just to the intestine, but it should also include the health state of the whole gastrointestinal tract (Bischoff, 2011).

The primary function of the intestinal tract is nutrient digestion and absorption. However, the intestine also plays a crucial barrier function protecting against antigens and pathogens that enters the organism towards the ingestion of food (Zhou et al., 2018). In association with an equilibrated intestinal microbiota, the integrity of the intestinal barrier is essential to maintain a healthy gut (Bischoff, 2011). The intestinal barrier was once considered to act simply as a mechanical barrier. However, the intestinal barrier is now known to refer to a complex defence mechanism that includes epithelial cells, the mucosal immune system and the enteric nervous system. Gastrointestinal barrier dysfunction is being implicated in the pathogenesis of many gastrointestinal and extraintestinal diseases. Likewise, most of these diseases show concurrent changes in microbial composition, which implies interlinkage between intestinal microbial composition and barrier function integrity (Bischoff, 2011; Camilleri et al., 2012; Redfern et al., 2017).

In humans, barrier function integrity may be assessed *in vivo* by measuring urinary excretion of ingested test substances (usually mono or disaccharides). This non-invasive technique is used in clinical practice and research to gather information about malnutrition and gastrointestinal disease (Bjarnason et al., 1995). However, integrity testing only considers the mechanical protective role exerted by the intestinal barrier (Bischoff, 2011). Furthermore, the lack of standardization prejudices the comparison among studies (Bjarnason et al., 1995; Peeters, et al., 1994). Similar techniques were also used to determine intestinal permeability in

dogs, and although some researchers report positive results, others found considerable variability among breeds (Garden et al., 1997; Randell et al., 2001).

Host and intestinal microbiota co-evolved to build a complex symbiotic relationship that is crucial to keep a healthy intestinal environment. The inhabiting intestinal microbes compete for niche and nutrients with potential pathogens (Suchodolski, 2011a). In addition, some bacteria may also produce antimicrobial substances to overcome competition (Garcia-Gutierrez et al., 2019). Besides protecting against pathogens, intestinal microbes also generate metabolites that benefit the host. For instance, intestinal bacterial fermentation yields amino acids (arginine, cysteine and glutamine), SCFAs (acetate, propionate and butyrate) and several other substances that not only are an important source of nutrients but are also important for intestinal homeostasis. Furthermore, resident microbes modulate the immune system minimizing unnecessary and exaggerated immune responses that could result in chronic inflammation and food sensitivity (Barko et al., 2018; Cencic and Chingwaru, 2010; Redfern et al., 2017; Tuddenham and Sears, 2015).

Intestinal microbiome is the term used to define all the microorganisms that inhabit the intestinal tract of mammals (bacteria, archaea, fungi, protozoa and viruses) plus their respective genetic material (Barko et al., 2018). Technological advances involving DNA sequencing and computational biology have expanded the knowledge of the composition of the intestinal microbiome as well as its relationship to health and disease (Redfern et al., 2017; Suchodolski, 2011a). Despite all the recent advances, the study of the intestinal microbiome still an area of active research in many species. No specific parameters are available to define what typifies a "healthy" microbiome, but in general, a "healthy" microbial community is formed by a diverse, rich and even microbial community that is resilient to changes in the intestinal microenvironment (Tuddenham Sears, 2015).

Richness and evenness are different approaches used to quantify biological diversity. In microbiome studies, a rich microbial community is composed of a high number of different microbial species, regardless of how many individuals of each species are present. In contrast, evenness considers how uniform the population size is for each species present in the intestinal tract (Ashton et al., 2016; Claesson et al., 2017; Kim et al., 2017). The immunological state of the host, age, genetics and drugs (especially antibiotics) may change the intestinal microbial

composition and have an impact on the biological diversity found in the gut, which consequently impacts intestinal health (Dave et al., 2012; Tuddenham and Sears, 2015) .

The number of microbes in the gastrointestinal tract increases aborally, and the vast number of intestinal microbes are found in the colon (Suchodolski, 2011a). According to a study using 16S rRNA gene clone sequencing, the microbiome present in the colon of healthy dogs is composed primarily of Fusobacteria, Bacteroidetes and Firmicutes (Hooda et al., 2012). Similar studies show that Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria and Actinobacteria are, respectively, the most common bacteria phylum found in feces of cats (Minamoto et al., 2012).

The vast community of bacteria inhabiting the intestinal tract may be differentiated into two major groups according to how they impact the health status of the host. For instance, Bifidobacterium and Lactobacillus are considered beneficial bacterial species, while Enterobacteriaceae and Clostridium spp. are considered to be detrimental to intestinal health (Bischoff, 2011; Redfern et al., 2017). However, the role of some bacteria considered as enteropathogens (Campylobacter jejuni, Clostridium difficile, Clostridium perfringens, and Salmonella) is still under investigation, since the microorganisms can also be found in the gastrointestinal tract of healthy animals. Furthermore, studies show that a reduction in the diversity of Clostridium cluster XIVa and IV is related to inflammatory bowel disease in humans, dogs and cats (Honneffer, 2014).

1.6.1   Relationship between Intestinal Health and Metabolic Status

Differences in gut microbial abundance and composition were reported in several studies comparing the microbiome of lean subjects to their obese counterparts. Obese mice, for instance, show a higher proportion of Firmicutes to Bacteroides when compared to lean mice. Similar changes were found when comparing the intestinal microbiome of obese and lean humans subjects (Clarke et al., 2012; Kallus and Brandt, 2012). It is possible that bacteria that belong to the phylum Firmicutes are more efficient energy extractors than Bacteroides, which may explain the higher proportion of Firmicutes to Bacteroides observed in obese subjects (Kallus and Brandt, 2012). A study comparing the microbiome of obese-prone rats to

obese-resistant rats shows that obese-prone rats have specific microbial groups that are absent in obese-resistant rats. The bacterial groups found in obese-prone rats are clustered within the Firmicutes phylum and generally related to Clostridium Cluster XIVa, Clostridium Cluster IV and Oscillibacter. These findings support the role of the intestinal microbiome in obesity onset. However, more studies are needed to fully understand the link between microbiome composition and metabolic health (de Clercq et al., 2017; Dave et al., 2012; Duca et al., 2014)

Besides harvesting energy from otherwise indigestible compounds, the gut microbiome may also interfere with host metabolism through a bidirectional signalling pathway called the microbiome-gut-brain axis. The microbiome-gut-brain axis pathway plays a role in appetite regulation and also interferes with blood glucose level, adipocyte function and energy expenditure, which further impacts metabolic health (Hussain and Bloom, 2013).

The inhabiting intestinal microbes can metabolize resistant starch and fiber, generating SCFAs. Short-chain fatty acids are used as an energy source by the host and by the intestinal microbiota. They are also important to keep a healthy intestine, not just by keeping a low intestinal pH, but also participating in several metabolic processes. Butyrate, propionate and acetate correspond to the majority of SCFA produced in the gut. Butyrate is essential for growth and differentiation of colonocytes and optimal butyrate concentration has been traditionally associated with a healthy gut. Propionate generated in the colon is transported to the liver, where it can be used to synthesize protein or can be used as a precursor for gluconeogenesis and lipogenesis. Acetate is used to generate cholesterol and other fatty acids (Gagné et al., 2013; Kallus and Brandt, 2012; Saad et al., 2016). Additionally, acetate and butyrate enhance fatty acid oxidation and increase energy expenditure (Topping and Clifton, 2001).

Despite most evidence pointing to increased production of SCFAs as intestinal health is enhanced, studies comparing the SCFA concentration of obese and lean subjects show a higher concentration of SCFA in the intestine of obese individuals. Furthermore, obesity-related intestinal microbes are more efficient carbohydrate fermenters than intestinal microbes that are commonly found in lean subjects, leading to the observed higher production of SCFA (Clarke et al., 2012). The intestine of genetically obese mice and obese humans, for instance, shows an increased concentration of SCFA when compared to their lean counterparts (Kallus

and Brandt, 2012; Murugesan et al., 2018). More studies are needed to understand the paradoxical link between microbiome composition, SCFA production and intestinal health. It has been hypothesized though that the increased concentrations of fatty acids found in obese subjects may result from decreased mucosal absorption and/or reduced rate of transit time in the intestine (Kallus and Brandt, 2012; Kotzampassi et al., 2014; Murugesan et al., 2018).

When compared to humans, fewer studies have investigated the correlation between microbiome composition, intestinal health and metabolic status of dogs and cats. A study evaluating the effects of weight loss on SCFA and microbiome composition in dogs concluded that weight loss reduced the abundance of Megamonas and Rumminococcaceae and, similar to human studies, SCFA production is positively correlated with body weight in dogs (Kieler et al., 2017). Another study showed that Firmicutes was the predominant phylum in the intestine of lean dogs, while Proteobacteria was the predominant phylum found in the intestine of obese dogs. The same study also shows that obese dogs have higher leptin concentrations than lean dogs (Park et al., 2015). Likewise, differences in microbiome composition were found between obese and lean cats. However, the authors did not find a link between obesity and a specific group of bacteria in the cat intestine (Kieler et al., 2017).

1.6.2   Yeast Fermented Foods and Intestinal Health

Besides improving food flavour, fermentation is also known as a processing method that enhances the functional properties of foods. Functional foods are defined as foods that exceed their nutritional role by preventing or treating diseases (Di Cerbo et al., 2017). Yeast fermented foods are considered as functional foods. Yeast produces carbohydrate modifying enzymes that act in carbohydrate rich substrates generating bioactive oligosaccharides (Rai et al., 2019). These bioactive compounds favour the growth of Bifidobacterium, Lactobacillus and other bacteria species that are considered beneficial for intestinal health. Furthermore, the yeast cell wall is rich in fiber and β-glucan, and the consumption of both substances is associated with lower blood cholesterol levels, reduced inflammation and an improved immune system (Redfern et al., 2017).

Advances in the technologies used for DNA sequencing combined with reduced costs made the study of intestinal microbial composition more reliable and accessible. Bacterial phylogeny and taxonomy may be assessed by different methodologies. However, the sequencing of the 16SrRNA bacterial gene is the most common method used to determine microbial profile. The 16SrRNA gene is found in the 30S subunit of prokaryotic ribosomes and contains hyper variable regions that can be target and amplified allowing taxonomic microbial identification in a variety of types of samples (Ashton et al., 2016; Claesson et al., 2017; Janda and Abbott, 2007). Researchers have investigated the intestinal microbiome of healthy dogs and cats and its association with many diseases. However, a limited number of studies evaluated the effects of dietary changes and/or metabolic status on the intestinal microbial composition of dogs and cats (Hooda et al., 2012; Suchodolski, 2011).

In 2010 a study compared the intestinal microbiome of dogs fed either, a control diet or a diet supplemented with fiber (beet pulp). In this study, the intestinal bacterial composition was determined using 454 pyrosequencing of the V3 hypervariable region of the microbial 16SrRNA gene. Fusobacteria, Firmicutes and Bacteroidetes were the phyla most commonly found in dog intestine. Dogs fed the diet supplemented with fiber showed increased concentration of Firmicutes and decreased concentration of Fusobacteria (Middelbos et al., 2010). Another study used 454 pyrosequencing to compare the microbiome of dogs fed raw diets before and after inclusion of yeast cell wall extract or inulin. Although yeast cell wall extract increased Bifidobacterium and inulin increased Lactobacillus, the authors concluded that no strong prebiotic effect was observed with the inclusion of yeast cell wall extract or inulin (Beloshapka et al., 2013).

A study compared the intestinal microbiome of dogs before and after weight loss. By using a MiSeq based tag encoded 16SRNA gene high-throughput amplicon sequencing (V3-V4 region), the authors concluded that intestinal concentration of Megamonas and Rumminococcaceae decreased with weight loss. Furthermore, this study showed an inverse correlation between intestinal SCFA concentration and dog body weight (Kieler et al., 2017). A study comparing the microbiome of obese and lean dogs shows reduced bacterial biodiversity in obese dogs. In this study, the phylum Proteobacteria was the predominant bacteria found in the intestine of obese dogs, while more than 85% of the intestinal microbiome

of lean dogs was comprised of bacteria belonging to the phylum Firmicutes. This study also investigated blood adipokines concentration in both lean and obese dogs and the authors concluded that leptin increases, while adiponectin decreases with body weight (Park et al., 2015).

Fewer studies are available for cats. A previous study shows that inclusion of yeast cell wall extract in the diet of cats reduced the concentration of potentially pathogenic bacteria (Clostridium perfringes and Escherichia coli), while the concentration of bacteria often associated with health in the gut (Bididobacterium spp. and Lactobacillus spp) increased (Santos et al., 2018). Studies conducted with humans and mice show that obesity leads to an increase in Firmicutes to Bacteroidetes ratio (Clarke et al., 2012; Kallus and Brandt, 2012). However, a study comparing the intestinal bacterial profile of lean and obese neutered cats concluded that lean neutered cats have higher Firmicutes to Bacteroidetes ratio than obese neutered cats (Fischer et al., 2017) Another study investigating the effects of dietary fiber supplementation on cat microbiome did not find significant differences between the control and test groups. The authors concluded that high individual variation prejudices the investigation of changes in the intestinal microbial composition of cats (Barry et al., 2012).

In conclusion, few studies are available, and more research is needed to understand how prebiotic supplementation and dietary changes influence dog and cat intestinal microbial profile and metabolic status. This thesis will evaluate the influence of diets formulated with different carbohydrate sources (corn starch, pea starch and yeast-fermented pea starch) on intestinal microbial composition and intestinal health of dogs and cats.

1.7   Obesity, Inflammation and Adipokines

Obesity is characterized by chronic low-grade inflammation and is considered the most common nutritional disease affecting more than half of the population of pets worldwide (German, 2006; Tvarijonaviciute et al., 2012a). The adipose tissue produces hormones and factors that are collectively known as adipokines. Adiponectin and leptin are the adipokines most well studied in dogs and cats. Both adipokines are important in glucose metabolism, energy balance, inflammatory processes and immune function (Zoran, 2010). In humans, leptin

levels are known to positively correlate with body weight, fat mass, glucose and triglycerides levels. Conversely, adiponectin decreases with obesity in humans, which might seem paradoxical as both adipokines are produced by the adipose tissue. In addition, despite being found in higher concentrations in obese patients, the primary leptin role is inhibition of appetite in healthy humans, but this suppression of appetite becomes blunted in obesity (Park et al., 2015; Torres et al., 2019; Verkest et al., 2011).

These controversies are explained by looking at the effects that obesity has on other organs, which further impacts adipokine production and functions. In normal physiological conditions, leptin potentiates anorexigenic peptides and inhibits orexigenic neuropeptides. As body fat mass increases, leptin production also increases. However, the brain of obese patients become unresponsive to the anorexic effects of leptin. Furthermore, leptin has proinflammatory effects, which contribute to the low grade inflammation associated with obesity (Münzberg and Heymsfield, 2015; Torres et al., 2019). Unlike leptin, adiponectin production is impaired in obese patients, proposed to occur due to dysfunctional adipocytes that cannot produce adiponectin in obesity. Besides anti-inflammatory properties, adiponectin has also been shown to enhance glucose metabolism and fatty acid oxidation in humans or animal models of human disease. Therefore, the low levels of adiponectin observed in obese patients further compromise their metabolic status (Dutheil et al., 2018; Ishioka et al., 2009; Torres et al., 2019).

Less is known in dogs and cats. Most researches agree that similar to humans; obesity leads to an increase in leptin and a decrease in adiponectin levels in dogs and cats (Hoenig et al., 2007; Ishioka et al., 2006; Ishioka et al., 2009; Park et al., 2015; Piantedosi et al., 2016). However, some studies report similar adiponectin levels regardless of dog and cat metabolic status (Coradini et al., 2013; Verkest et al., 2011; Wakshlag et al., 2011). Combined with changes in blood concentration of adipokines, researchers also report that obese dogs and cats tend to show abnormalities in blood biochemistry profile that are similar to what is observed in obese humans. These abnormalities may include increased levels of total cholesterol, triglycerides, lactate dehydrogenase, total serum proteins, α-globulins, total bilirubin, alkaline phosphate and alanine aminotransferase (Forster et al., 2018; Piantedosi et al., 2016). This thesis aims to investigate the effects of dietary changes on the metabolic status of dogs and

cats and to determine if there is a correlation between metabolic status, diet and intestinal health.

CHAPTER 2

INTRODUCTION

2.1 Rationale

To address the growing demand for higher quality food, the pet food industry is investing in new ingredients and formulations that can provide health benefits and potentially increase pet longevity. Among these ingredients are peas, a low glycemic index ingredient that has become popular over the past few years in large part due to the popularity of grain free diets. One of the challenges of using peas as an ingredient in pet food formulation is the high levels of antinutritional factors that give peas a bitter taste. Yeast fermentation is a processing technique used to improve food flavour and texture. Moreover, due to the positive effects on intestinal health and metabolic status, fermented foods are classified as functional foods, a concept that is becoming a trend in the pet food market.

Saccharomyces cerevisiae yeast extract is currently being used in the pet food industry to improve food flavour. However, because *S. cerevisiae* fermentation yields alcohol, this yeast strain is not suitable to be used to ferment pet food ingredients. Differently from *S. cerevisiae*, *C. utilis* does not produce alcohol and grows easily in a variety of substrates, including foods that contain high concentrations of sugar. Therefore, *C.utilis* yeast fermentation might decrease the amount of rapidly available carbohydrate contained in starchy food ingredients, positively impacting the glycemic index. Moreover, *C. utilis* fermentation might enhance food flavour through the production of umami amino acids and reduction of antinutrients in the food. In addition, C. *utilis* cell wall has prebiotic effects, which may lead to the growth of beneficial bacteria, thereby improving the intestinal health of dogs and cats.

2.2 Objectives

The overall objective of this study is to develop a yeast fermented pea starch product that can be used in diets for dogs and cats. Once developed, the fermented pea starch will be

used to determine the effects on palatability, glycemic index, metabolic health and intestinal health in dogs and cats fed diets formulated with yeast fermented pea flour compared to unfermented pea flour. Study 1 refers to the methodology used to develop the yeast fermented pea starch. Eight beagle dogs and seven mixed breed cats were included in the other three studies where glycemic index, palatability and health status were assessed. In study 2, the postprandial glucose response to fermented and unfermented pea starches and diets were determined on both species. Three lab made diets (corn, unfermented pea and fermented pea diet) and one commercial diet (Legacy Horizon Pet Food) were used to assess palatability in study 3. The three lab made diets (corn, unfermented and fermented diet) were also used in study 4 to assess and compare the effects of carbohydrate source and yeast fermentation on metabolic status and intestinal health of dogs and cats.

The specific objectives of each study are as follows:

Study 1

1. To develop a technique to ferment pea starch using the yeast *C. utilis*
2. To formulate test diets with 30% inclusion of either unfermented or fermented pea starch.
3. To determine changes in the macronutrient composition of pea starch after fermentation

Study 2

1. To determine the effects of fermentation on the glycemic response after feeding dogs and cats unfermented or fermented pea starch, alone and when included in whole diets.
2. To calculate the glycemic index of unfermented and fermented starches and diets

Study 3

1. To develop a new consumption test to assess palatability in dogs and cats
2. To determine the palatability effects of yeast fermentation of peas in dogs and cats

Study 4

1. To determine the digestibility of the unfermented and fermented pea diets
2. To determine the health effects of a yeast fermented pea diet on weight management and general health of dogs and cats
3. To determine the health effects of a yeast fermented pea diet on intestinal health of dogs and cats

2.3 Hypotheses

The overall hypothesis for this research was that fermentation enhances the palatability of pea-based diets and improves the general and intestinal health of dogs and cats.

