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POTATO PULP AS A DIETARY FIBER SOURCE IN HIGH QUALITY DOG FOODS

BY

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THESIS

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ABSTRACT

The objective of this thesis was to evaluate potato pulp (PP) as a functional fiber source in high quality dog diets by investigating *in vitro* digestion and fermentation characteristics and *in vivo* responses to graded levels of PP in the diet. Raw and cooked PP both contained 55% total dietary fiber (TDF) with a favorable ratio (1.4:1) of insoluble:soluble fiber. Fermentation characteristics of the substrates suggested that both cooked and raw PP were moderately fermentable through 9 h. Differences in fermentability between raw and cooked PP was revealed at the 12 h time point of fermentation, where raw PP was still fermentable while cooked PP was not. The *in vivo* experiment revealed that graded level inclusion of PP had no adverse effects on dry matter, organic matter, crude protein, acid hydrolyzed fat, or gross energy digestibilities. Total dietary fiber digestibility increased as dietary concentration of PP increased, as did concentrations of fecal SCFA. Furthermore, fecal consistency, total fecal output, fecal protein catabolites (except spermidine), and fecal dry matter output were not affected by increased inclusion levels of PP. Overall, PP was characterized as a moderately fermentable fiber source with potential to serve as a novel fiber source in high quality dog foods.

To my Grandpa, Robert Panasevich

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TABLE OF CONTENTS

	Page
LIST OF TABLES AND FIGURES	vii
CHAPTER 1: INTRODUCTION	1
Literature cited	3
CHAPTER 2: LITERATURE REVIEW	4
Introduction	4
Dietary fiber	5
Health benefits of dietary fiber	8
Fiber in companion animal diets	14
The gut microbiota	15
Starch	18
Hindgut fermentation	21
Protein fermentation	29
Potatoes and potato fractions as dietary ingredients	33
Thesis objective	39
Literature cited	40
CHAPTER 3: POTATO PULP AS A DIETARY FIBER SOURCE IN HIGH QUALITY DOG FOODS	54
Abstract	54
Introduction	55
Materials and methods	56
Results	60
Discussion	63
Literature cited	69

LIST OF TABLES AND FIGURES

	Page
Table 3.1: Chemical composition of raw and cooked potato pulp.....	72
Table 3.2: Ingredient and chemical composition of experimental diets fed to dogs.....	73
Table 3.3: Nutrient digestibility and fecal scores from dogs fed diets containing graded levels of potato pulp.....	74
Table 3.4: Short-chain fatty acid (SCFA), branched-chain fatty acid (BCFA), ammonia concentrations, and fecal pH values for dogs fed potato pulp.....	75
Table 3.5: Biogenic amine, phenol, and indole concentrations in fecal samples from dogs fed diets containing graded levels of potato pulp.....	76
Figure 3.1: pH and concentrations of acetate, propionate, butyrate, and total short-chain fatty acid SCFA during a 12 h <i>in vitro</i> fermentation	77

CHAPTER 1: INTRODUCTION

Dogs are considered important parts of society, and the demand for high quality pet foods is high. Pet owners desire to feed their pets high quality ingredients to maximize the health and well-being of their dogs and cats. According to the American Pet Products Association (2012), the number of households in the United States that own a pet increased 2.1% from 2011 to 2012, reaching an all-time high of 72.9 million pets. In 2011, pet-related expenditures accounted for an estimated \$52.9 billion USD, with \$20.5 billion USD of that amount spent on pet food. Pet food companies explore different ingredient sources (e.g., organic and natural), high quality ingredients (food-grade ingredients), and novel dietary supplements to meet consumer demands (e.g., fiber sources; Faber et al., 2011). Ultimately, ingredients must be evaluated for their functionality, with functional ingredients defined as those that support or improve health and physiology of the animal, thereby justifying health claims (Roberfroid, 2000). In this respect, functional ingredients must be evaluated for their effects on animal physiology broadly defined.

Dietary fiber is a food component that has been found to improve gut and overall host health. Many dietary fiber sources are by-products or co-products of the human food industry. Potato pulp, as a co-product of potato starch isolation, has potential to serve as a functional fiber source in pet foods. Potato pulp consists mainly of residual starch and the peel, which is rich in cellulose, hemicelluloses, and galactan- branched rhamnogalacturonan I type pectin (Thomassen et al., 2011). The total dietary fiber (TDF) concentration is about 55%, with 32% insoluble fiber and 23% soluble fiber (Panasevich, unpublished data). The insoluble to soluble fiber ratio found in potato pulp makes it a promising high quality fiber source in pet foods. Research on potato pulp as a fiber source is limited, but human and animal feeding studies have shown some beneficial health effects. Laerke et al. (2007) found that the soluble fiber fraction of potato pulp

resulted in higher cecal SCFA concentrations of 173.5 μ mole compared to 83.0 μ mole for cellulose controls ($P < 0.05$). Furthermore, solubilized potato pulp decreased ($P < 0.05$) postprandial plasma triacylglycerol from 2.79 mmol/L in the cellulose control diet to 1.82 mmol/L in the solubilized potato pulp diet. Potato peels also have phytochemicals, flavonoids, and carotenoids that may serve antioxidant, anticarcinogenic, antimutagenic, and low-glycemic roles (Friedman, 1997).

Along with having a high TDF, potato pulp also has considerable amounts of resistant starch, which has been known to have favorable fermentation characteristics, in particular butyrate formation (Topping and Clifton, 2001). Potato pulp has both RS2 (native starch granule that is resistant to hydrolytic/enzymatic digestion) and RS3 (retrograded resistant starch), both of which have been shown to be fermentable and capable of improving gut health. Potato pulp, with its high TDF content, favorable insoluble to soluble fiber ratio, and considerable concentration of resistant starch, is a potentially high quality fiber source in dog foods.

The goal of this thesis is to evaluate whether potato pulp could serve as a functional fiber source in high quality dog foods. This was accomplished by investigating chemical composition, *in vitro* digestion and fermentation characteristics, and *in vivo* effects of potato pulp in the dog. In this thesis, Chapter 2 includes a literature review that encompasses topics pertaining to potato pulp and dietary fiber, while Chapter 3 presents the chemical composition, *in vitro* digestion and fermentation, and *in vivo* data.

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CHAPTER 2: LITERATURE REVIEW

Introduction

According to the American Pet Products Association (2012), the number of households in the United States that own a pet increased 2.1% from 2011 to 2012, reaching an all-time high of 72.9 million pets. In 2011, pet-related expenditures accounted for an estimated \$52.9 billion USD, with \$20.5 billion USD of that amount spent on pet food. The majority of the pet households in the US own either a cat or dog, which means the majority of pet food spending is done on these species. With this increase in pet spending, consumers have become more concerned with their pet's nutrition and health and have increased their demand for high quality pet foods. Pet owners often base quality on what they perceive is healthy for themselves, and will purchase pet food in this manner. This consumer trend is referred to as the "humanization of pet food" and is largely responsible for the pet food industry's steady economic growth (Domblaser, 2011). Pet food companies seek out novel, high quality ingredients to put into pet foods. These would include fiber sources with prebiotic potential, or the addition of a probiotic and other supplements thought to improve the health and well-being of the animal. Pet owners desire high quality ingredients to maximize health of their dogs or cats. High quality ingredients include dietary fibers such as beet pulp and indigestible oligosaccharides, non-byproduct