The specific hypotheses of each study were:

Study 1

1. Pea starch protein content will increase after fermentation
2. Total and RDS pea starch content will decrease after fermentation

Study 2

1. Fermented pea starch and fermented pea diet will generate a lower peak post prandial glycemic response than unfermented pea starch and diet
2. Fermentation will decrease the glycemic index of pea starch and pea diet

Study 3

1. When offered the unfermented and the fermented pea diets simultaneously (two-bowl test), dogs and cats will show preference through increased food intake for the fermented pea diet
2. When offered the four test diets simultaneously (corn diet, Horizon/Legacy pet food, unfermented and fermented pea diet) in a limited time frame (four-bowl test), dogs and cats will show a preference for the fermented pea diet over the unfermented pea diet

Study 4

1. Diets formulated with unfermented or fermented pea starches have similar digestibility
2. Dogs and cats fed diets formulated with peas (unfermented and fermented pea diets) will show better metabolic status reflected by lower leptin, but higher adiponectin levels than dogs and cats fed a diet formulated with corn
3. Dogs and cats fed diets formulated with peas (unfermented and fermented pea diets) will show improved intestinal health reflected by beneficial changes in intestinal microbial profile than dogs and cats fed a corn diet

4. Dogs and cats fed a yeast fermented pea diet will show increased production of butyrate and increased diversity and abundance of intestinal microbes than dogs and cats fed an unfermented pea diet

CHAPTER 3

Consumption of peas (*Pisum sativum L.*) has been associated with a variety of health benefits in humans (Dahl et al., 2012; Mudryj et al., 2014) and pets (Adolphe et al., 2015; Carciofi et al., 2008; Mitsuhashi et al., 2010). However, cats tend to refuse the bitter taste of peas (Briens, 2018). Yeast fermentation is a common technique used to improve food quality and flavour (Kieliszek et al., 2017). In this study, *Candida utilis* yeast (ATCC 9950) was used to ferment pea starch (Parrheim Foods, Saskatoon, SK). Although *Saccharomyces cerevisiae* is the yeast commonly used in pet food formulation (Martins et al., 2014), *Candida utilis* might be a more suitable option because *Candida utilis* easily grow on different substrates and under variable conditions without producing alcohol. Ethanol produced during *Saccharomyces cerevisiae* fermentation is thought to be a detractor and a toxic compound for feeding in pets (Kovalkovičová et al., 2009). Furthermore, *C. utilis* yeast has high nutritional value, being rich in proteins, nucleotides, and vitamins; which results in a final product of higher quality and better flavor (Buerth et al., 2016; Kieliszek et al., 2016). The yeast fermented pea starch produced in this study was used as the carbohydrate source of one of the test diets, namely the fermented pea diet, that are discussed in subsequent chapters in this thesis. The pure starch glycemic response in dogs and cats was investigated in Chapter 4 of this thesis, the effects of fermentation on palatability are described in Chapter 5, while the feeding trial that used fermented pea starch as an ingredient can be found in Chapter 6.

This chapter will be submitted to the Journal of Animal Science as supplemental material to the manuscript based on Chapter 5 of this thesis. Co-authors on this manuscript will be A. Kilgour, M. Loewen, M.D. Drew, and L.P. Weber. The specific contributions of each co-author are as follows: P. Curso-Almeida executed all experiments including animal work, performed all biochemical assays, data analyses and wrote the manuscript; A. Kilgour helped with the fermentation process; M. Loewen was co-PI and supervised A. Kilgour; M.D. Drew was co-PI and helped with study design; L.P. Weber was PI, supervised P. Curso-Almeida, helped with study design and editing of manuscripts.

FERMENTATION OF PEA STARCH USING CANDIDA UTILIS

3.1 Introduction

Yeasts have been used in animal nutrition for different purposes. They may be used as a nutritional enhancer due to their high concentration of vitamin B, nucleotides and amino acids or as a functional ingredient, due to the presence of mannooligosaccharides and *B*-glucans present in the yeast cell wall. Moreover, many species showed an increase in food intake when offered yeast-fermented foods, which may be related to the 'umami' taste trigged by the high concentration of glutamic acid and 5`-ribonucleotide present in the yeast extract (Martins et al., 2014). *Saccharomyces cerevisiae,* also known as brewer's yeast, is the traditional yeast used in the pet food industry (Martins et al., 2014). Yeast nutritional composition varies widely depending on factors like the medium and substrate used for yeast grown, but when similar growth conditions are provided, *Candida utilis* and *S. cerevisiae* share some desirable nutritional features (Brown et al., 1996). Both yeasts can *grow* in aerobic conditions. However, brewer's yeast growth requires an environment limited in sugar concentration, while *C. utilis* can assimilate pentoses and hexose, which makes *Candida utilis* easily adaptable to growth on a wider variety of substrates. Moreover, the *S. cerevisiae* fermentation process may lead to alcohol production, which does not happen when *C. utilis* is used (Bekatorou et al., 2006). Besides being toxic, alcohol is undesirable in pet feeds because it is considered as a feed detractor in several species (Kovalkovičová et al., 2009)

Candida utilis is a robust-growing yeast with high respiratory activity and fast metabolism (Buerth et al., 2016). When optimal conditions are provided, the yeast growth can be potentiated. Temperature, oxygen, and nitrogen availability are some of the factors to be considered. A temperature range of 30 to 35ºC stimulates *C. utilis* growth. Microbial protein synthesis can be optimized by adding nitrogen sources, either organic or inorganic, to the substrate. Furthermore, continual microbial access to oxygen can be assured by aeration, which also favours yeast growth (Reihani and Khosravi-Darani, 2019).

Pea starch is a type C starch that has a considerable amount of amylose, substantial protein content, and is rich in anti-nutritional factors. Although these characteristics are related to some of the health benefits attributed to peas consumption, the presence of antinutritional

factors gives peas an undesirable bitter taste (Thorne et al., 1983). Tannins, phytic acid, trypsin inhibitors, and flatulence-causing oligosaccharides are among the antinutritional factors found in peas. Research shows that *S. cerevisiae* yeast fermentation efficiently reduces the levels of antinutritional factors in peas (Khattab and Arntfield, 2009). To our best knowledge, no previous study using *C. utilis* to ferment peas is available. The objective of this study was to develop a fermentation technique using *C. utilis* with pea starch, creating a novel carbohydrate source to be included in diets for dogs and cats.

3.2 Material and Methods

3.2.1 Pea Starch

Pea starch (Parrheim Foods, Saskatoon, SK, Canada) was used as the fermentation substrate. The ingredient proximate analysis is shown in Table 3.1.

3.2.2   *Candida utilis*

Candida utilis (ATCC 9950) was maintained in sterile 80% (v/v) glycerol solution at -80ºC (Cedarlane, Ontario, Canada) until it was reactivated on YGC agar plates (Yeast Extract Glucose Chloramphenicol Agar -95765 Sigma Aldrich). The plates were kept at 30ºC for 72 hours (see Figure 3.1 for a schematic of the steps for yeast growth, then fermentation). Two loops of colonies were transferred using a platinum needle to a 250 ml sterile conical flask containing 100 ml of YPD liquid medium (Yeast Peptone Dextrose- A1374501 ThermoFisher). The flask was kept in a horizontal shaking incubator at 120 rpm and 30ºC for 12 to 15 hours. After this period, 10 ml of the cultured yeast mass was transferred into a 500 ml sterile conical flask containing 250 ml of YPD liquid medium. The medium containing the yeast was then incubated on a horizontal shaker at 120 rpm and 30ºC for another 12 to 15 hours.

3.2.3   Fermentation process

Forty kilo batches of pea starch were fermented at the Canadian Feed Research Center (CRFC North Battleford, SK). For each batch, 27.5 l of water, 4.0 l of activated yeast culture in YPD broth and 200 g of ammonia were added to the mixture. This protocol was adapted from Zhang & Drew that developed a similar technique to ferment canola meal using *C. utilis* (unpublished). However, due to the differences in canola meal and pea starch dry matter, the water content was adjusted according to the following equation:

$$\left. \frac{\text{Pea starch (Kg)} \times 0.93}{} \middle/ \text{Pea starch (Kg)} + X = 0.52 \right.$$

Where X is the amount of water in liters, 0.93 corresponds to the expected pea starch dry matter (93%) and 0.52 the desired moisture content of the final mixture (52%). The aerobic fermentation took place in an adapted vacuum coater machine at the Canadian Feed Research Centre (CFRC, North Battleford, SK – see Figure 3.1 middle panel for a picture of the coater used). The temperature was kept at 30ºC, and the ingredients were mixed for 3 minutes every hour for 72 hours. Samples were collected every 24 hours, and serial dilutions were performed. Agar plates containing the mixture samples were cultured at 30ºC for 72 hours to verify yeast growth through the process.
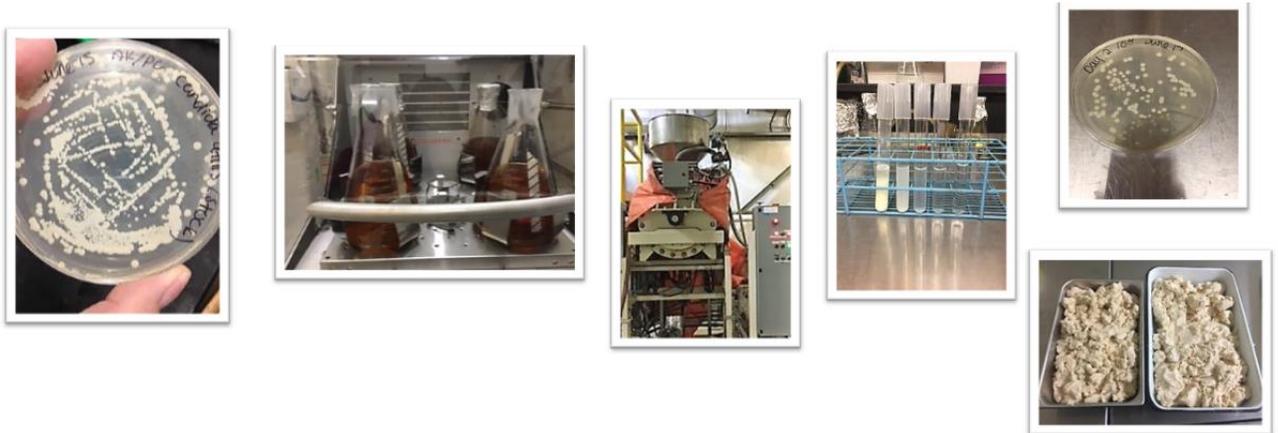
Figure 3.1 Schematic of the pea starch yeast fermentation process. YPD= Yeast extract peptone dextrose broth

3.2.4 Proximate analyses of fermented and unfermented pea starches

After fermentation, the different batches of the product were combined and air-dried on metal sheets in a room warmed to 25°C for 3 days, then ground in a hammer mill using a 9/64-inch screen. Moisture and macronutrient content were analyzed by a commercial lab in sub-samples of the fermented and unfermented pea starches (Central Testing Inc., Winnipeg, MB).

3.2.5 Analyses of inorganic compounds of fermented and unfermented pea starches

The levels of inorganic compounds present in unfermented and fermented pea starches were determined by a commercial lab (Central Testing Inc., Winnipeg, MB).

3.3 Results and Discussion

Yeast colonies were grown from samples taken at all time points during the incubation, indicating that fermentation was successful during the entire 72-hr incubation period. Based on dilution testing, the yeast concentration was estimated to be approximately 10^4 colony forming units (CFU)/ml of incubation medium. Moreover, the yeast biomass generated its own heat and fermentation and was overly successful at certain points. This resulted in an overflow of the incubation medium out of the incubation container and onto the floor in several instances. Based on this, subsequent batches were no longer pre-heated or provided heat during incubation, which minimized overflow but did not prevent fermentation from proceeding. The final fermented product was similar in appearance to the unfermented product but had an appealing 'bread-like' smell.

Table 3. 1 Proximate analyses of unfermented and fermented pea starches

Analysis	Unfermented pea starch	Fermented pea starch
Moisture (%)	5.5	11.1
Dry matter (%)	94.5	88.9
Crude protein (% dry matter)	7.7	10.6
Crude fibre (% dry matter)	0.8	1.7
Fat (% dry matter)	1.0	1.1
Non-fibre carbohydrate (% dry matter)	88.3	83.3
Total starch content (enzymatic) (% dry matter)	75	77
Metabolizable energy for dogs (Kcal/Kg)	3,805	3,668

Table 3. 2 Level of inorganic compounds found in pea starch before and after fermentation

Inorganic compounds (DM)	Unfermented pea starch	Fermented pea starch
Calcium (%)	0.04	0.04
Phosphorus (%)	0.14	0.57
Magnesium (%)	0.05	0.05
Potassium (%)	0.55	0.60
Sodium (%)	0.00	0.01
Sodium chloride (%)	0.00	0.02
Sulphur (%)	0.07	0.08
Copper (mg/Kg)	2.51	3.08
Iron (mg/Kg)	45.0	87.9
Manganese (mg/Kg)	4.03	7.73
Zinc (mg/Kg)	18.9	25.2
Cobalt (mg/Kg)	0.10	0.12

DM= dry matter

The fermented pea starch produced in this study was used to formulate the fermented pea diet used in the following chapters. Statistics cannot be performed on the proximate since all fermented pea batches were pooled and the unfermented pea starch was purchased as a single batch, thereby eliminating replication. Nonetheless, we qualitatively observed a slight increase in crude protein and crude fibre after pea starch fermentation (Table 3.1). In contrast, the change in starch content is less clear. Indirect calculation of starch content, calculated by industry standard by subtracting the protein, fat and fibre content from 100%, suggests fermentation decreased pea starch content. In contrast, the direct enzymatic measurement of total available starch content suggests instead that starch content remained relatively constant or increased slightly (Table 3.1). Our results agree with a previous study showing a slight increase in protein content and in vitro digestibility of pea meal after *S. cerevisiae* fermentation (Khattab et al., 2009). Another study showed that fermentation of pea starch by *Lactobacillus plantanarum* also resulted in a final product with higher crude protein content (Çabuk et al., 2018). Therefore, fermentation may be a desirable tool to improve the protein content of peas and pea related products. Another interesting finding was the increased concentration of inorganic compounds found in pea starch after fermentation (Table 3.2). This might be explained by the ability that yeasts have to bind with minerals and metals present in the fermentation substrate and incorporate it on its own cell wall (Boze et al., 1992; Kieliszek et al., 2017; Rai et al., 2019).

Taken together, the results of this chapter show that we can successfully ferment pea starch with the yeast, *C. utilis*. While proximate analyses suggest no remarkable change in macronutrient composition, this does not preclude the potential for significant changes after fermentation on health end-points, which will be tested in subsequent chapters. In contrast, the aroma of the pea starch did change, and this may produce differences in palatability which again, will be tested in subsequent chapters.

CHAPTER 4

To investigate the effects of pea starch yeast fermentation on dog and cat post prandial glycemic response, four different pea products were included in this study (unfermented pea starch, fermented pea starch - from study 1; unfermented pea diet and fermented pea diet – formulated with pea starches from study 1). Glucose responses after feeding pure starches and diets were compared to investigate how fermentation, industrial processing and diet formulation change glucose metabolism in dogs and cats.

This chapter will be submitted to the Journal of Animal Science. Co-authors on this manuscript will be M. Loewen, M.D. Drew, J.A. Adolphe and L.P. Weber. The specific contributions of each co-author are as follows: P. Curso-Almeida executed all experiments including animal work, performed all biochemical assays, data analyses and wrote the manuscript; M. Loewen and M.D. Drew were co-PIs and helped with study design; J.A. Adolphe helped with diet formulations; L.P. Weber was PI, supervised P. Curso-Almeida, helped with study design and editing of manuscripts.

POST PRANDIAL EFFECTS OF SINGLE FEEDING OF PURE UNFERMENTED AND
FERMENTED PEA STARCHES AND WHOLE FORMULATED DIETS ON GLYCEMIC
RESPONSE

4.1 Introduction

Peas are low glycemic index ingredients that are associated with a variety of health benefits in humans and dogs (Adolphe et al., 2015; Dahl et al., 2012; Mudryj et al., 2014; Nguyen et al., 1998). Over the past few years, peas have become a common ingredient in pet food formulation (Skerrit Jen, 2018). However, research has found that cats tend to refuse the taste of pea based diets (Briens, 2018). Anti-nutritional factors prejudice nutrient absorption and are also responsible for the bitter taste of peas (Drewnowski and Gomez-Carneros, 2000; Parca et al., 2018). Paradoxically, the consumption of some anti-nutritional factors has been associated with health benefits in humans (Frias et al., 2017; Thompson, 1993; Parca et al., 2018). For instance, although phenolic compounds (flavonoids, tannins, saponins and phenolic acid) reduce protein digestibility, they may also have antioxidant activity. Another antinutrient, phytic acid, well-known for reducing mineral absorption, may benefit health due to its anticarcinogenic and hypocholesterolemic properties (Campos-Vega et al., 2010; Champ, 2002; Thompson, 1993; Mohan et al., 2016).

A previous study showed that yeast fermentation leads to a significant reduction in phytic acid and lectin levels of peas. Generally, foods that contain high levels of anti-nutritional factors are associated with lower postprandial glycemic response (Dvořák et al., 2011; Khattab et al., 2009; Thompson, 1993; Parca et al., 2018). It follows that pulse fermentation may increase the glycemic index. Conversely, yeasts consume carbohydrates present in the substrate, which may counterbalance the effects of fermentation on the glycemic index of pulse based foods (Adebo et al., 2017; Shurson, 2018). Therefore, the effect of yeast fermentation on the glycemic index of peas is unknown and hard to predict, with either an increase or decrease possible, based on the literature.

Therefore, the goal of this study was to characterize the effect of yeast fermentation on the glycemic index of pea starch, either alone or when formulated in a whole diet in both dogs

and cats. To achieve this objective, glycemic index tests were conducted using previously validated methods in our group in dogs and cats (Adolphe et al., 2012; Briens, 2018).

4.2 Material and Methods

4.2.1 Animals

Eight adult neutered/spayed beagle dogs weighting 10.8 (± 0.8) and seven adult mixed breed cats weighting 4.8 kg (± 0.5) were included in this study (both mixed sex). The animals were kept at the Animal Care Unit (ACU) at the Western College of Veterinary Medicine, Saskatoon, SK, Canada. The dogs were housed together during the day and in their individual kennels at night. The cats were allowed to roam freely during the day and were housed in their individual kennels at night. Freshwater was provided *ad libitum,* and except for the trial period, the animals were fed a commercial dry species-specific pet food (Hill's Science Diet, Hill's Pet Nutrition, Inc. Topeka, USA). The dogs were walked daily and both groups of animals had free access to an outdoor play area. Each animal's calorie requirement was calculated to keep an optimal body score condition following the National Research Council guidelines (National Research Council, 2006). This work was approved by the University of Saskatchewan Animal Research Ethics Board following the Canadian Council on Animal Care guidelines.

4.2.2 Diets

To investigate the effects of *C. utilis* yeast fermentation on pea product post prandial glucose response, dogs and cats were fed 1 g/kg available carbohydrate from the following: unfermented pea starch (Pahrreim Foods), fermented pea starch (produced in study 1), pea diet with 30% inclusion of unfermented pea starch (unfermented pea diet) or pea diet with 30% inclusion of fermented pea starch (fermented pea diet). See Table 5.2 for ingredients and inclusion rate for the fermented and unfermented pea diets. Proximate analyses for the fermented and unfermented pea starches alone or formulated as whole diets were performed on singlet pools for each sample type, shown in Table 4.1, by Central Testing (Winnipeg, MB).

A 20% (v/v) glucose solution was used as control at 1 g/kg. The amount of available carbohydrate was determined for each test food using a commercially available colorimetric assay kit (Megazyme International, Wicklow, Ireland). After fasting for 12 hours (overnight), the dogs and cats were fed voluntarily or through a syringe into the back of the mouth, respectively, so that all of the meal was consumed within a maximum of 5 minutes. A single feeding was conducted for each diet/starch on a given day and the order was randomized. The glucose standard was tested twice in each animal. Animals were maintained on a normal husbandry diet between test days and after testing was finished on a given day.

Table 4. 1 Proximate analyses of starches and diets included in the glycemic index trial

Analysis	Unfermented pea starch	Fermented pea starch	Unfermented pea diet	Fermented pea diet
Crude protein (% dry matter)	7.7	10.6	37.4	37.9
Crude fibre (% dry matter)	0.8	1.7	4.8	4.9
Fat (% dry matter)	1.0	1.1	15.7	12.7
Non-fibre carbohydrate (% dry matter)	88.3	83.3	30.9	32.7
Metabolizable energy for dogs (Kcal/Kg)	3,805	3,668	3,748	3,550

4.2.3 Blood collection

Before feeding, dogs and cats were aseptically catheterized using an intravenous catheter inserted into the cephalic vein. Dogs and cats were allowed to acclimatize to the environment for one hour after the vein catheterization to minimize the effects of stress on blood glucose concentration. Blood was then collected at time zero to determine baseline (fasting) levels and at times determined to be optimal for that species in previous studies from our group (Adolphe et al., 2014, Briens, 2018): 15, 30, 45, 60, 90, 120, 150 and 180 minutes for dogs and 15, 30, 60, 120, 180, 240 and 300 minutes for cats. Catheters were flushed with

2% citrate in normal saline after each collection. Collected blood was put into EDTA tubes and stored on ice until centrifuge at 5000 x g for 10 min to collect plasma. Plasma was frozen at -80°C until analyzed for glucose.

4.2.4 Plasma glucose analysis

Plasma glucose analysis was determined using a colorimetric, glucose oxidase assay method (Sigma–Aldrich, Oakville, ON, Canada). Incremental area under the curve was calculated using the trapezoidal method, and the glycemic index was calculated by expressing the AUC for glucose for each food as a percent of the mean AUC to the mean of the two glucose control determinations for that animal (Brouns et al., 2005). Serum glucose peak concentration and time to peak were also determined for each starch or diet tested in each animal.

4.2.5 Englyst Method

In vitro digestibility (rapidly digestible starch or RDS, slowly digestible starch or SDS and resistant starch or RS) of starches and diets was determined as described in the literature (Englyst et al., 1992).

4.2.6 Statistical Analyses

Statistical analyses were performed using IBM SPSS version 24 (IBM Corp., Armonk, NY). Data collected was determined to be normally distributed using the Kolmogorov-Smirnov test. Two-way repeated measures ANOVA (extrusion processing and yeast fermentation as factors) was used to determine the effect of processing the starches into whole diets and to determine the effects of fermentation on glycemic index, area under the curve, peak time and time to peak. All post-hoc analyses were performed using Tukey's test. Differences were considered significant at $P < 0.05$.

4.3 Results

4.3.1 *In Vitro* Digestibility testing

Qualitatively, pea starch fermentation increased SDS, decreased RS and had no real effect on RDS (Table 4.2). Different effects were observed in the diets, with the fermented pea diet having lower levels of SDS, higher RS and again little effect on RDS levels. Comparing the changes from starch to extruded diets with 30% inclusion of either fermented or unfermented pea starch also reveals large effects on starch fractions. Specifically, most SDS and RS appear to have been converted to RDS in the diets (Table 4.2). While the total of all three starch fractions (RDS + SDS + RS) for both starches was approximately 75%, this should have produced about 75% of the 30% pea starch inclusion for the total of all starch fraction, which would be equal to 22.5%, a value very close to what was measured for both diets, indicating no starch was lost.

Table 4. 2 In vitro digestibility of unfermented and fermented pea starches and diets

Sample	RDS (%DM)	SDS (% DM)	RS (% DM)
Unfermented pea starch	14.7	28.9	31.3
Fermented pea starch	15.5	34.9	26.7
Unfermented pea diet	22.0	1.7	0.2
Fermented pea diet	22.6	0.8	0.5

RDS= rapidly digestible starch, SDS=slowly digestible starch, RS=resistant starch, DM= dry matter.

4.3.2   *In vivo* post prandial glucose response (Glycemic Index)

No statistically significant differences were found in peak glucose concentration, time to reach the peak, area under the curve and glycemic index in dogs or cats fed any of the four test foods (Tables 4.3 and 4.4). Despite the lack of statistical significance, the time to reach the peak glucose is longer for the fermented pea starch for both species (1.5 times longer for dogs, >2 times greater in cats) compared to the unfermented pea starch, a change that would have biological significance. In addition, cats also show increased time to reach the glucose peak when they were fed the fermented pea diet over the unfermented pea diet (2 times longer), but again this difference was not statistically significant (Table 4.4).

Table 4. 3 Dog post prandial glycemic response to unfermented and fermented pea starches and diets

	Glucose	UPS	FPS	UPD	FPD
Peak (mmol/L)	6.5 ± 0.4	4.0 ± 0.4	4.2 ± 0.1	4.1 ± 0.2	4.0 ± 0.1
Time to peak (min)	34 ± 4	40 ± 6	60 ± 4	42 ± 8	45 ± 9
Area under the curve (mmol/L min)	95 ± 8	37 ± 6	21 ± 3	28 ± 5	28 ± 4
Glycemic index		44 ± 9	27 ± 6	35 ± 9	34 ± 9

Values are mean $\pm$ SEM n=8. Two-way repeated measures ANOVA. No significant differences were found. UPS=unfermented pea starch, FPS=fermented pea starch UPD=unfermented pea diet and FPD=fermented pea diet.

Table 4. 4 Cat post prandial glycemic response to unfermented and fermented pea starches and diets

	Glucose	UPS	FPS	UPD	FPD
Peak (mmol/L)	6.2 ± 0.2	3.9 ± 0.1	3.9 ± 0.2	3.7 ± 0.2	3.6 ± 0.2
Time to peak (min)	47 ± 5	52 ± 8	120 ± 24	54 ± 10	104 ± 15
Area under the curve (mmol/L min)	90 ± 9	22 ± 6	31 ± 7	34 ± 8	40 ± 9
Glycemic index		25 ± 6	39 ± 9	38 ± 9	46 ± 9

Values are mean $\pm$ SEM n=7. Two -way repeated measures ANOVA. No significant differences were found. UPS=unfermented pea starch, FPS=fermented pea starch, UPD=unfermented pea diet and FPD=fermented pea diet

4.4   Discussion

4.4.1 Post prandial Glycemic Response of Dogs and Cats to Unfermented and Fermented Pea Starch and Pea Diets

Unlike post prandial responses to fermented pea products, the post prandial response of dogs and cats to unfermented pea starch and diet has been evaluated in previous nutritional studies (Adolphe et al., 2012; Briens 2018). The values found for the time to reach the peak blood glucose after feeding unfermented pea starch is much shorter for this current study at 40 min for dogs versus 94 or 54 min in dogs previously (Adolphe et al., 2012; Briens, 2018). This difference likely reflects differences in the purity and purification method used to produce the pea starch tested in the different studies, with the Adolphe et al. (2012) study having used crude, unpurified pea flour, the Briens (2018) study having used dry-processed, semi-purified pea starch and the current thesis having used highly purified, wet-processed pea starch. Greater processing would be expected to degrade the native pea starch structure, removing proteins associated with the starch granules (Bach Knudsen, 2013; Hall and Mertens, 2017) and thus increasing the efficiency of digestion. What is surprising is that these modifications did not seem to change the peak glucose or the glycemic index in dogs. In comparison, cat peak glucose and time to peak in this thesis after feeding unfermented pea starch were more comparable to that observed previously (Briens, 2018). Additionally, as observed in these previous studies (Adolphe et al., 2012; Briens, 2018), post prandial glucose response changes after processing and inclusion of starch products into whole diets, obscured differences in glycemic responses or index among different starch sources. Despite the loss of measurable difference in glycemic properties when formulated as a whole diet, pea-based diets have been shown to still exert health benefits on metabolic hormones, insulin sensitivity and/or weight loss (Adolphe et al., 2014; Briens, 2018). This highlights the importance of measuring the glucose response to individual ingredients and diets when determining the glycemic index of diets formulated for pets.

Blood glucose peak values of dogs and cats were similar regardless of fermentation. This is a desirable result since the goal of producing a fermented pea product was primarily to improve palatability but without a loss in the health benefits of peas, which included its low glycemic index properties (Adolphe et al., 2014; Briens, 2018). Pea starch consumption led to a non-significant tendency to delay the time to reach the peak glucose in dogs when compared to the unfermented pea starch. Cats, on the other hand, show a similar tendency for increased time to reach the peak glucose, but when fed either fermented pea product (starch or diet). Although these differences are not statistically significant, they may have biological significance, as increased time to reach the glucose peak improves satiety (Lehmann and Robin, 2007) .Both the dogs and cats would feel full for twice the time after being fed fermented pea products, thereby inhibiting overfeeding and eventually preventing obesity. Unexpectedly, but likely as a consequence of the longer time to reach the peak glucose, the area under the curve and the glycemic index both tended to increase with the fermentation of pea starch, despite retaining low peak glucose responses in both species. Based on these findings, we refute the hypothesis that *C. utilis* yeast fermentation decreases pea starch and pea diet glycemic index. Nevertheless, based on the results of this study, fermented pea products are likely to have retained low glycemic properties that would benefit the metabolic status of pets.

4.4.2 Comparison Among Glycemic Index, Englyst Method and Proximate Analyses Results

Research suggests that the Englyst method is a reliable *in vitro* technique used to predict the *in vivo* glucose response in humans (Tas and El, 1999; Anderson et al., 2010; Araya et al., 2002; Englyst et al., 1992, 1999). The starch fractions that contribute greatest to *in vivo* glycemic responses in humans have been found to be first the SDS, then the RDS, while RS and other fibre sources have a complicated interaction with the glycemic response (Meynier et al., 2015). Accordingly, the results of the Englyst method show that fermentation had little effect on the RDS + SDS content in the whole diets (23.7% for unfermented versus 23.4% for fermented), but with a shift to more RDS. Thus, the Englyst results would predict a quicker time to peak glucose response after feeding diets, but this was not observed. Instead, an

increased time to reach the peak after consumption of fermented pea diet was observed in both dogs and cats. What is consistent with the similar SDS + RDS content of fermented versus unfermented pea starch was a lack of change in peak glucose response in both cats and dogs. However, if the values for SDS + RDS are examined for the unfermented versus fermented pea starches alone (43.6% versus 50.4%), there is a clear expectation from these Englyst results for a greater peak blood glucose or faster time to peak glucose. However, the peak blood glucose, time to peak glucose and glycemic index all do not change in either dogs or cats, highlighting a disconnect between the *in vivo* and *in vitro* glycemic test results.

The lack of change in glycemic response with processing to produce a whole diet differs from previous studies from our group, where the whole diet increased glycemic responses (Adolphe et al., 2012, 2014; Briens, 2018). However, as discussed above, this difference in findings likely reflects the differences in the purity and processing method used to produce the pea starch in the current study versus previous ones. The decrease in SDS content is expected and might be explained by changes in starch structure due to gelatinization (Baller et al., 2018; Niba, 2002; Tran et al., 2007; Yao et al., 2010).

Discrepancies in carbohydrate content were found between the proximate analyses results (commonly used in commercial pet food labelling to show carbohydrate and all other macronutrients concentration in the diet) and the Englyst method results (compare Tables 4.1 and 4.2). Proximate analyses determine carbohydrate content by deducting the sum of other macro ingredients (ash, protein, moisture, fat and crude fiber). As a result, the values found and named as carbohydrates include any type of carbohydrate plus other substances such as lignin that are not accounted as crude fiber. Furthermore, the so-called carbohydrate content value is susceptible to interference by analytical errors from any of the assays performed to determine the value of other macronutrients (Cummings and Stephen, 2007; Southgate, 1969; Englyst and Englyst, 2005; Mccance and Lawrence, 1929). Therefore, the difference between proximate analyses and Englyst measurement of carbohydrate content was expected and raised the question of whether proximate analyses should remain the method of choice to be used in pet food labelling.

In summary, C. *utilis* yeast fermentation does not exert a significant effect on glycemic responses to pea starch or pea diet glycemic index in dogs and cats. Additionally, it might

delay postprandial glucose response in both species, but particularly in cats, which may improve satiety and help to prevent obesity. These results support the use of fermented pea starch on pet food formulation as it might be a tool to improve the palatability of pea base diets without compromising the beneficial low glycemic index associated with peas consumption. Furthermore, *in vitro* (Enlgyst method) and *in vivo* (glycemic index methodology) results disagree, highlighting the need to test potential feed ingredients *in vivo* before conclusions about glycemic properties can be made for dogs and cats.

CHAPTER 5

Chapter 5 will investigate whether dogs and cats show a preference for a diet formulated with yeast fermented pea starch over a diet formulated with regular unfermented pea starch. Moreover, this chapter also investigates whether dogs and cats can show preference when four different diets are offered simultaneously (four-bowl test). Besides the unfermented and fermented pea diets, in the four-bowl test dogs and cats were also offered a lab made corn diet and a commercial diet containing pea starch (Legacy/Horizon). Before starting palatability preference testing, the acceptability of the four diets included in this study was assessed using the one-bowl test.

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DETERMINING THE EFFECTS OF YEAST FERMENTATION ON PALATABILITY OF
A PEA BASED DIET FOR DOGS AND CATS AND DEVELOPING A NOVEL
CONSUMPTION TEST TO ASSES PALATABILITY IN PETS

5.1 Introduction

It is estimated that a third of worldwide households own at least one pet, and the numbers are growing (Surie, 2014). The global pet food market was worth $98.3 billion USD in 2018, and it should reach a value of $128.4 billion USD in 2024 (Research and Market 2019). The strong emotional link that bonds pets and owners have created a novel and profitable market for high-quality pet food that increased 170% in the past 15 years (Petfood Industry, 2018). Research shows that pet owners are willing to pay for diets that offer health benefits that might increase pet life span. Furthermore, pet food palatability has also been emphasized as an important feature, and most pet owners perceived palatability as the most important factor determining the repurchase of a diet (Di Cerbo et al., 2017; Petfood Industry, 2018; Surie, 2014). Studies have shown that after (perceived) pet food quality, the most important criteria used by pet owners when choosing a diet for their pets is the preference of the pet. Therefore, to be well accepted in the market, the pet food industry must formulate pet foods that possess both high-quality ingredients and a pleasant taste, making palatability assessment a crucial step in the development of new products (Aldrich and Koppel, 2015; Surie, 2014).

Palatability may be assessed through either consumption or non-consumption techniques. There are two standard consumption tests used by the pet food industry: the one-bowl and the two-bowl test. The one-bowl test aims to determine food acceptability, while the two-bowl test assesses preference (Tobie, Péron, and Larose 2015; Aldrich and Koppel 2015; Li et al. 2018, Vondran 2013). Although peas have been used as a carbohydrate source in many commercial pet food formulations, previous work from our group has shown that cats refuse the taste of pea-based diets (Briens et al., 2018). *Candida utilis*, also known as torula yeast, is a common yeast used by the human food industry to ferment foods (Bekatorou et al., 2006; Boze et al., 1992; Kieliszek et al., 2017). Yeast extracts in general, including torula yeast, are rich in protein, including amino acids like lysine, threonine, valine, and glutamic acid (Bekatorou et al., 2006). Glutamic acid chemically co-exists as glutamate and is responsible

for the pleasant 'umami' or meaty taste of foods (Kulkarniet al., 2005). For over 60 years now, the human food industry has been using *Candida utilis* as a meat substitute due to its high protein content and meaty flavour (Bekatorou et al., 2006). While this study did not use *C. utilis* as a feed additive and instead used this yeast to ferment pea starch, the yeast residues left in the fermented product would likely improve the pea starch palatability. With fermentation, we also hypothesize that *C. utilis* would reduce the amount of anti-nutritional factors thought to be responsible for the bitter taste in pea starch, similar to that observed after fermentation with *C. utilis* or other yeast/bacteria and other plant-based feed ingredients (Drewnowski and Gomez-Carneros, 2000; Khattab and Arntfield, 2009; Millar et al., 2019; Mohan et al., 2016; Gupta, 1987), thereby potentially further enhancing palatability of pea starch.

Studies show that the addition of yeast extract increases food consumption in dogs and cats (Martins et al. 2014; Swanson and Fahey 2003). However, *Candida utilis* is still a new food ingredient for the pet food industry. The objective of this study is to determine if replacing pea starch for a *Candida utilis* yeast-fermented pea starch increases food intake of a pea based diet for dogs and cats. In order to test the hypothesis that *C. utilis* will increase the palatability of pea starch, an experiment to test first acceptability, then preference for test diets was conducted. Laboratory-made test diets with 30% inclusion of fermented pea starch versus unfermented pea starch or corn starch were compared to a commercial diet that had high levels of pea starch inclusion in laboratory beagles and mixed breed domestic cats using one-bowl, two-bowl and four-bowl tests.

5.2 Material and Methods

5.2.1 Animals

Seven adult, neutered/spayed mixed breed, mixed sex cats weighing 4.8 ± 0.5 kg and eight adult, neutered, mixed sex beagle dogs weighing 10.8 ± 0.8 kg were used to determine palatability. The dogs and the cats were obtained from a certified scientific breeder (Marshall Farms, North Rose, NY) and were kept at the Western College of Veterinary Medicine at the University of Saskatchewan (Saskatoon, Saskatchewan, Canada). The dogs were group-housed

during the day, but kept in individual kennels during feeding and at night. Fresh water was provided *ad libitum*. Each individual dog kennel has free access to an outdoor area. In addition, the dogs had daily playtime with volunteers and daily walks outdoors on leash. During the day, the cats were allowed to roam freely within the common areas of the animal care facility and had access to a common outdoor courtyard with grass and scratching/climbing structures. At night the cats were housed individually where they were fed and with fresh water *ad libitum.* Both species were fed species-specific whole balanced standard commercial dry pet food (Hill's Science Diet, Hill's Pet Nutrition, Inc. Topeka, USA), except for the feeding trial periods where test diets were fed. The daily food amount was individually adjusted to keep the animals in an optimal body score condition following the National Research Council guidelines (National Research Council, 2006). This work was approved by the University of Saskatchewan Animal Research Ethics Board following the Canadian Council on Animal Care guidelines.

To assure animal health status before the palatability trial, a physical exam was performed, then blood samples collected for complete blood count/differential and biochemistry profile. All animals were healthy at the time of testing. For validation of palatability methods, all animals were submitted to an obvious choice test before starting the trials. In the obvious choice, the animals received a highly palatable food (commercial wet canned food) and a less attractive choice (dry food) simultaneously. After consistently choosing the high palatable food, all animals were included in the palatability trials.

5.2.2 Diets

Three lab-made diets and one commercial diet (Legacy Horizon dog and Legacy Horizon cat dry foods, Rosthern, SK) were used in this study. The lab-made diets were formulated to have similar composition, differing only in the carbohydrate source. The same diets were fed to both dogs and cats; thus, the diets were formulated to meet the higher minimum protein, taurine and other nutritional needs of cats. While the lab-made diets exceeded nutritional requirements for dogs, they resembled many of the premium dog diets on the commercial market (Carciofi et al., 2006; Dzanis, 1994, 2008). Corn starch was the

carbohydrate source used in the control lab-made diet at 30% inclusion, while the test diets were formulated with pea starch (Parrheim Foods, Saskatoon, SK; fermented or unfermented) as the carbohydrate source at 30% inclusion. See Tables 5.1 and 5.2 for a complete list of lab-made test diet ingredients and inclusion rates compared to the ingredient list for the commercial diets. Although the carbohydrate source of the commercial pet food used was also pea starch, the other ingredients included in its formulation differ from the lab made diets. Proximate analyses for macronutrient composition and available energy for each lab-made and commercial diet (see Table 5.3) were done at a commercial testing lab (Central Testing, Winnipeg, MB).

5.2.3 Measurement of Inorganic compound and Total Phenols and Tannins

The levels of inorganic compounds present in the test diets were determined by a commercial lab (Central Testing Inc., Winnipeg, MB).

Total phenol and tannins content of unfermented and fermented pea diets were determined according to the Folin-ciocalteu Method, as described in the literature (FAO/IAEA, 2000)

Table 5. 1 Lab-made diets formulated for the palatability trial with ingredients listed in decreasing order of inclusion

Ingredient	Corn Diet (%)	Unfermented Pea Diet (%)	Fermented Pea Diet (%)
Cornstarch	30	0	0
Unfermented pea starch	0	30	0
Fermented pea starch	0	0	30
Chicken meal	35.78	25.46	25.46
Soy protein concentrate	9.31	15	15
Chicken fat with Dadex T	7.75	9.76	9.76
Pea fibre (PetFine®)	6.88	4.96	4.96
Fish meal, mixed	5.0	5.0	5.0
Liquid palatant (AFB LC647 LQDGST)	2.0	2.0	2.0
Fish oil	2.0	2.0	2.0
Celite	1.0	1.0	1.0
Potassium chloride	0.9	0.88	0.88
Sodium chloride	0.3	0.5	0.5
Dry palatant (AFB F24047 Dry cat)	0.5	0.5	0.5
Calcium carbonate	-	0.41	0.41
Choline chloride	0.1	0.2	0.2
Methionine/DL	0.1	0.1	0.1
Mineral premix, dog cat	0.1	0.1	0.1
Vitamin premix, cat	0.1	0.1	0.1
Taurine	0.1	0.1	0.1
Dicalcium phosphate	-	0.01	0.01

Diets were formulated by Dr. Jennifer Adolphe using Concept 5 software (Creative Formulation Concepts, LLC, MD, USA) according to AAFCO nutrient profiles (The Association of American Feed Control Officials, 2014). The diets were extruded at the Food Science and Technology Centre (FSTC) in Brooks, Alberta, Canada, using a Coperion ZSK57 extruder (Werner & Pfleiderer, Ramsey, NJ, USA). The extruder consisted of 8 barrels on which the temperature was independently controlled by Mokon oil heaters (HC5X12-Q1, Mokon, Buffalo, NY, USA). At the end of the process, the pellets were coated with a mixture of chicken fat and fish oil with a fluidizing paddle blender (FPB-20, American Process Systems, Gurnee, IL, USA). Finally, the diets were coated first with a commercial liquid palatant (AFB LC647 LQDGST), followed by a dry palatant (AFB F24047 Dry Cat) at the Canadian Feed Technology Research Facility (CFTRF) in North Battleford, SK.

Table 5. 2 Ingredient composition of commercial diets used in the palatability trial (shown in decreasing order of inclusion), as listed on the product labels by the manufacturer. Exact inclusion rates are not known

Dog Legacy diet*	Cat Legacy diet*
Chicken	Chicken
Chicken meal	Chicken meal
Peas	Turkey meal
Pea starch	Peas
Turkey meal	Pea Starch
Chicken fat	Chicken fat
Salmon meal	Salmon
Pea fibre	Salmon meal
Flaxseed, Egg Product, Salmon Oil (preserved with mixed tocopherols, vitamin E), Carrots, Apples, Broccoli, Bok choy, Cabbage, Blueberries, Salt, Fructooligosaccharides, Yuucca Shidiger A extract, Dried Apergillus Niger Fermentation Extract, Dried Aspergilus Fermentation Extract, Pineapple, Dried Trichoderma Longibrachiatym Fermentation Extract, Dried Rhizopus Oryzae Fermentation Extract, Dried Enterococcus Faecacium Fermentation Product, Dried Lactobacillus casei Fermentation Product, Dried Lactobacillus Acidophilus Fermentation Product, Dried Lactobacillus Plantarum Fermentation Product, Vitamin A acetate, Vitamin D3 Supplement, Vitamin E Supplement, Vitamin E Supplement, Vitamin 812 Supplement, Riboflavin, Niacin, Vitamin D3, Folic Acid, Pyridoxine, Hydrochloride, Thiamine Mononitrate, D-Calcium Pantothenate, Biotin, L-Ascorbyl-2-Polyphosphate (Source of Vitamin C), Ferrous Manganous Oxide, Manganese Proteinate**, Copper Sulphate, Copper Proteinate**, Calcium Iodate, Selenium Yeast, Magnesium Oxide	Egg Product, Herring oil (preserved with mixed tocopherols, source of Vitamin E), Frucctooligosaccharides, Yucca Shidigera Extract, Dried Aspergillus Nigger Fermentation Extract,, Dried Aspergillus Oryzae Fermentation Extract, Pineapple, Dried Trichoderma Longibrachiatum Fermentation Extract, Dried Rhizopus Oryzae Fermentation Extract, Dried Enteroccocus Faecium Fermentation Product, Dried Lactobacillus Casei Fermentation Product, Dried Lactobacillus casei Fermentation Product, Dried Lactobacillus Acidophilus Fermentation Product, Dried Bifidobacterium Product, Dried Lactobacillus Plantarum Fermentation Product, Taurine, Vitamin D3 Supplement,, Vitamin A Acetate, Vitamin E Supplement, Vitamin 812 Supplement, Riboflavin, Niacin, Folic Acid, Pyridoxine Hydrochloride, Thiamine Mononitrate, D-Calcium, Panthothenate, Biotin, L- Ascorbyl-2l Polyphosphatase (source of Vitamin C) , Ferrous Sulphate, Zinc proteinate, Manganous Oxide,Manganese Copper Sulphate, Copper Proteinate, Calcium Iodate, Selenium Yeast, Magnesium Oxide

*Manufactured by Horizon Pet Foods (Rosthern, SK); **Chelated minerals

Table 5. 3 Proximate analyses of test diets formulated for and commercial diets used in the palatability trial

Nutrient (%DM)	Control (corn) diet	Unfermented pea diet	Fermented pea diet	Legacy dog food	Legacy cat food
Protein	38.4	37.4	37.9	34.0	40.0
Fat	14.2	15.7	12.7	15.0	19.0
Carbohydrates	32.0	30.9	32.7	22.0	15.6
Fibre	3.2	4.8	4.9	3.5	2.4
ME (Kcal/Kg)	3,760	3,748	3,550	3,650	3,870

DM = dry matter; ME = metabolizable energy

Table 5. 4 Analyses of total phenol and tannin content of lab made diets used in the palatability trial

Sample (%DM)	Total phenols (mg/g phenolic functional group equivalents)	Tannins (mg/g tannic acid equivalents)	Condensed tannins (mg/g leucocyanidin equivalents)
UPD	5.66	0.74	Not detectable
FPD	7.82	3.60	0.04

DM= dry mater; UPD= unfermented pea diet and FPD=fermented pea diet

Table 5. 5 Level of inorganic compounds present in diets used in the palatability trial

Inorganic compounds (DM)	Corn diet	UPD	FPD
Calcium (%)	2.48	1.95	2.16
Phosphorus (%)	1.48	1.20	1.41
Magnesium (%)	0.14	0.15	0.17
Potassium (%)	1.11	1.11	1.10
Sodium (%)	0.51	0.69	0.73
Sodium chloride (%)	1.30	1.75	1.86
Sulphur (%)	0.45	0.37	0.37
Copper (mg/Kg)	7.43	2.77	8.87
Iron (mg/Kg)	239	218	235
Manganese (mg/Kg)	11.4	14.2	17.2
Zinc (mg/Kg)	109	80	110
Cobalt (mg/Kg)	0.27	0.37	0.32

DM=dry matter, UPD= unfermented pea diet, FPD= fermented pea diet

5.2.3 Palatability trials

5.2.3.1 One-bowl test

To determine acceptability, dogs and cats were offered one of the four test diets for five consecutive days in a slight excess amount from the daily requirement to ensure that food would remain uneaten. The remaining food was weighed and subtracted from the total offered to calculate food intake. The total of food offered was equal in weight for all four diets, and it was calculated to meet or exceed the requirements of each animal. The normal feeding routine at the Animal Care Unit facility was for dogs to be fed in their individual kennels twice a day (between 8:30 – 9:00 am and between 4:00 - 4:30 pm), while cats were fed once daily in their individual kennels between 4:00 pm - 8:00 am. To keep as much consistency as possible

between species, palatability testing occurred only in the afternoon for dogs and overnight for cats.

A previous pilot study conducted with the same panel of animals included in palatability testing determined the average time necessary for the consumption of a regular meal containing the daily energy requirements of each animal. The times to complete the one-bowl, two-bowl and four-bowl tests were based on the results of this pilot study (Curso-Almeida and Weber, unpublished). Although the literature states that dogs should complete the test within 15 to 30 minutes for one-bowl and two-bowl tests (Tobie et al.,2015; Aldrich and Koppel 2015), in this study, the dogs were allowed just 90 seconds to complete the one-bowl test. The shorter time allowance for completing the test assured that at least some food would remain uneaten, and the dogs would not exceed their daily calorie intake (beagles are excessively eager to eat any food). In accordance with the literature and the pilot study results, cats were allowed more time due to their slower, grazing behavior and given 16 hours to complete the tests (Becques and Niceron 2014; Bradshaw et al. 1996; Tobie at al., 2015; Curso-Almeida and Weber, unpublished). The test was performed blinded by the same individual using glass bowls that were daily washed with an unscented dish soap using methods adapted from previous studies (Aldrich and Koppel 2015; Tobie et al., 2015). Each diet was tested five times in a randomized order and mean values per diet used in statistical analyses.

5.2.3.2 Two-bowl test

To determine preference between the unfermented and fermented pea diets, dogs and cats were offered two diets simultaneously for five consecutive days, and the remaining food was weighed to calculate food intake. A similar testing routine as for the one-bowl test was followed for the two-bowl test with respect to frequency of testing, location of testing and time of day, except for the following details where the two-bowl test differed. Each bowl contained the same amount in weight of food, and each diet was offered in sufficient amount to meet or exceed the animals calorie requirements for that meal. The calorie requirement of each animal was calculated according to the equation below (Wills, 1991):

RER= 70* (BW) $^{0.75}$

Dogs : MER= 1.6 * RER

Cats : MER= 1.2 * RER

Where RER= Resting energy requirement, MER = Maintenance energy requirement, and BW= body weight (Kg).

The bowl position was randomized to avoid side bias. To ensure that dogs would have enough time to investigate both food options, the time allowed to complete the two-bowl was slightly greater than the time allowed to complete the one-bowl test. Preliminary experiments showed that allowing the dogs to have 2 minutes to complete the two-bowl test was optimal (Curso-Almeida and Weber, unpublished), while preliminary cat testing determined optimal time needed for the food to be available was 18 hours (Becques and Niceron 2014; Tobie et al., 2015). Each diet was tested five times and mean values per diet used in statistical analyses.

5.2.3.3 Four-bowl test

This method was developed by our group for this study to allow comparison of preference among multiple diets at the same time. To determine preference among the four test diets, dogs and cats were simultaneously offered the corn diet, the unfermented pea diet, the fermented pea diet, and the Legacy pet food for five consecutive days. A similar testing routine as for the two previous tests was followed with respect to frequency of testing, location of testing and time of day, except for the following details where the four-bowl test differed. Any the remaining food was weighed to calculate the respective food intake. The quantity of food offered in each bowl was equal for all diets, and the total amount consumed for each diet was calculated to meet or exceed the requirements of each animal (see Methods section for calculation of feeding amount). The feeding time followed the normal feeding routine, and the test was performed blinded by the same individual using glass bowls daily washed with unscented dish soap. The position of the bowls was randomized to avoid side bias. To provide enough time for the dogs to investigate all the food options and show preference for more than

one diet, the time allowed to complete the four-bowl test was greater than the time allowed to complete the one and two bowl tests. The dogs had 3 minutes to finish the test, and the cats were allowed 18 hours with the bowls.

5.2.4 Statistical Analyses

Statistical analyses were performed using IBM SPSS version 24 (IBM Corp., Armonk, NY). Data collected was determined to be normally distributed using the Kolmogorov-Smirnov test. A paired T-test was used to compare the difference in food intake in the two-bowl test. One-way repeated measures ANOVA was used to compare differences in food intake in the one-bowl and in four-bowl tests. All post-hoc analyses were performed using Tukey's test. Differences were considered significant at $P < 0.05$.

5.3 Results

5.3.1 Inorganic compounds and Total Phenols and Tannins

Measurement of inorganic compounds of diets formulated with fermented pea starch or regular pea starch shows that fermentation increased the concentration of most inorganic compounds (except for potassium, Sulphur and cobalt) found in the pea starch (see Table 5.5)

Likewise, compared to the diet formulated with unfermented peas, total phenol and tannin concentrations increased in the diet formulated with fermented pea starch (see Table 5.4).

5.3.2 One-bowl test

For either species, food intake did not differ among the diets in the one-bowl test (Figure 5.1 and 5.2, left panels). Dog food intake in the one-bowl test showed the following

rank order from highest to lowest, albeit with no significant differences among diets: unfermented pea diet > Legacy > fermented pea diet > corn diet (Figure 5.1). Similarly, cat food intake in the one-bowl test showed the following rank order from highest to lowest, albeit with no significant differences among diets: corn diet > Legacy > fermented > unfermented pea diet (Figure 5.2).

5.3.3 Two-bowl test

Both species show a preference for the fermented pea diet compared to the unfermented pea diet in the two-bowl test (paired T-test, $p < 0.05$; Figures 5.1 and 5.2, middle panels). When given the choice between the two diets, dogs consumed 1.5 times more of the fermented pea diet compared to the unfermented pea diet (Figure 5.1). In contrast, when given the choice between the two diets, cats showed a higher preference and consumed over 2 times more of the fermented pea diet compared to the unfermented pea diet (Figure 5.2).

5.3.4 Four-bowl test

For both species, while the one-bowl test showed no difference among diets, the four-bowl test showed clear differences in preferences (Figures 5.1 and 5.2, right panels). For dogs, the four bowl test showed the following rank order in preference from highest to lowest: Legacy > fermented pea diet > unfermented pea diet > corn diet (Figure 5.1). However, the difference in dog food intake showed only the Legacy and fermented pea diet was statistically higher than either the unfermented pea or corn diets (one-way repeated measures ANOVA followed by Tukey's posteriori tests, $p < 0.01$).

The order of preference shown in the cat four-bowl test was different from that of dogs, with cats showing the following: corn diet > fermented pea diet > Legacy > unfermented pea diet (Figure 5.2). Despite these differences, a common finding between species was that the fermented pea diet was preferred to a significantly greater extent compared to the unfermented pea diet (compare right panels of Figures 5.1 and 5.2). The average consumption of the corn diet was statistically higher in cats compared to any other diet (one-way repeated measures

ANOVA followed by Tukey's posteriori tests, p<0.01). In contrast, the Legacy and fermented pea diet showed intermediate levels of consumption but were statistically higher than the unfermented pea diet consumption in cats (Figure 5.2).
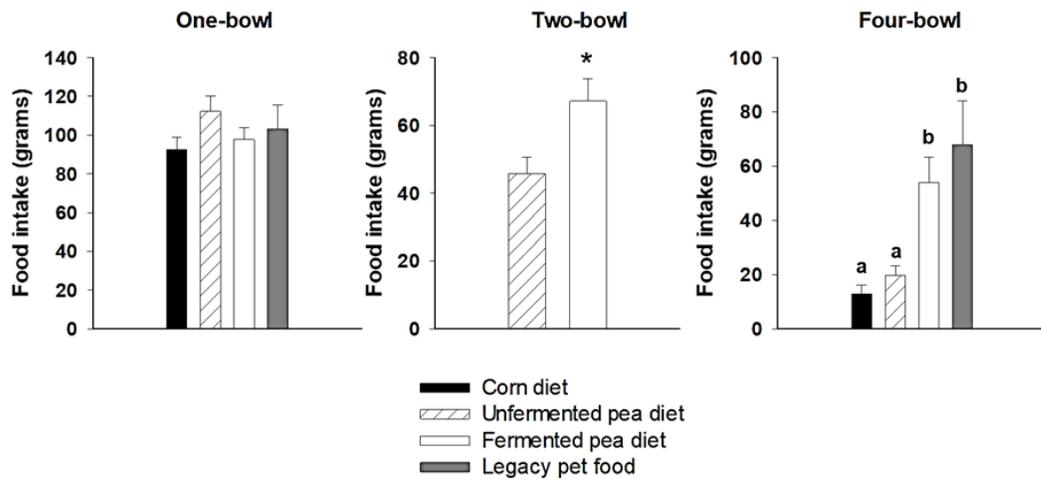
Figure 5. 1 Dog food intake in the one-bowl, two-bowl, and four-bowl palatability tests. Values are mean ± SEM, n=8. One-way repeated measures ANOVA was used for one-bowl and four-bowl tests, while a paired T-test was used for the two-bowl test. * $p<0.05$ compared to the unfermented pea diet. Bars with superscripts with a different letter are significantly different ($p<0.05$) from each other.
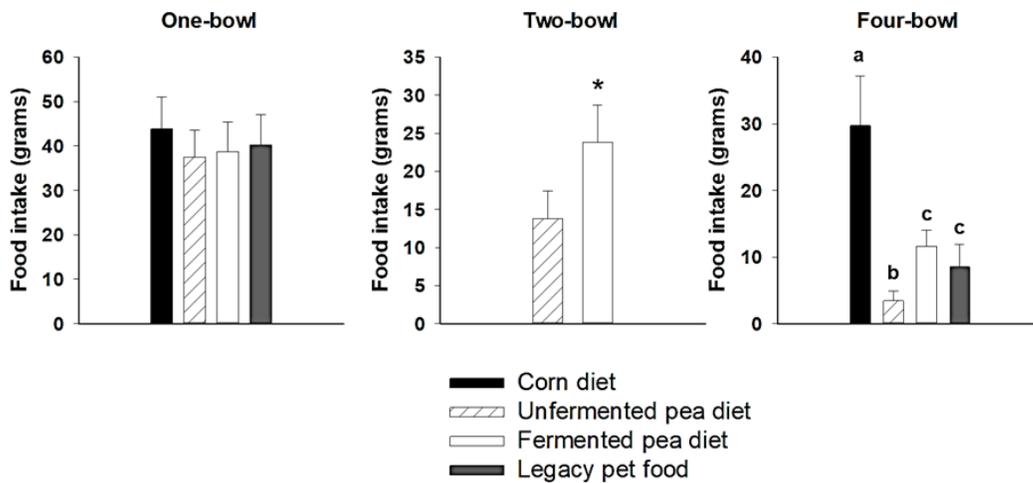
Figure 5. 2 Cat food intake in the one bowl, two-bowl and four bowl palatability tests. Values are mean ± SEM, n=7. One-way repeated measures ANOVA was used for one-bowl and four-bowl tests, while a paired T-test was used for the two-bowl test. * $p<0.05$ compared to the unfermented pea diet. Bars with superscripts with a different letter are significantly different ($p<0.05$) from each other.

5.4 Discussion

The main objective of this study was to determine whether yeast fermentation of pea starch increases the food intake of pea-based diets formulated for dogs and cats. Over the past few years, peas have been used as a carbohydrate source in pet food formulation and, despite all the scientific evidence linking the consumption of peas to a variety of health benefits in dogs and humans (Adolphe et al., 2014, 2015; Dahl et al., 2012; Jenkins et al., 1981; Mudryj et al., 2014; Nguyen et al., 1998; Thomas MS Wolever et al., 1991), few studies are available in cats. The lack of cat studies is probably due to cats refusal to eat pea products (Briens, 2018), which would compromise the nutritional investigation of the effects of consumption of peas in this species. Considering that the cat pet food market comprises approximately 40% of the total market of pet foods, addressing potential palatability issues would positively impact the market of pet diets containing peas (Progressive Grocer, 1993). Furthermore, research shows that pet food palatability is the principal factor that owners considered in pet food repurchase, which highlights the importance of palatability testing and techniques to enhance the flavour of foods developed for dogs and cats (Aldrich and Koppel, 2015; Surie, 2014).

To test the hypothesis that yeast fermentation improves the palatability of pet diets formulated with pea starch, two diets were formulated to contain 30% of inclusion of either unfermented or fermented pea starch. A control diet formulated with 30% corn starch and a commercial diet containing peas (Horizon Legacy pet food, Rosthern, SK) were also included in the four-bowl test. The one-bowl test is designed to determine acceptability and diets that fail this basic criterion should not be included in preference tests. In this study, all of the four test diets were accepted by both species and were therefore included in the preference testing (Tobie, Péron, and Larose 2015; Vondran, 2013). When given the option between the fermented and the unfermented pea diet in the two-bowl test, both dogs and cats showed a clear preference for the fermented pea diet. When the four diets were offered simultaneously, Horizon/Legacy pet food and the fermented pea diet showed a similar preference in both species. However, cats preferred the corn diet over the Horizon/Legacy and fermented pea diet, while dogs preferred Horizon/Legacy and fermented pea diet over corn and unfermented pea diet. These results support the hypothesis that yeast fermentation of pea starch increases the food intake of pea-based diets in dogs and cats.

The bitter taste of peas that is described by human consumers correlates to the presence of anti-nutritional factors in the grain. Peas contain trypsin, chymotrypsin, oxalates, phytates, oligosaccharides, phenolic compounds, tannins, and lectins, all of which are bioactive compounds that impart both bitter taste and well as impair protein digestion and mineral absorption (Drewnowski and Gomez-Carneros 2000; Mohan et al. 2016; Khattab and Arntfield 2009; Gupta 1987). In the current study, the total phenol and tannin content was found to be higher in the diet formulated with fermented pea starch than in the diet formulated with unfermented pea starch (Table 5.4). This clearly contradicts the literature that supports the use of pulse fermentation as a processing method to improve food taste and quality as well as to reduce the concentration of anti-nutritional factors (Drewnowski and Gomez-Carneros, 2000; Iqbal et al., 2006; Khattab and Arntfield, 2009; Kieliszek et al., 2017; Rai et al., 2019). Nevertheless, this apparent contradiction might be explained by further looking at the methodology used to measure total phenols and tannin in the samples. The Folin-ciocalteu method measures the sample total reducing capacity and is, therefore, affected by several non-phenolic substances such as organic acids and amines (Hinojosa-Nogueira et al., 2017; Turkmen et al., 2006). One of the biotechnological uses of yeast is the formation of bioplexes in which the yeast absorbs metal ions present in the environment and incorporates it to its own cell wall (Bekatorou et al., 2006; Kieliszek et al., 2017; Rai et al., 2019). After fermentation, pea starch apparent concentration of inorganic compounds increased (see table 3.2). Similarly, higher levels of inorganic compounds were found in the fermented pea diet when compared to the unfermented pea diet (table 5.5).), which might explain the higher values for total phenols and tannin found in the fermented pea diet (Table 5.4) if the yeast was simply making ions that cross-reacted with the reagent more chemically accessible. Furthermore, the ammonia added in the fermentation process (see description of study 1) might have also contributed to the increased quantification of phenols and tannins in the fermented pea diet. More importantly, we were unable to measure other anti-nutritional factors present in the samples, which makes it difficult to determine the real impact that yeast fermentation had on pea starch antinutrients level. What can be concluded is that if anti-nutritional factors were reduced by yeast fermentation and if this were linked to the increase in palatability, it was due to anti-nutritional factors other than tannins or phenols.

Besides the reduction of anti-nutritional factors and associated bitter taste of peas, yeast fermentation may also improve flavour through the addition of substances that enhances palatability. Yeasts are rich in glutamic acid and ribonucleotides; both substances are linked to the umami taste in humans, dogs and cats (Kurihara and Kashiwayanagi 2000; Yamaguchi and Ninomiya 2000; Zhang et al. 2017; Swanson and Fahey 2003; Kieliszek et al. 2017). However, studies evaluating the effects of yeast supplementation in pet food palatability are scarce and most studies used the same yeast (*S. cerevisiae*) in different inclusion rates. Studies show increased food intake in dogs fed diets supplemented with industrial strains of *S. cerevisiae* (Swanson and Fahey, 2003; Martins et al., 2014). In contrast, despite the high yeast content of umami ingredients, studies evaluating the effects of *S. cerevisiae* yeast extract supplementation in cats show a reduction in food intake in this species (Aquino et al., 2010; M. Lima et al., 2015; Ogoshi et al., 2014). The divergence among studies might be explained by some peculiarities in food preference observed in cats. Cats tend to refuse the taste of "bitter" amino acids, such as leucine, arginine, isoleucine, phenylalanine and tryptophan (Zaghini and Biagi, 2005). *S. cerevisiae* yeast extract is rich in leucine, which may explain why cats show reduced food intake in studies using yeast extract in unprocessed diets (M. Lima et al., 2015). Another possible explanation relates to the rate of inclusion of yeast extract in the test diets. Research shows that a high inclusion of umami substances leads to a paradoxical impairment in food palatability (Martins et al., 2014; Zaghini and Biagi, 2005; Zhang et al., 2017). Therefore, high inclusion of yeast extract, an ingredient that is rich in umami substances, may prejudice food taste. Our results agree with other studies showing increased palatability of pet foods containing yeast in dogs and cats (Swanson and Fahey 2003; Martins et al. 2014). However, the current study evaluated palatability of a diet formulated with *C. utilis* fermented pea starch, and to our best knowledge is the first study comparing palatability of diets formulated with a fermented ingredient, instead of using diets supplemented with yeast products in pet foods.

The traditional methodology used to assess food preference in dogs and cats requires feeding exaggerated amounts of food, which may lead the test subjects to develop obesity and other correlated diseases (Basque et al., 2019; Li et al., 2018; Rashotte and Smith, 1984; Thompson et al., 2016; Tobie et al., 2015). This issue is probably more relevant to palatability trials conducted with dogs as studies show that dogs are more prone to overfeeding than cats,

probably due to differences in feeding behavior (Bradshaw, 2006; Bradshaw et al., 1996; Zoran, 2010). In the current study, the time allowed for dogs to complete the preference test was significantly reduced compared to the literature or compared to cats but was based on the results of a pilot study performed with the same group of animals. The reduction in the time allowed to the dogs to complete the tests with sufficient sensitivity in our results but minimized undesirable weight gain because they did not consume all of the food offered. Although the consumption time used to this study might not be applicable for all palatability studies, performing a pilot study to determine the specific time of consumption needed for the group of animals being used in preference trials may be an alternative to simply overfeeding.

The two-bowl test is the test of choice used by the pet food industry to determine the palatability of commercial diets. As opposed to non-consumption tests, the two-bowl test does not require any training. However, as only two choices are available to the animals, the preference for one diet is intrinsically related to the taste of its competitor (Vondran 2013; Tobie et al. 2015; Li et al. 2018; Aldrich and Koppel 2015). All the tests currently available to assess the palatability of more than two diets simultaneously demand animal training, which increases the time and the cost of palatability trials (Aldrich and Koppel, 2015; Thompson et al., 2016). Furthermore, as cats are difficult to train, the current methodologies limit palatability assessment to dogs, with puppies generally used, that can be easily trained (Becques et al., 2014b; Savolainen et al., 2016; Tobie et al., 2015). Offering more food choices makes the animal's decision less dependent on the taste of the other diets and may be a more reliable way to asses pet food palatability. The four-bowl test was included in this study to investigate whether dogs and cats are able to make a decision when more than two diets are offered simultaneously, with results showing that this method is robust in its ability to detect preference.

In the four bowl- test in the current study, both species again showed a significant preference for the fermented pea diet over the unfermented pea diet. Moreover, both species showed similar food intake of the fermented pea diet compared to the Legacy/Horizon pet food. Although pea starch is also the carbohydrate source present in the Legacy/Horizon pet food, there are many differences in the formulation of this commercial diet and the lab-made unfermented pea diet. Legacy/ Horizon pet food is enriched with different protein sources,

which might explain why the dogs and cats preferred the Legacy/Horizon over the unfermented pea diet. Furthermore, Legacy/Horizon contains a different palatant than that used in the current study, which may have led to this diet being the preferred food choice compared to the unfermented pea diet.

Another interesting finding of the four-bowl test was the difference between dogs and cats on the consumption of the corn diet. The results of the current study suggest that cats have a preference for corn-based diets over pea-based diets, while dogs seem to prefer all the pea-based diets over corn. A possible explanation would be the recent discovery of functional bitter taste receptors in cats and its relationship with evolutionary defence mechanisms. Although dogs also have the ability to sense the bitter taste derived from peas, cats are genetically closest to their wild ancestors and therefore more prone to correlate the presence of anti-nutritional factors in the food with potential harm (Lei et al. 2015; John W. S. Bradshaw, 1996). Furthermore, studies show that food preference in adult cats can be highly influenced by pre-exposure to a diet and/or ingredient at earlier life stages (Becques et al., 2014b; Hepper et al., 2012; Stasiak, 2002). The cats included on the palatability trial were fed a husbandry diet containing corn prior to all experiments, which may also explain the preference that cats showed towards the corn diet in the four bowl test. Despite the fact that slight differences were found between dogs and cats in food preference, the four-bowl test results show consistent and definitive results for both species. Although more research is needed to validate the test, our results encourage the use of the four-bowl test as a new methodology to assess palatability in pets.

A strength of this study was that multiple methods were used to determine the palatability of test diets, with both the two-bowl and four-bowl tests supporting each other. Furthermore, all methods were validated with an obvious choice test in both cats and dogs. It is also important to note that a potential limitation of this study was the fact that palatants were used in all lab-made diets. This was necessary because preliminary studies showed that cats refused to consume any unfermented pea-containing diet that was made without palatant for periods of up to 5 days, even when this was offered as the only choice for all meals for the entire period (Briens 2018). In fact, the commercial pea-containing Horizon diets also contain palatants for similar reasons. However, the fact that preferences were still detectable in both

species despite the use of palatants provides evidence of the sensitivity of the methods used and strengthens our conclusions. Another limitation was the small sample size and use of a single dog breed (beagles) that are not known for being picky eaters. Furthermore, despite our efforts to answer whether yeast fermentation reduces the high levels of antinutrients present in pea starch, the reasons behind dog and cat preference for the fermented pea diet is still unclear. As mentioned before, food palatability may be enhanced by adding yeast umami compounds (Kelly S. Swanson and George C. Fahey, 2003; Kieliszek et al., 2017; Oliveira et al., 2016; Shurson, 2018). It is possible that dogs and cats were merely attracted by those substances instead of being driven by a diet containing both lower concentration of antinutrients and higher concentration of flavour enhancers. In this case, starch fermentation would be an unnecessary step, as the simple addition of *C. utilis* yeast extract would probably lead to similar positive results. Nevertheless, preferences were clearly evident, suggesting that the methods used were robust to these limitations.

In conclusion, our results suggest that *C. utilis* pea starch yeast fermentation increases food intake of pea based diets fed to dogs and cats and might be an alternative to increase pet food palatability and reduce the rate of inclusion of other ingredients used to enhance pet food palatability (e.g., yeast extract, palatants, diverse protein sources). Also, this study shows an alternative methodology to overfeeding and encourages a pre-palatability trial pilot study to customize the time allowed to complete palatability tests to the specific panel of animals available, therefore reducing gain weight and metabolic disorders derived from the excess of food consumed in preference tests. Moreover, this study found consistent results in an alternative four-bowl test to determine food preference in dogs and cats and propose further investigation to validate the test.

CHAPTER 6

This chapter compares the metabolic status and intestinal health of dogs and cats after consumption of diets formulated with 30% inclusion of corn, unfermented pea starch or fermented pea starch. The aim of this study was to investigate whether a diet formulated with yeast fermented pea starch has health benefits over either a diet formulated with regular unfermented pea starch and a diet formulated with a corn diet as a control in dogs and cats.

This chapter will be submitted to PLOS ONE. Co-authors on this manuscript will be M. de Oliveira Costa, M. Loewen, M.D. Drew, J.A. Adolphe, and L.P. Weber. The specific contributions of each co-author are as follows: P. Curso-Almeida executed all experiments including animal work, performed all biochemical assays, data analyses and wrote the manuscript; M. de Oliveira Costa performed the computational microbiome data analyses, M. Loewen and M.D. Drew were co-PIs and helped with study design; J.A. Adolphe helped with diet formulations; L.P. Weber was PI, supervised P. Curso-Almeida, helped with study design and editing of manuscripts.

EFFECTS OF FEEDING WHOLE DIETS FORMULATED WITH 30% INCLUSION OF UNFERMENTED AND FERMENTED PEA STARCH ON OVERALL AND INTESTINAL HEALTH OF DOGS AND CATS

6.1 Introduction

The gastrointestinal tract is responsible for many vital functions in the body, including food digestion and nutrient absorption. Digestion happens mainly in the stomach and duodenum, while absorption happens in the intestine. Digested nutrients will be absorbed into the bloodstream in the small intestine, while non-digestible nutrients may be fermented by microbes in the large intestine generating SCFAs such as acetate, butyrate, and propionate (Barko et al., 2018; Clarke et al., 2012; Redfern et al., 2017).

The gastrointestinal tract harbours a diverse and abundant population of microbes comprised of bacteria, archaea, fungi, protozoa, and viruses. Along with their genetic material, these resident microbes make up the gastrointestinal microbiome. In a healthy intestine, microbes and hosts develop a symbiotic relationship. The microbes contribute to gut epithelial health, immunology activity, energy homeostasis, and host metabolism, while the host offers the nutrients required for microbial growth and survival. Intestinal microbial communities are dynamic and can be changed by many factors, including diet (Barko et al., 2018; Dave et al., 2012; Suchodolski, 2011b; Tuddenham and Sears, 2015).

As a general rule, greater microbial abundance and diversity of species favours microbe-host homeostasis and benefits intestinal health. Dysbiosis is the term used to describe detrimental changes in microbial diversity and composition that compromises the harmonic host-microbe relationship. Although some of the mechanisms are still unclear, dysbiosis is associated with many gastrointestinal and systemic diseases, including obesity (Ashton et al., 2016; Bickenbach, 2015; Clarke et al., 2012). Obesity and other conditions that cause low-grade chronic inflammation may lead to changes in blood cell number and/or ratio. For instance, obese humans show higher white blood cell (WBC) count, higher neutrophil/leukocyte (NLR) ratio and higher platelet/lymphocyte ratio (PLR) (Furuncuoğlu et al., 2016; Karakaya et al., 2019). Despite being used in human medicine, few studies evaluated the use of NLR and PLR in a dog, and no studies are available for cats (Macfarlane et al., 2016;

Mutz et al., 2015). Furthermore, previous research shows that blood concentrations of adipokines (especially leptin and adiponectin) are reliable and useful tools to predict the metabolic status of humans, dogs and cats (Deng and Scherer, 2010; German et al., 2010; Ishioka et al., 2006; Ishioka et al., 2009; Zoran, 2010). If the intestinal microbiome is linked to changes in obesity in dogs and cats, then this may occur through alterations in leptin and adiponectin, although no studies to date have examined this potential linkage.

In the past, the study of the intestinal microbiome was limited to bacterial culture techniques that have now been replaced by molecular tools. High-throughput next-generation sequencing and taxonomic classification of 16S ribosomal RNA (16SrRNA) genes help to identify bacterial communities and to determine the number and diversity of species present in the intestinal tract (Kim et al., 2017). In this technique, microbial genetic material (RNA or DNA) is extracted from intestinal samples, and a conserved target region of the gene is amplified using universal primers. Subsequently, phylogenetic identification is achieved by sequencing this amplified target region. Because the 16SrRNA gene is present in almost all bacteria and its function has not changed over time, the 16S rRNA is the gene commonly targeted to study bacterial communities (Claesson et al., 2017; Janda and Abbott, 2007; Kim et al., 2017; Suchodolski, 2011b). The 16SrRNA gene is composed of nine hypervariable regions separated by nine highly conserved regions. The current sequencing techniques do not allow full-length sequencing of the 16SrRNA gene, and therefore, hypervariable regions must be selected for phylogenetic analysis and taxonomic classification (Claesson et al., 2017; Janda and Abbott, 2007). Different hypervariable regions have been used in studies evaluating the intestinal microbial community of dogs and cats, which presents difficulties in comparing among studies (Hooda et al., 2012; Suchodolski, 2011a). Although some debate exists about the most efficient hypervariable region, research has pointed to the V4 region as the most sensitive marker for bacterial analysis (Janda and Abbott, 2007).

Candida utilis is an important yeast used in biotechnology food processing, and it is used in the development of human functional foods. The yeast cell wall corresponds to 20-30% of its total biomass, and it is constituted by polysaccharides such as beta-glucans (1,3 and 1,6), mannoproteins, chitin and glucomannans (Boze et al., 1992; Kieliszek et al., 2017). Yeast-derived beta-glucans have been shown to benefit human health by lowering cholesterol levels

and displaying anti-inflammatory properties (Kieliszek et al., 2017). Likewise, glucomannans prevent cellular damage by reducing the production of reactive oxygen species (ROS). In addition, components of the yeast cell wall (glucomannan, beta-glucan, and mannoproteins) are recognized as pathogen-related patterns by receptors present in the intestinal immune system, therefore, enhancing immunological functions (Kieliszek et al., 2017). The fiber components found in the yeast cell wall cannot be digested by the enzymes present in the gastrointestinal tract of mammals and are then fermented by intestinal microbes, generating SCFA (e.g. acetate, propionate and butyrate) that are important for intestinal health (Rai et al., 2019). All of these factors justify investigating the use of *Candida utilis* in the processing of functional foods for dogs and cats, with the fermentation of pea starch to be included at 30% in whole diets as the test substrate being the focus of this study.

Diets formulated with pea starch have been previously shown to exhibit health benefits compared to diets formulated with grains such as corn or rice in dogs and cats (Adolphe et al., 2014; Briens et al., 2018). The hypothesis of the current study is that the use of fermented pea starch will further improve health benefits compared to unfermented pea starch diets, both of which will be better than corn-based diets. This lab-based study used a cross-over design in beagle dogs and domestic cats, with each feeding period on each diet being 20 days, to investigate changes in general health (blood biochemistry, CBC), metabolic status (weight, plasma leptin and adiponectin) and intestinal health (fecal SCFA and microbial communities).

6.2 Material and Methods

6.2.1 Animals and feeding

Seven adult, neutered/spayed mixed breed, mixed sex cats weighing 4.8 ± 0.5 kg and eight adult, neutered, mixed sex beagle dogs weighing 10.8 ± 0.7 kg were included in this study. The dogs were obtained from certified scientific breeders (Marshall Farms, North Rose, NY and Liberty Research, Waverly, NY, respectively) and were kept at the Western College of Veterinary Medicine at the University of Saskatchewan (Saskatoon, Saskatchewan, Canada). The dogs were group-housed during the day, but kept in individual kennels during

feeding and at night. Fresh water was provided *ad libitum*. Each individual dog kennel has free access to an outdoor area. In addition, the dogs had daily playtime with volunteers and daily walks outdoors on a leash. During the day, the cats were allowed to roam freely within the common areas of the animal care facility and had access to a common outdoor courtyard with grass and scratching/climbing structures. At night the cats were housed individually with fresh water *ad libitum.* Both species were fed species-specific whole balanced standard commercial dry pet food (Hill's Science Diet, Hill's Pet Nutrition, Inc. Topeka, USA), except for the feeding trial periods where test diets were fed. Prior to the start of the study, the daily caloric need was individually calculated based on previous experience and was limited to the calories/day needed to maintain that animal in an optimal body score condition following the National Research Council guidelines (National Research Council, 2006).

Each animal was fed daily portions of test diets with equivalent ME to that calculated to maintain optimal body condition prior to the start of experiments. During the trial, dogs and cats received either a diet with 30% inclusion of corn (control), unfermented pea starch or fermented pea starch for 20 days. Dogs and cats received the corn diet for 15 days in a washout period before each test of the two pea-based diets. This work was approved by the University of Saskatchewan Animal Research Ethics Board following the Canadian Council on Animal Care guidelines.

6.2.2 Diets

Three lab-made diets were used in this study (see Table 6.1 for ingredients and inclusion rates). The lab-made diets were formulated to have similar composition, differing just in the carbohydrate source. The same diets were fed to dogs and cats. Thus, the diets were formulated to meet the higher minimum protein, taurine and other nutritional needs of cats. While the lab-made diets exceeded nutritional requirements for dogs, they resembled many of the premium dog diets on the commercial market (Carciofi et al., 2006; Dzanis, 1994, 2008). Test diets were formulated with unfermented pea starch (Parrheim Foods, Saskatoon, SK) or used the fermented pea starch used previously in this thesis (see study 1). Cornstarch was purchased from a local grocery (Whole Sale Club, Saskatoon, SK Canada). See Table 6.1 for

a complete list of lab-made test diet ingredients and inclusion rates. Proximate analyses for macronutrient composition, available energy and acid insoluble ash (Celite) for each lab-made (see Table 6.2) were done at a commercial testing lab (Central Testing, Winnipeg, MB).

Table 6. 1 Lab-made diets formulated for the feeding trial with ingredients listed in decreasing order of inclusion

Ingredient	Corn Diet (%)	Unfermented Pea Diet (%)	Fermented Pea Diet (%)
Carbohydrate source	30.08	31.92	31.92
Chicken meal	35.78	25.46	25.46
Soy protein concentrate	9.31	15	15
Chicken fat with Dadex T	7.75	9.76	9.76
Pea fibre (PetFine®)	6.88	4.96	4.96
Fish meal, mixed	5.0	5.0	5.0
Liquid palatant (AFB LC647 LQDGST)	2.0	2.0	2.0
Fish oil	2.0	2.0	2.0
Celite	1.0	1.0	1.0
Potassium chloride	0.9	0.88	0.88
Sodium chloride	0.3	0.5	0.5
Dry palatant (AFB F24047 Dry cat)	0.5	0.5	0.5
Calcium carbonate	-	0.41	0.41
Choline chloride	0.1	0.2	0.2
Methionine/DL	0.1	0.1	0.1
Mineral premix, dog cat	0.1	0.1	0.1
Vitamin premix, cat	0.1	0.1	0.1
Taurine	0.1	0.1	0.1
Dicalcium phosphate	-	0.01	0.01

Table 6. 2 Proximate analyses of test diets formulated for the feeding trial

Nutrient (%DM)	Control (corn) diet	Unfermented pea diet	Fermented pea diet
Protein	38.4	37.4	37.9
Fat	14.2	15.7	12.7
Carbohydrates	32.0	30.9	32.7
Fibre	3.2	4.8	4.9
ME (Kcal/Kg)	3,760	3,748	3,550

DM = dry matter; ME = metabolizable energy

Diets were formulated by Dr. Jennifer Adolphe using Concept 5 software (Creative Formulation Concepts, LLC, MD, USA) according to AAFCO nutrient profiles (The Association of American Feed Control Officials, 2014). The diets were extruded at the Food Science and Technology Centre (FSTC) in Brooks, Alberta, Canada, using a Coperion ZSK57 extruder (Werner & Pfleiderer, Ramsey, NJ, USA). The extruder consisted of 8 barrels on which the temperature was independently controlled by Mokon oil heaters (HC5X12-Q1, Mokon, Buffalo, NY, USA). At the end of the process, the pellets were coated with a mixture of chicken fat and fish oil with a fluidizing paddle blender (FPB-20, American Process Systems, Gurnee, IL, USA). Finally, the diets were coated first with a commercial liquid palatant (AFB LC647 LQDGST), followed by a dry palatant (AFB F24047 Dry Cat) at the Canadian Feed Research Centre in North Battleford, SK.

6.2.3 Sample collection and handling

6.2.3.1 Total apparent digestibility testing

Animal feces were collected at the end of each feeding trial period (day 15 to 20) using the index method (Zhang and Adeola, 2017). The samples were dried in a forced-air oven at 65ºC for 72 hours and then ground (Vasconcellos et al., 2007).

Apparent digestibility was determined according to the following equation (Zhang and Adeola, 2017):

$$Digestibility\ (\%) \ = \ 100 \ - \ \left[100 \ x \ \left(\frac{M\ feed\ x\ C\ feces}{M\ feces\ x\ C\ feed} \right) \right]$$

Where *Mfeed* and *Mfeces* represent concentrations of index compound in feed and feces, respectively; *Cfeed* and *Cfeces* represent concentrations of components in feed and feces, respectively.

6.2.3.2 Short-chain fatty acids (SCFAs)

From day 15 to 20, a portion of the fresh feces collected was used to measure SCFAs. To avoid loss of volatile compounds, 1 gram of feces were immediately placed in a sealed tube containing a mixture of water and 25% m-phosphoric acid (4:1). After vortexing, samples were allowed to precipitate for 30 minutes and then centrifuged at 20,000 x g for 20 minutes (Gagné et al., 2013). The samples were kept at -80ºC before being sent to the College of Agriculture and Bioresources (University of Saskatchewan, SK, Canada), where SCFA concentrations were determined by gas chromatography (Friedt et al., 2014).

6.2.3.3 Food Intake, Body Weight and Body Condition Score

At day 20 of each trial period, animals body weight was obtained and body condition score (BCS) was assessed by the same individual using a 9 point scale (Laflamme DP, 1997a, 1997b). The remaining food was weighed and subtracted from the total offered to determine daily food intake.

6.2.3.4 Blood Collection

After 20 days of feeding each diet, blood collected from the cephalic vein of fasted dogs and cats was sent to Prairie Diagnostic Services (University of Saskatchewan, SK, Canada) for total blood cell count and biochemistry profile. Additional blood samples were placed in EDTA tubes, centrifuged at 5000 x g for 10 min and plasma frozen at -80°C until used to determine adipokine levels and total triglycerides.

6.2.3.4.1 Adipokines

Dog adiponectin was determined using a species-specific ELISA kit (Circulex dog adiponectin ELISA kit, MBL international, Massachusetts, USA). Cat adiponectin was determined using a human ELISA kit validated for cats (Tvarijonaviciute et al., 2012c; Adiponectin Sandwich High Sensitivity, Biovendor, Brno, Czech Republic). Dog and cat leptin plasma concentrations were determined using a canine ELISA assay (Canine Leptin ELISA kit, Millipore Sigma, Oakville, ON, Canada).

6.2.3.4.2 Total Triglycerides

Plasma total triglycerides of dogs and cats were determined using a glycerol kinase-based method for the colorimetric determination of triglycerides (McGowan et al., 1983; Sigma-Aldrich, Oakville, ON, Canada).

6.2.3.5 Microbiome Analyses

Sterile dry transport swabs were used to collect rectal samples from dogs and cats. Samples were kept at -80ºC until processing at the University of Minnesota Genomics Center (UMGC- Minnesota, MN, USA). DNA content from swabs was extracted using the PowerSoil® Max DNA Kit (Mo Bio, Carlsbad, California, USA) following the manufacturer's instructions. Reagent blanks were included in the extraction process and remained free from DNA contamination during this process. Total DNA extracted from samples was measured prior to library preparation using both NanoDrop spectrophotometer (ND-1000, NanoDrop Technology, Wilmington, Delaware, USA) and PicoGreen dsDNA assay (Life Technologies, Carlsbad, CA, USA). Sequencing was performed using a protocol previously described (Callahan et al., 2016). Briefly, amplification of the 16S rRNA gene V3-V4 hypervariable region used a KAPA HiFidelity Hot Start Polymerase (Kapa Biosystems Inc., Wilmington,

MA, USA) and Nextera primers (Meta_V3_F_Nextera: 5′-CCTACGGGAGGCAGCAG-3′, Meta_V4_806_R: 5′-GACTACHVGGGTWTCTAAT-3′, Integrated DNA Technologies, Coralville, IA, USA). The first round of amplification used the following cycling parameters: one cycle of 95°C for 5 minutes, followed by 20 cycles of 98°C for 20 seconds, 55°C for 15 seconds, and 72°C for 1 minute. The second round used indexing primers (F: 5′-AATGATACGGCGACCACCGAGATCTACAC[i5]TCGTCGGCAGCGTC-3′, R: 5′-CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG-3′) and the following cycling conditions: 1 cycle at 95°C for 5 minutes, followed by 10 cycles of 98°C for 20 seconds, 55°C for 15 seconds, and 72°C for 1 minute. We chose to verify whether amplification was successful by performing electrophoresis of amplicons on 1% agarose. Pooled, size-selected samples were denatured with NaOH, diluted to 8 pM in Illumina's HT1 buffer, spiked with 15% PhiX, and heat denatured at 96°C for 2 minutes immediately prior to loading. The MiSeq 600 (2×300 base pairs, bp) cycle v3 kit (Illumina, San Diego, CA, USA) system was used to sequence DNA libraries. Data were analyzed using QIIME2 (Bolyen et al., 2019).

6.2.4 Data analyses

Sequencing data were demultiplexed and sorted by barcodes to generate fastq files for each individual sample. Raw amplicon reads were filtered by trimming the first 13 bp, truncating forward and reverse reads at 150 bp using the Dada2 plug-in(Callahan et al., 2016). Filtered reads were dereplicated and denoised using parameters estimated for this dataset. Forward and reverse sequences were merged using a minimum overlap of 20 bp and 0 mismatches allowed with Dada2. Representative sequences were classified against the SILVA SSU Ref NR dataset v.128 at 98% sequence similarity using a classifier algorithm trained for this work's dataset(Wang et al., 2007). Next, contaminant sequences (non-bacterial, mitochondrial or chloroplast DNA) and sequences not classified beyond the kingdom level were removed from the dataset using the quality control plug-in.

Statistical analyses were performed using IBM SPSS version 24 (IBM Corp., Armonk, NY). When data was determined to be normally distributed using a Kolmogorov-Smirnov test, a paired t-test (digestibility) or one-way repeated measures ANOVA (SCFA, blood work, BW,

food intake, triglycerides, NLR and PLR) were conducted. Friedman's ANOVA was performed on data that failed normality (BCS). Post-hoc analyses were performed using Fisher's LSD or Tukey's test. Differences were considered significant at $P<0.05$. DNA. Sequencing data was analyzed separately using Phyloseq, as described in the literature (McMurdie and Holmes, 2013). Alpha diversity was determined using Friedman's test, followed by Dunn's multiple comparison post hoc tests. Beta diversity analyses were conducted using PERMDISP and PERMANOVA. Differences in taxa abundance were determined using Friedman's test, followed by Dunn's multiple comparison tests. False discovery ratio (FDR > 2-fold) correction was applied to the calculated p values. Differences were considered significant at p and q <0.05.

6.3 Results

6.3.1 Total tract apparent digestibility

In comparison to the digestibility values determined for the unfermented pea diet, dogs fed the fermented pea diet show significantly increased protein and starch digestibility, but no change in fat digestibility (Table 6.3). Digestible energy was also significantly higher in dogs fed the fermented pea diet compared to the unfermented pea diet. Compared to the unfermented pea diet, cats show significant increases in only starch digestibility when fed the fermented pea diet (Table 6.3).

Table 6. 3 Apparent digestibility coefficient for dogs and cats fed the unfermented or fermented pea diets

Species	Diet	Protein (%DM)	Fat (% DM)	Starch (% DM)	DE (kcal/kg)
Dog	UPD	84 ± 0.8^a	98 ± 0.1	81 ± 1.0^a	83 ± 0.5^a
	FPD	86 ± 0.4^b	98 ± 0.1	86 ± 0.2^b	84 ± 3.0^b
Cat	UPD	83 ± 0.7	95 ± 1.0	85 ± 1.0^a	82 ± 1.0
	FPD	85 ± 0.4	97 ± 0.2	89 ± 0.4^b	84 ± 0.3

Data is mean $\pm$ SEM. Dogs n=8, cats n=7. P <0.05, paired-sample T-test. UPD=unfermented pea diet, FPD=fermented pea diet, DE= digestible energy, DM= dry matter. Values in a column with superscripts without a common letter differ

6.3.2 Food intake, body weight and body condition score

No difference in dog food intake was found regardless of the diet being fed. Cats showed significantly reduced food intake when fed the unfermented pea diet compared to both corn and fermented pea diets (Figure 6.1) . No significant differences in body weight and body condition score were found in dogs fed either pea based diets or the control corn diet for 20 days. Conversely, in comparison with the corn diet, significant weight loss was observed when cats were fed the unfermented pea diet, with the fermented pea diet producing an intermediate body weight. Despite the differences observed in body weight, no significant difference was found in cats body condition score after feeding the different diets (Tables 6.4 and 6.5).
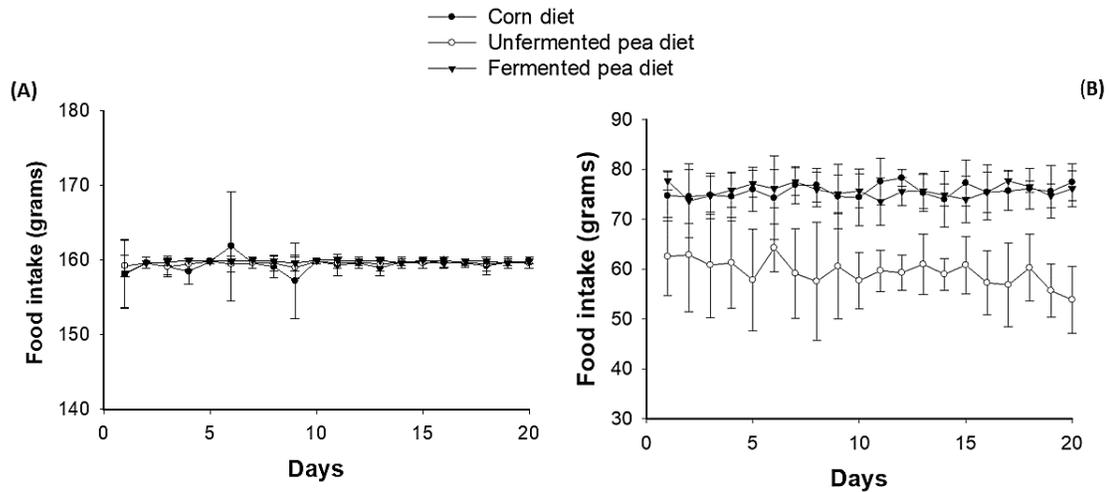
Figure 6. 1 Daily food intake. Values are mean ± SEM for (A) dogs, n=8 and (B) cats, n=7. P < 0.05. One-way repeated measures ANOVA followed by Tukeys test.

Table 6.4 Body weight of dogs and cats after being fed test diets for 20 days

Species	Corn diet	UPD	FPD
Dogs	10.8 ± 0.2	10.6 ± 0.2	10.7 ± 0.2
Cats	4.8 ± 0.2[a]	4.6 ± 0.3[b]	4.7 ± 0.2[ab]

Data is mean ± SEM, n=8 for dogs and n=7 for cats. One-way repeated measure ANOVA, p< 0.05. Values in a row with superscripts without a common letter differ. UPD=unfermented pea diet, FPD=fermented pea diet

Table 6.5 Body condition score of dogs and cats after being fed test diets for 20 days

Species	Corn diet	UPD	FPD
Dogs	6.1 ± 0.2	5.8 ± 0.2	5.6 ± 0.2
Cats	5.5 ± 0.2	5.0 ± 0.3	5.2 ± 0.2

Data is mean ± SEM, n=8 for dogs and n=7 for cats. Friedman's ANOVA test, p< 0.05. No significant differences. UPD=unfermented pea diet, FPD=fermented pea diet

6.3.3 Total blood cell count and biochemistry profile

Despite some significant changes in blood biochemistry and cell counts, except for globulin, all values for all dogs after being fed all diets for 20 days were within the reference range. In cats, all the values except for cholesterol were within the reference range. The values for cholesterol in cats were already outside the reference range before starting this feeding trial. For changes, some of the most notable was that dogs showed a significant decrease in serum amylase concentration with the fermented pea diet (Table 6.6). Because amylase concentration has no clinical application for cats, this parameter was not determined in cats (Xenoulis and Steiner, 2008). In comparison to the corn diet and the fermented pea diet, dog plasma cholesterol levels were significantly lower when they received the unfermented pea diet (Table 6.6). Likewise, cats also showed lower cholesterol levels when fed the unfermented pea diet followed by the fermented and corn based diet. Fasting blood glucose concentration was higher in cats fed the fermented pea diet followed by the unfermented and corn based diet. No differences in fasting dog blood glucose concentration were observed. In comparison to both pea based diets, the blood concentration of urea increased, while creatinine levels decreased in cats after eating the corn diet compared to either pea diet (Table 6.7). No difference was observed in blood urea or creatinine concentration in dogs regardless of diet. Cats fed the corn diet show lower bilirubin concentration than when they consumed the pea based diets. No difference in blood bilirubin concentration was found in dogs. Total white blood cell count (WBC) was lower in dogs fed both pea based diets in comparison to corn. No difference in WBC count was observed in cats regardless of diet.

Table 6.6 Dog blood cell count and biochemistry profile after feeding test diets for 20 days

Blood parameters	Reference range	Corn diet	UPD	FPD
Sodium (mmol/l)	140 - 153	147 ± 0.4	147 ± 0.3	148 ± 0.2
Potassium (mmol/L)	3.8 - 5.6	4.6 ± 0.06	4.6 ± 0.05	4.6 ± 0.06
Chloride (mmol/L)	105-120	112 ± 0.4	111 ± 0.4	111 ± 0.4
Bicarbonate (mmol/L)	15 -25	20 ± 0.7	20 ± 0.6	22 ± 0.5
Anion gap (mmol/L)	12-26	19 ± 0.6	20 ± 0.8	19 ± 0.4
Calcium (mmol/L)	1.91-3.03	2.4 ± 0.02	2.5 ± 0.02	2.5 ± 0.02
Phosphorus (mmol/L)	0.63-2.41	1.2 ± 0.05	1.1 ± 0.05	1.2 ± 0.04
Magnesium (mmol/L)	0.70-1.16	0.8 ± 0.01	0.7 ± 0.01	0.8 ± 0.01
Urea (mmol/L)	3.5-11.4	5.3 ± 0.2	5.2 ± 0.2	5.5 ± 0.4
Creatinine (mmol/L)	41-121	65 ± 1	62 ± 2	60 ± 5
Amylase (U/L)	343-1375	523 ± 24^a	516 ± 27^a	468 ± 17^b
Lipase (U/L)	25-353	56 ± 7	52 ± 8	43 ± 5
Glucose (mmol/L)	3.1-6.3	4.8 ± 0.10	4.9 ± 0.10	4.8 ± 0.08
Cholesterol (mmol/L)	2.7- 5.94	4.5 ± 0.2^a	4.1 ± 0.2^b	4.4 ± 0.2^a
Total bilirubin (μmol/l)	1.0-4.0	1.3 ± 0.3	1.4 ± 0.1	1.7 ± 0.2
Direct bilirubin (μmol/l)	0.0- 2.0	0.3 ± 0.10	0.6 ± 0.06	0.6 ± 0.09
Indirect bilirubin (μmol/l)	0.0- 2.5	1 ± 0.1	0.8 ± 0.1	1 ± 0.1
ALK phosphatase (U/L)	9-90	29 ± 5	25 ± 3	25 ± 3
GGT (U/L)	0-8	0.8 ± 0.4^a	2.3 ± 0.4^b	2.7 ± 0.3^b
ALT (U/L)	19-59	27 ± 4	23 ± 1	24 ± 2
CK (U/L)	51-418	188 ± 25	180 ± 15	182 ± 16
Total protein (g/L)	55-71	56 ± 0.8	56 ± 0.8	56 ± 0.7
Albumin (g/L)	32-42	35 ± 0.6	36 ± 0.5	36 ± 0.6
Globulin (g/L)	20-34	20 ± 0.5	19 ± 0.5	20 ± 0.3
A:G ratio	1.06-1.82	1.7 ± 0.05	1.8 ± 0.04	1.7 ± 0.04
WBC (x10^9/L)	4.9-15.4	6.0 ± 0.3^a	4.7 ± 0.2^b	4.7 ± 0.3^b
RBC (x10^{12}/L)	5.8-8.5	6.0 ± 0.2	6.0 ± 0.1	6.0 ± 0.1

Data is mean ± SEM, n=8, $p < 0.05$; One-way repeated measures ANOVA post hoc LSD. Values in a row with superscripts without a common letter differ. UPD = unfermented pea diet; FPD = fermented pea diet; ALK= alkaline phosphatase, GGT= gama-glutamyl transferase, ALT= alanine transaminase, CK= creatine kinase, A:G ratio= albumin/globulin ratio, WBC= white blood cells, RBC= red blood cells

Table 6.7 Cat blood cell count and biochemistry profile after feeding test diets for 20 days

Blood parameters	Reference range	Corn diet	UPD	FPD
Sodium (mmol/l)	147-160	152 ± 0.6^a	154 ± 0.6^b	153 ± 0.4^b
Potassium (mmol/L)	3.9-5.5	4.7 ± 0.05	4.7 ± 0.09	4.5 ± 0.10
Chloride (mmol/L)	111-125	116 ± 0.9^a	118 ± 0.7^b	116 ± 0.4^{ab}
Bicarbonate (mmol/L)	11 -22	14 ± 0.8^a	17 ± 0.4^b	17 ± 0.2^b
Anion gap (mmol/L)	15-30	27 ± 0.9^a	23 ± 0.4^b	23 ± 0.5^b
Calcium (mmol/L)	2.26-2.86	2.4 ± 0.02	2.4 ± 0.02	2.5 ± 0.03
Phosphorus (mmol/L)	1.08-2.21	1.5 ± 0.06	1.4 ± 0.04	1.4 ± 0.05
Magnesium (mmol/L)	0.74-1.12	0.9 ± 0.01^a	0.9 ± 0.01^{ab}	0.8 ± 0.01^b
Urea (mmol/L)	6-11.4	7.6 ± 0.3^a	6.8 ± 0.4^b	7.2 ± 0.3^{ab}
Creatinine (mmol/L)	78-178	118 ± 2.8^a	149 ± 3.4^b	153 ± 2.3^b
Glucose (mmol/L)	3.5-8.1	3.9 ± 0.2^a	4.3 ± 0.2^{ab}	4.5 ± 0.2^b
Cholesterol (mmol/L)	1.62-4.32	7.1 ± 0.5^a	4.5 ± 0.4^b	5.0 ± 0.5^c
Total bilirubin (μmol/l)	0.0-3.0	0.3 ± 0.06^a	0.7 ± 0.07^b	0.7 ± 0.10^{ab}
GGT (U/L)	0-6	0	0	0
ALT (U/L)	22-90	64 ± 8	59 ± 7	52 ± 4
GLDH (U/L)	1-5	2.7 ± 0.4	4.2 ± 0.4	3.1 ± 0.8
CK (U/L)	75-471	281 ± 59^{ab}	151 ± 16^a	125 ± 16^b
Total protein (g/L)	53-84	77 ± 2^a	69 ± 3^b	76 ± 2^a
Albumin (g/L)	28-43	35 ± 0.6^{ab}	35 ± 1.0^a	38 ± 0.9^b
Globulin (g/L)	23-45	41 ± 1^a	34 ± 2^b	38 ± 1^a
A:G ratio	0.77-1.64	0.8 ± 0.03^a	1 ± 0.05^b	1 ± 0.03^b
WBC (x10^9/L)	4.2-13	10 ± 0.8	11 ± 0.8	12 ± 1.0
RBC (x10^{12}/L)	6.2-10.6	10 ± 0.4	10 ± 0.5	10 ± 0.5

Data is mean ± SEM, n=7, $p < 0.05$; One-way repeated measures ANOVA post hoc LSD. Values in a row with superscripts without a common letter differ. UPD = unfermented pea diet; FPD = fermented pea diet; GGT= gama-glutamyl transferase, ALT= alanine transaminase, GLDH= glutamate dehydrogenase, CK= creatine kinase, A:G ratio= albumin/globulin ratio, WBC= white blood cells, RBC= red blood cells

6.3.4 Total Triglycerides

When compared to the values obtained after receiving the corn diet, plasma levels of total triglycerides from dogs and cats significantly decreased after consuming both pea based diets for 20 days (Table 6.9).

Table 6.8 Total triglycerides of dogs and cats after feeding test diets for 20 days

Triglycerides (mg/dL)	Reference range*	Corn diet	UPD	FPD
Dogs	29-291	481.5 ± 50^a	225.3 ± 30^b	186.6 ± 19^b
Cats	25-160	340.0 ± 66^a	167.5 ± 29^b	161.2 ± 15^b

*(Klaassen, 1999). Values are mean ± SEM, n=8 for dogs and n=7 for cats, $P < 0.05$, One-way repeated measures ANOVA, post-hoc LSD. Values in a row with superscripts without a common letter differ UPD = unfermented pea diet; FPD = fermented pea diet

6.3.5 Neutrophil-lymphocyte ratio (NLR) and platelet-lymphocyte ratio (PLR)

Higher total counts of blood neutrophils and lymphocytes were found in dogs fed the corn diet compared to both pea based diets, without any difference between fermented and unfermented pea diet results (Table 6.10). In contrast, no significant differences were observed in total blood platelets count and NLR regardless of diet. Dogs fed both pea based diets show similarly higher PRL than dogs fed corn (Table 6.10). In cats, no significant differences were observed in the total count of blood neutrophils, lymphocytes or NLR among diets. However, the PLR value was lower when cats consumed the fermented pea diet compared to either corn and unfermented pea diet (Table 6.11).

Table 6.9 Dog neutrophil, lymphocyte and platelet counts plus NLR (neutrophil:lymphocyte rate) and PLR (platelets:lymphocyte rate) after feeding test diets for 20 days

Blood Parameter	Corn diet	UPD	FPD
Neutrophils (x10^9/L)	3705 ± 234[a]	2882 ± 277[b]	3009 ± 244[b]
Lymphocytes (x10^9/L)	1909 ± 216[a]	1390 ± 106[b]	1358 ± 112[b]
Platelets (x10^9/L)	224 ± 19	228 ± 26	225 ± 36
NLR	2.0 ± 0.2	2.2 ± 0.3	2.3 ± 0.2
PLR	0.12 ± 0.1[a]	0.17 ± 0.2[b]	0.17 ± 0.1[b]

Data is mean ± SEM, n=8, $p < 0.05$; One-way repeated measures ANOVA. Values in a row with superscripts without a common letter differ. UPD = unfermented pea diet; FPD = fermented pea diet; NLR= neutrophil/ lymphocyte ratio, PLR= platelet/ lymphocyte ratio.

Table 6.10 Cat neutrophil, lymphocyte and platelet total counts plus NLR (neutrophil: lymphocyte rate) and PLR (platelets:lymphocyte rate) after feeding test diets for 20 days

Blood Parameter	Corn diet	UPD	FPD
Neutrophils (x10^9/L)	5177 ± 778	4960 ± 472	5321 ± 532
Lymphocytes (x10^9/L)	4584 ± 790	4918 ± 748	5504 ± 1044
Platelets (x10^9/L)	224 ± 19	228 ± 26	225 ± 36
NLR	1.5 ± 0.5	1.2 ± 0.3	1.1 ± 0.2
PLR	0.06 ± 0.07[a]	0.06 ± 0.10[a]	0.04 ± 0.09[b]

Data is mean ± SEM, n=7, $p < 0.05$; One-way repeated measures ANOVA. Values in a row with superscripts without a common letter differ. UPD = unfermented pea diet; FPD = fermented pea diet; baseline = corn diet; NLR= neutrophil/lymphocyte ratio, PLR= platelet/ lymphocyte ratio.

6.3.6 Adipokines

No significant changes in adiponectin concentration were observed in dogs or cats regardless of diet). Conversely, dog leptin concentration significantly decreased after 20 days of consumption of the unfermented pea diet compared to the control corn diet, but after feeding the fermented pea diet, leptin was further decreased in dogs after 20 days (Figure 6.2). In contrast, in cats, both pea diets decreased leptin concentrations to a similarly significant extent compared to the corn based diet (Figure 6.2).
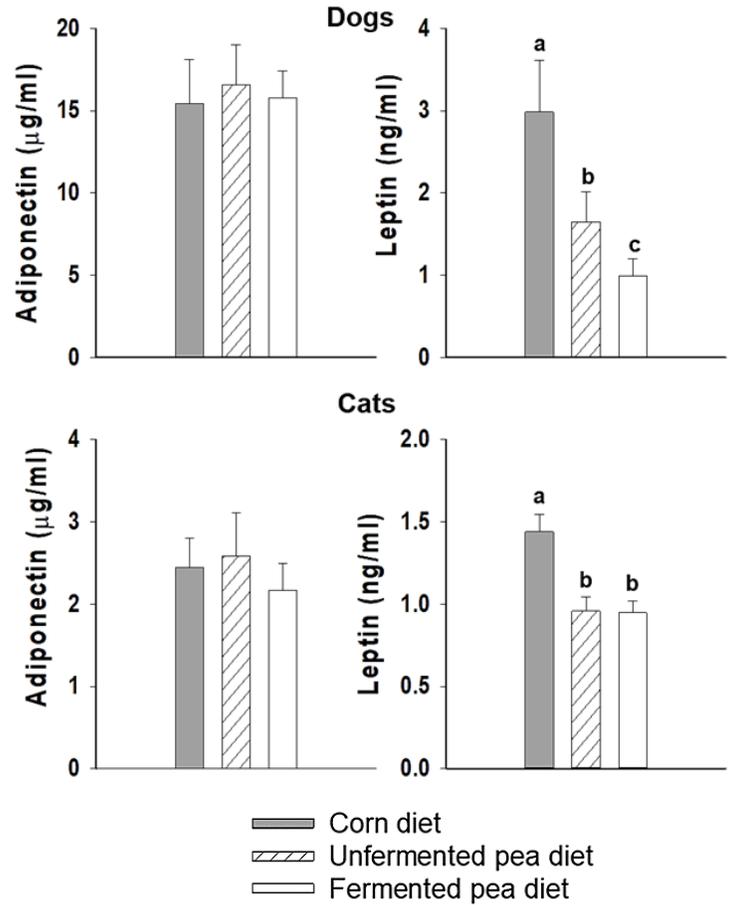
Figure 6. 2 Adipokine levels of dogs and cats after feeding test diet for 20 days. Values are mean +/- SEM, n= 8 for dogs and n=7 for cats. $P < 0.05$. One-way repeated measures ANOVA, post-hoc LSD. Bars with superscripts without a common letter differ

6.3.7 Short-Chain Fatty Acids

No significant differences were found in fecal SCFA concentration from dogs fed either test diet compared to the corn based diet for 20 days (Figure 6.3). Fecal acetate and propionate levels also did not significantly differ in cats after being fed pea based diets or the control diet for 20 days. However, compared to both pea based diets, cats show higher concentrations of fecal butyrate after consuming the corn diet for 20 days. No significant differences in fecal butyrate concentrations were found in cats fed fermented versus unfermented pea based diets (Figure 6.4).
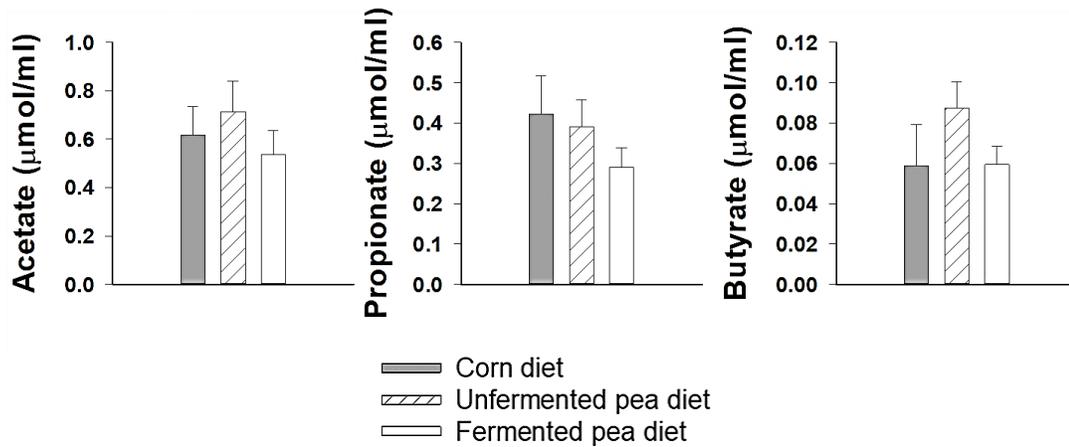
Figure 6. 3 Fecal short-chain fatty acid concentration of dogs after being fed test diets for 20 days. Values are mean ± SEM, n=8. No significant differences detected using one-way repeated measures ANOVA, p< 0.05.
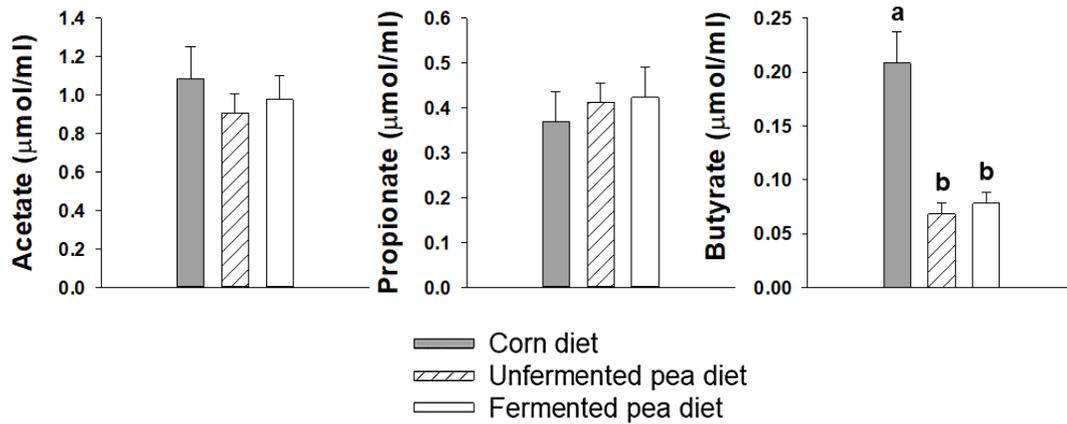
Figure 6. 4 Fecal short-chain fatty acids concentration of cats after being fed test diets for 20 days. Values are mean ± SEM, n=7. One-way repeated measures ANOVA, post-hoc LSD, p< 0.05. Bars with superscripts without a common letter differ.

6.3.8 Microbiome Sequencing

6.3.8.1 Relative frequency of each major bacteria phylum present in the intestine of dogs and cats fed corn, unfermented or fermented pea diet

Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria and Proteobacteria were the major bacteria phylum found in the intestine of dogs and cats (Figures 6.5 and 6.6, respectively). Dogs showed an increased population of intestinal bacteria belonging to the phylum Fusobacteria when fed both pea diets compared to the corn diet. No apparent differences in relative frequency was observed in intestinal bacterial composition of cats fed corn or unfermented peas. However, similar to dogs, cats fed the fermented pea diet show increased intestinal Fusobacteria.
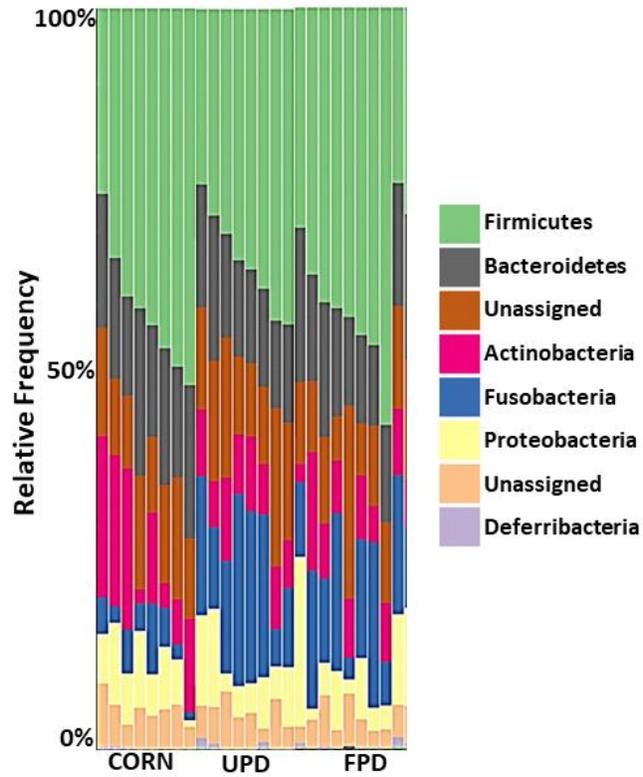
Figure 6. 5 Median percent of Operational Taxonomic Unit (OTU) representing bacterial phylum in the stool of dogs (n=8) fed different diets for 20 days: UPD= unfermented pea diet, FPD= fermented pea diet or corn. The relative abundances are inferred from 16S rRNA. The X and Y axes represent the sample name and percentages of bacterial taxa, respectively.
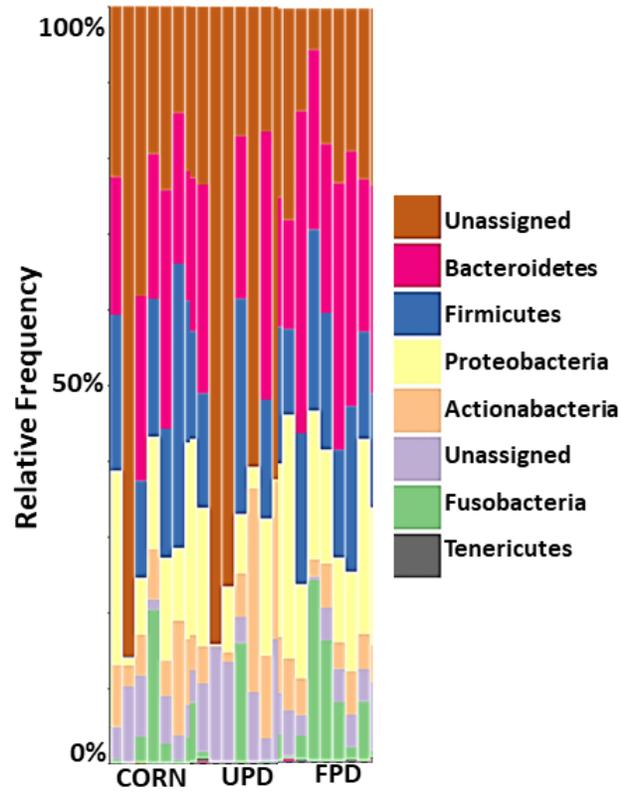
Figure 6. 6 Median percent counts of Operational Taxonomic Units (OTU) representing bacterial phylum in the stool of cats (n=7) fed different diets for 20 days: UPD = unfermented pea diet, FPD = fermented pea diet or corn = control diet. The relative abundances are inferred from 16S rRNA. The X and Y axes represent the sample name and percentages of bacterial taxa, respectively.

6.3.8.2 Alpha and beta diversity

Alpha diversity (richness and evenness) of species comprising the intestinal microbiome increased in dogs fed both pea diets in comparison to corn (Figure 6.7A). Likewise, beta diversity differed when dogs consumed both pea diets in comparison to corn (Figure 6.8A). No differences were found in alpha or beta diversity of cats fed either of the pea based test diets (Figures 6.7 B and 6.8B).
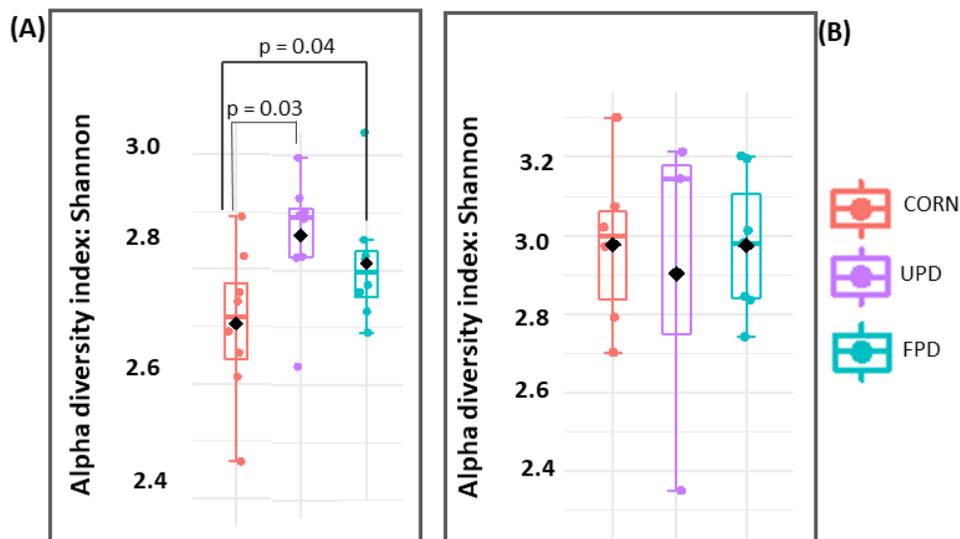
Figure 6. 7 Alpha diversity of intestinal microbiome after 20 days of feeding test diets: Shannon Index based on Operational Taxonomic Unit (OTU) abundance for (A) dogs (n=8) and (B) cats (n=7) fed different diets, UPD= unfermented pea diet, FPD= fermented pea diet. P < 0.05, Friedman test followed by Dunn's multiple comparison. The black diamond represents the mean and the middle line represents the median.
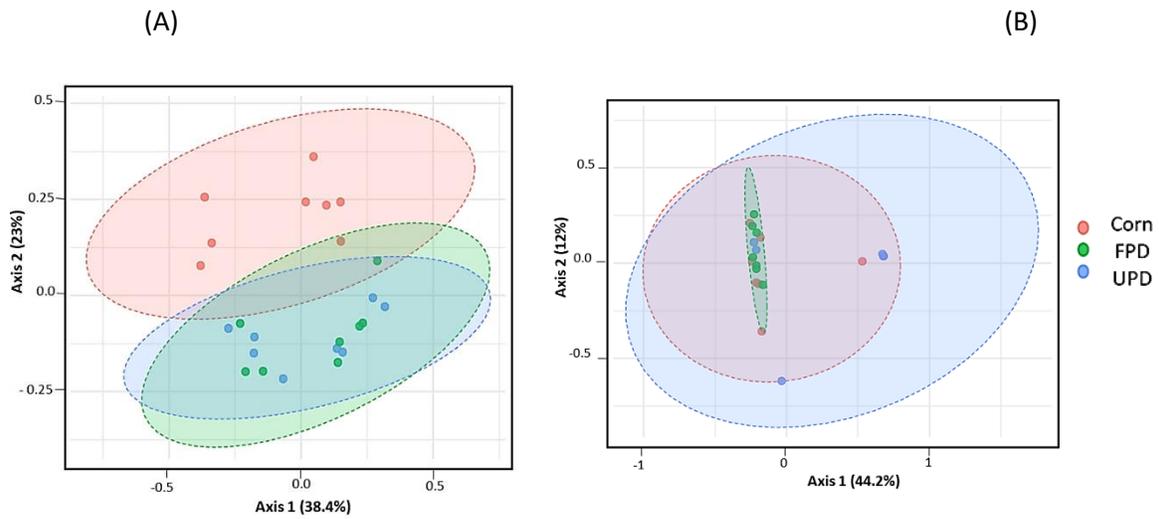
Figure 6. 8 Beta diversity of intestinal microbiome after 20 days of feeding test diets: Principal Components Analysis plot of weighted UniFrac distances for (A) dogs (n=8) and cats (n=7) fed different diets, UPD= unfermented pea diet, FPD= fermented pea diet. A significant (p < 0.05, PERMDISP and PERMANOVA) difference among diets was found in dogs but not cats.

6.3.8.3 Differentially abundant taxa

Differences in taxa abundance of intestinal microbiome after 20 days of feeding test diets were only determined in dogs due to the low quality of the samples collected from cats fed the unfermented pea diet. The microbes (genus) included in this section were the ones that presented significant differences in abundance among testing groups. In comparison to the corn diet, Lactobacillus and Prevotela decreased in dogs fed both pea diets (Figure 6.9). Conversely, Faecalibacterium and Coprabacillus increased when dogs consumed either pea based diet compared to when they were fed the corn based diet (Figure 6.8).
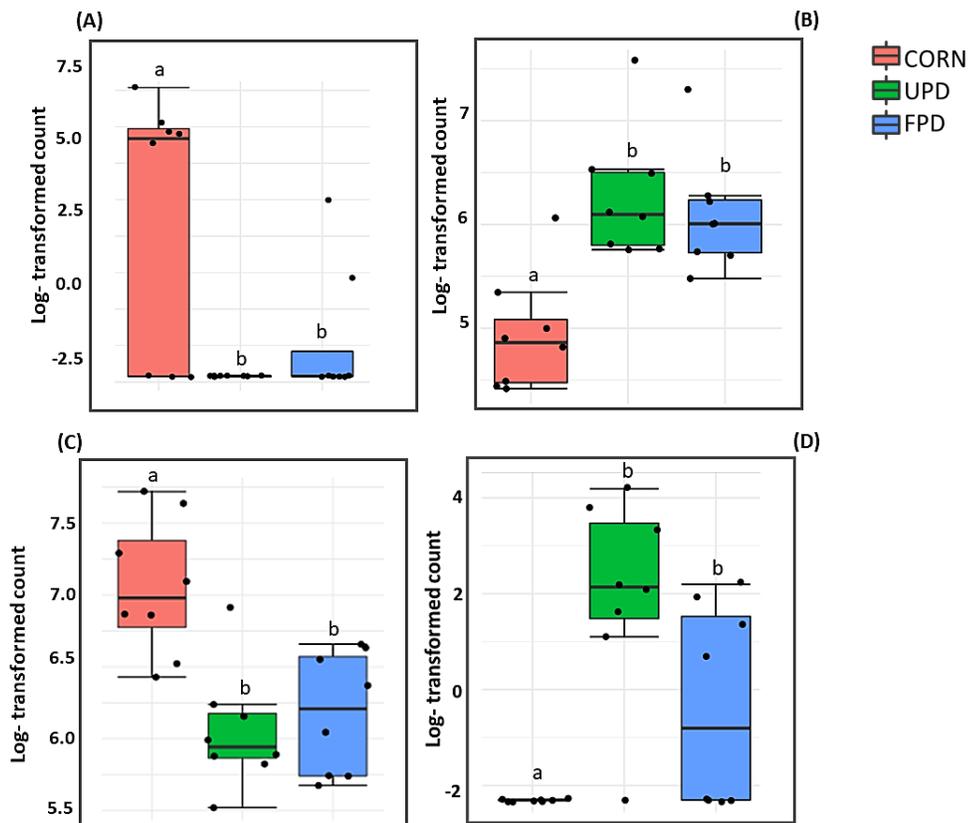
Figure 6. 9 Absolute abundance of bacteria in feces of dogs (n=8) after being fed different diets UPD= unfermented pea diet, FPD=fermented pea diet and corn for 20 days. (A) Lactobacillus, (B) Faecalibacterium, (C) Prevotella and (D) Coprobacillus. Different letters indicate significant differences after Friedmans test followed by Dunn's multiple comparison , P < 0.05 (adjusted FDR).

6.4 Discussion

The most important finding of this study is that, when compared to corn, pea based diets enhance dog and cat metabolic status, indicated by reductions in leptin, inflammation, triglyceride and cholesterol levels. Furthermore, diet and metabolic status appear to be linked to intestinal microbial composition and SCFA production in dogs and cats, but which of these is cause and which is effect is unclear.

6.4.1 Metabolic Status and Overall Health

In the current study, dog body weight was not affected by dietary changes. Conversely, compared to the corn and to the fermented pea diet, a significant weight loss was observed in cats fed the unfermented pea diet. However, rather than being a health benefit, in this case, it is more likely related to cats refusing to eat unfermented pea based diets due to palatability issues, similar to what has been observed in previous studies from this laboratory (Briens, 2018). Indeed, a significant reduction in average of food intake was observed when cats were fed the unfermented pea diet compared to either the control corn or fermented pea diets (Figure 6.1), agreeing with the palatability testing results of Chapter 4 in this thesis. Clearly, fermentation with *C. utilis* improves palatability of pea-based diets, particularly in cats. While beneficial body weight changes were not detected in the current study, both dogs and cats showed significant diet-related changes in leptin concentration. This supports the hypothesis that independent of weight loss, diet influences the metabolic status of dogs and cats. Nevertheless, diet had no influence on adiponectin levels of neither species, which differs from previous studies showing that similar to humans, poor metabolic status increases leptin and decreases adiponectin concentrations of dogs and cats (Appleton et al., 2000; Deng and Scherer, 2010; German et al., 2010; Hoenig et al., 2007; Katsumi Ishioka et al., 2009; Münzberg and Heymsfield, 2015; Park et al., 2014, 2015; Yadav et al., 2013). However, most studies analyzing adipokine concentrations in dogs and cats were conducted in animals suffering from obesity or other metabolic conditions, while the current study determined adipokine concentrations in normal weight dogs and cats in response to consumption of different diets. A previous study conducted with lean cats concluded that diet has no influence

on leptin or adiponectin concentration (Coradini et al., 2013). However, in this previous study, the cats received only one meal of each test diet before samples were collected to measure adipokines. The same authors reported that after weight gain, cats showed a different response to the test diets that was characterized by increased concentration of plasma leptin, while no changes were observed in adiponectin levels (Coradini et al., 2013). Thus, it might be possible that adiponectin and leptin respond differently to dietary changes. Moreover, different responses may be obtained according to the individual animal metabolic status and length of exposure to the test diets.

In addition to adipokines, C-reactive protein and other chronic inflammation markers have been used in humans and dogs to determine obesity related inflammation and assess metabolic status (Deng and Scherer, 2010; Tvarijonaviciute et al., 2012c; Wakshlag et al., 2011). However, due to inconsistent results reported in studies conducted with pets, inexpensive, alternative markers (NLR and PLR) were used instead in the current study. Based on the lack of change in NLR in either species with diet, the NLR does not appear to be a sensitive indicator of inflammation in dogs and cats. Dog PLR increased with both pea based diets. In contrast, cats showed reduced PRL with the fermented diet compared to both the unfermented pea and corn diet. This effect with PRL to indicate reduced inflammation was not as sensitive as total lymphocyte and neutrophil numbers, both of which were significantly reduced after feeding both fermented and unfermented diets in dogs, but not cats. While reduced lymphocytes and neutrophils clearly indicate reduced inflammation, the species differences are puzzling. However, more research is needed to investigate the usefulness of NLR and PLR compared to WBC counts as an inflammation biomarkers for dogs and cats as some researchers suggest that these parameters show high variability among breeds (Bahadir et al., 2015; Furuncuoğlu et al., 2016; Gürağaç and Demirer, 2016; Karakaya et al., 2019; Macfarlane et al., 2016; Mutz et al., 2015).

Despite some differences found in blood biochemistry of dogs and cats fed different diets, most values were still within the reference range, indicating minimal, if any, clinical significance of those findings. In contrast, globulin levels were below the reference range in dogs fed the unfermented pea diet. Hypoglobulinemia related to the diet is often related to low protein ingestion or poor absorption (Borges et al., 2014; Coles and Macdonald, 1963; Tothova

et al., 2016). Because the rate of protein inclusion was virtually identical among diets formulated with corn compared to peas in this study, the differences in serum globulin concentration between the control and the test diets cannot be attributed to differences in dietary protein. However, serum globulin levels of dogs fed fermented peas were higher than dogs fed unfermented peas, which might be explained by the increase in protein digestibility observed after fermentation of the pea starch. Similarly to dogs, cats fed unfermented peas showed lower serum globulin levels than cats fed corn and fermented peas. However, for cats, the values remained within the reference range.

Similar to obese humans, obese dogs and cats show higher blood concentrations of glucose, cholesterol and triglycerides compared to their lean counterparts (Diez et al., 2004; Forster et al., 2018; Heffron et al., 2016; Jeusette et al., 2005; Peña et al., 2008; Piantedosi et al., 2016; Radin et al., 2009). Among many other health benefits, consumption of peas reduces cholesterol and triglycerides levels in humans, probably due to the high concentration of some antinutrients found in peas (Campos-Vega et al., 2010; Dahl et al., 2012; Thompson, 1993; Millar et al., 2019). According to the Folin-ciocalteu method used to measure the levels of phenols and tannins present in the unfermented and fermented pea diet samples, fermentation increased pea diet concentration of tannins and phenols (see table 5.4). However, according to the literature, fermentation should decrease pea antinutrients levels,(Adebo et al., 2017; Champ, 2002; Khattab et al., 2009) which would explain why dogs and cats fed unfermented peas have lower cholesterol levels than dogs and cats fed the fermented peas or the corn diet. Specifically, cats fed corn showed cholesterol values above the reference range, and although these values were still above the reference range, both pea diets led to significantly lower cholesterol values. Compared to the corn diet, blood triglycerides levels remained lower when dogs and cats were fed either pea based diets. Taken together, these results support the hypothesis that compared to corn based diets, pea based diets enhance lipid handline and the metabolic status of dogs and cats.

6.4.2 Intestinal Health

The results of the current study are in agreement with previous studies showing that the vast majority of the dog and cat intestinal microbiome is composed of bacteria belonging to the phyla Firmicutes, Bacteroidetes, Fusobacteria, Proteobacteria and Actinobacteria (Hooda et al., 2012; Minamoto et al., 2012; Suchodolski, 2011b, 2011a). Previous studies report that consumption of fermented foods increases production of SCFAs, especially butyrate, which positively affects the intestinal health of dogs, cats and humans (Adebo et al., 2017; Aquino et al., 2010; Knudsen, 2015; Frias et al., 2017; Gagné et al., 2013; Santos et al., 2018; Topping and Clifton, 2001). In addition, human research suggests that increased microbial diversity is linked to improved intestinal health (Bischoff, 2011; Kim et al., 2017; Rinninella et al., 2019; Tuddenham and Sears, 2015).

Surprisingly, no changes in alpha or beta diversity were observed in cats and, even more unexpected was the finding that cats fed corn had a higher intestinal concentration of butyrate than cats fed peas (despite being fermented or not). Increased concentration of butyrate has been traditionally linked to improved intestinal health and is listed as one of the many health benefits attributed to the consumption of foods containing prebiotics (e.g. fermented pea diet) (Bach Knudsen, 2015; Gonçalves and Martel, 2013; Grześkowiak et al., 2015; Topping and Clifton, 2001). Therefore, it would be reasonable to assume that a higher concentration of butyrate would be found when cats were fed the fermented pea diet. However, the literature also states that high dietary fiber consumption increases the levels of butyrate in the intestine of dogs, cats and humans (Bach Knudsen, 2015; Gagné et al., 2013; de Godoy et al., 2013; Rochus et al., 2014; Topping and Clifton, 2001). Consequently, instead of being linked to differences in the source of carbohydrate included on each test diet (corn, peas or fermented peas) , the higher concentration of butyrate in the intestine of cats fed corn might be justified by the higher inclusion of pea fiber in the corn control diet when compared to both pea based diets (see Table 6.1). The lower levels of butyrate in the intestine of cats fed peas may also be explained by the concurrent changes in metabolic status. Despite being traditionally linked to improved intestinal health, obese individuals have recently been reported to have higher intestinal concentration of SCFAs than lean subjects (Clarke et al., 2012; Kallus and Brandt, 2012; Kieler et al., 2017; Kotzampassi et al., 2014; Murugesan et al., 2018). One

of the possible explanations provided in these previous studies is that obese individuals have an increased population of high-efficiency carbohydrate fermenting microbes in their intestine. It is also possible that SCFA absorption is impaired in individuals with poor metabolic status, leading to higher fecal levels (Clarke et al., 2012; Kallus and Brandt, 2012; Kieler et al., 2017; Kotzampassi et al., 2014; Murugesan et al., 2018). Consequently, the higher SCFA concentration in the intestine of cats fed corn might be due to the impaired metabolic status when compared to cats fed peas.

As mentioned before, diet had no effect on the intestinal microbial composition of cats. Although very few studies evaluating the effect of diet in cats intestinal microbiome are available, our results are in agreement with these few studies that concluded that high inter-individual variation in the domestic cat creates difficulties in studies targeting their intestinal microbiome (Barry et al., 2012; Minamoto et al., 2012). Moreover, in the current study, decreased food intake led to reduced stool production when cats were fed the unfermented pea diet. This is the likely explanation for the low yield of genetic material in the cat fecal samples and subsequently low numbers of reads for the DNA sequencing analyses. In addition, qualitatively, one of the study cats that was particularly prone to bouts of diarrhea, sometimes bloody, was much worse when fed the unfermented pea or corn based diets. However, this cat subsequently had no diarrhea and instead had nicely formed stools when fed the fermented pea diet. This qualitative finding is promising, but requires further study to confirm whether fermented pea diets can improve cat inflammatory bowel disease.

Despite the lack of diet related changes in SCFA concentration in the current study, increased alpha diversity of the intestinal microbiome was observed in dogs fed both pea based diets. This indicates that feeding dogs peas, either fermented or unfermented, benefits intestinal health when compared to diets formulated with corn. Moreover, intestinal microbiome beta diversity differed among dogs fed corn or pea based diets. Conversely, Lactobacillus was depleted in dogs fed peas instead of corn, which might be viewed as deleterious since Lactobacilli are often linked to enhanced intestinal health in different species including dogs (Grześkowiak et al., 2015; Losada and Olleros, 2002; Rinninella et al., 2019; Walter, 2008). The higher population of Lactobacillus in the intestine of dogs fed corn compared to dogs fed peas might be explained by the fact that pea fiber was included on all lab made diets, being

the levels of inclusion in the corn control diet higher than the levels of inclusion of pea fiber on both pea test diets (see Table 6.1). Consumption of diets rich in fiber positively correlates with intestinal levels of Lactobacillus in dogs, cats and humans . Therefore, the higher levels of pea fiber included in the control diet might have obscured potential health benefits linked to the consumption of fermented pea. Indeed, compared to the unfermented pea diet, dogs fed the fermented pea diet showed a modest improvement in the population of Lactobacillus, which might be related to the prebiotic compounds present in *C.utilis* cell wall (Cerbo et al., 2014; Kieliszek et al., 2017; Rai et al., 2019; Shurson, 2018).   Conversely, the intestinal Faecalibacterium population increased with the consumption of pea diets in dogs. Reduction in intestinal Faecalibacterium has been linked to obesity, diabetes and Chrone's disease in humans. Likewise, it has also been shown that dogs suffering from inflammatory bowel disease have a decreased population of Faecalibacterium (Al Shawaqfeh et al., 2017; Blake and Suchodolski, 2016; Honneffer, 2014; Schmitz and Suchodolski, 2016). Therefore, the higher concentration of Faecalobacterium found in the intestine of dogs fed peas might exert functional properties by preventing intestinal bowel disease. In the current study, intestinal Prevotella population was reduced when dogs consumed pea based diets instead of a corn based diet, which contrasts with human studies showing that consumption of a plant-rich diet leads to an increased concentration of intestinal Prevotella. The role of Prevotella in intestinal and overall health is not yet fully understood. Studies with mice revealed that intestinal Prevotella improves glucose handling. Conversely, increased Prevotella has been linked to some intestinal inflammatory diseases in humans. Because Prevotella genus includes many species, further studies are needed to understand the role of specific species in the dog gut (Ley, 2016; Ottman et al., 2012; Precup and Vodnar, 2019). Little information is available about the role of Coprobacillus in intestinal microbial ecology. However, in agreement with our results, supplementation with yeast cell wall led to decreased levels of Coprobacillus in dogs intestine (Kageyama and Benno, 2000; Santos et al., 2018). Taken together, the general trend for changes in abundance of specific bacteria seems to be toward beneficial in dogs fed pea based compared to corn based diets.

In summary, the results of this study show that intestinal microbial composition in dogs is influenced by diet and that cat metabolic status might have an impact on SCFA fecal concentration. Additionally, pea starch yeast fermentation seems to have no effect on the

intestinal microbial diversity or composition of dogs. However, in comparison to dogs fed corn, diets formulated with peas lead to a more rich and even intestinal microbiome, which is often consider as a positive marker of intestinal health (Blake and Suchodolski, 2016; Ottman et al., 2012; Rinninella et al., 2019; Suchodolski, 2011; Tuddenham and Sears, 2015). Taken together, the use of yeast-fermented pea starch should be further explored as a novel functional food that could be used to improve health benefits of pea based diets in the pet food industry.

CHAPTER 7

OVERALL DISCUSSION

7.1 Summary of Conclusions

The main objectives of this thesis were to develop a highly palatable diet for dogs and cats using yeast fermented pea starch as the carbohydrate source as well as investigate functional properties and potential health benefits linked to the consumption of this novel pet diet over more regular diet formulations with unfermented peas or corn. The first study describes a novel protocol using *C. utilis* to ferment pea starch. The fermented pea starch produced in study 1 was then included in the fermented pea diet that was tested in the subsequent studies. The second study revealed that fermentation has no significant effect on the postprandial glucose response of dogs and cats fed either, pea starch or pea diet. Therefore, the hypothesis that fermentation reduces the glycemic index of pea products was rejected. The third study confirmed the hypothesis that yeast fermentation enhances the palatability of pea based diets. The last study revealed beneficial effects of pea diets over corn diets on the metabolic status of dogs and cats. Moreover, this study concluded that dog intestinal microbiome diversity is positively affected by diets containing peas.

The following is a summary (Figure 7.1) of the most important findings of each study included in this thesis:

Pea Starch yeast fermentation

Final product with slight higher protein and crude fiber content

Diet formulation
(corn, unfermented and fermented peas)

Does pea starch yeast fermentation increase glycemic index of pea starch/diet?

Yes

No

Does pea starch yeast fermentation enhance palatability of pea based diets

No

Yes

Does pea starch yeast fermentation improve health?

Metabolic status

Intestinal Health

Yes

Yes

	Dogs	Cats
Adiponectin	Corn = UPD=FPD	Corn = UPD=FPD
Leptin	Corn > UPD >FPD	Corn > UPD=FPD
Triglycerides	Corn > UPD=FPD	Corn > UPD=FPD
Cholesterol	Corn = FPD >UPD	Corn > UPD >FPD

|	Dogs	Cats
Alpha diversity	Corn > UPD=FPD	Corn = UPD=FPD
Beta diversity	Corn ≠ UPD=FPD	Corn = UPD=FPD
Faecalibacterium	Corn < UPD=FPD	?
Lactobacillus	Corn > UPD=FPD	?

Figure 7. 1 Flowchart showing the order of trials and main findings of each chapter included in this thesis.

7.2 Strengths and Limitations

A strength of this study was the inclusion of two species that, although being popular pets, have different metabolic pathways to handle carbohydrates and different eating behaviors. Cats are exigent eaters, which makes them a challenging species to conducted nutritional research. Nevertheless, the popularity of cats as pets is increasing worldwide, which makes palatability an even more important feature in pet food formulation (Bradshaw, 1991; Bradshaw et al., 1996; MacDonald et al., 1984). The inclusion of both species in the palatability trials allows a fair comparison between species and reinforces the efficacy of yeast fermentation as a palatability enhancer. Furthermore, despite the fact that both species are generally fed diets containing high levels of carbohydrate, little is known about carbohydrate handling in cats (Zoran and Buffington, 2011; Laflamme, 2008; Verbrugghe and Hesta, 2017; Zoran, 2010). Therefore, the inclusion of both species in these studies evaluating glucose response and metabolic status adds more information about carbohydrate metabolism in cats and allows comparison between species.

To cite strengths of each study, study 2 included dogs and cats that had previously been used on another glycemic index trial. The animals were therefore used to the experimental procedures, which probably helped to minimize cortisol or catecholamine release related to stress, yielding reliable glucose measurements. Likewise, study 3 was conducted after a pilot palatability trial conducted in the same group of animals. The pilot study was important not just to acclimatize the animals to the procedures, but it was also crucial to determine the ideal time frame to be used in the four-bowl test. One strength of study 4 was the fact that the diets used were very similar in composition, which minimized interference of other feed compounds on metabolic status and intestinal health of the dogs and cats being tested.

The common limitation of all studies included in this thesis is the small sample size. The inclusion of more animals would produce better results and minimize the likelihood of type 2 error on statistical analyses. In addition to high inter-individual variation, the small sample size had a great negative impact on cats intestinal microbiome analysis. Study 4 was also compromised by the low palatability of the unfermented pea diet. The cats refusal to eat as much of the unfermented pea diet negatively impacted body weight and stool production. Moreover, the feeding trial period had to be shortened to 20 days so as not to put cat welfare

at risk. This shortened feeding period may have had an impact on the microbiome results of both species included in the trial. For instance, some studies suggest that more time might be needed to detect related dietary changes in the intestinal microbiome. However, no golden standard has been established for this finality and studies show that changes might be seen as early as 10 days after dietary intervention (Cencic and Chingwaru, 2010; Gerber, 2014; Singh et al., 2017; Turroni et al., 2017; Valdes et al., 2018).

7.3 Future Work

This thesis was focused on using *C. utilis* to ferment pea starch, and the findings suggest that yeast fermentation is an efficacious food flavor enhancer for pea based diets. More research is needed to investigate whether *C. utilis* fermentation could be used with other pet food ingredients, especially ingredients that, despite being healthier, have poor palatability. Furthermore, a comparison between *C.utilis* and other yeast or bacteria species could help to determine which is best to be used in pet food fermentation.

The palatability trial introduced a novel technique to assess food preference in dogs and cats. If other studies confirm the success of this methodology, the four-bowl test might become the only consummatory method to assess palatability in dogs and cats, which would make possible comparisons among different diets in an industrial setting.

The results of study 4 support the use of peas as a functional ingredient to be used in pet food. According to the findings of this study, peas help to modulate the metabolic status of dogs and cats. Prevention and treatment of obesity is a challenge in veterinary medicine and often requires the cooperation of owners. However, owner cooperation might be compromised when high-priced diets that are marketed as 'weight loss' turn out to have low palatability and their pets refuse to eat it (Bland et al., 2010; Burkholder and Bauer, 1998; Churchill and Ward, 2016; German, 2006; Sloth, 1992). The development of a tasteful diet that can prevent obesity without requiring a drastic reduction in meal size might facilitate obesity treatment by increasing the owners commitment to the treatment. Despite further evidence in this thesis that pea diets are linked to improved metabolic status, the link between metabolic status and intestinal microbiome is still unclear, especially in pets. Further research is needed to define a

core intestinal microbiome for each species before this can be linked to disease states such as obesity dogs and cats.

7.4 Final Conclusions

The goal of this study was to develop a healthier and tastier diet for dogs and cats. *C. utilis* yeast fermentation resulted in a final product that enhanced the palatability of pea diets formulated for dogs and cats. Previous studies showed that consumption of pea based diet benefits metabolic status and glucose handling in dogs and cats (Adolphe, 2013; Briens, 2018). Therefore, the finding that fermentation has no significant negative impact on dog and cat postprandial glycemic response to pea products can be considered beneficial. This thesis has produced a fermented functional food that resulted in better taste without compromising the health benefits of diets formulated with peas. Furthermore, when compared to a diet formulated with corn, dogs and cats fed unfermented or fermented peas show improved metabolic status and increased intestinal microbial diversity, which further supports the hypothesis that peas are a healthier carbohydrate option than corn.

In summary, the results of this thesis support the use of yeast fermented pea starch as an innovative ingredient to be used in the formulation of palatable functional foods for dogs and cats.

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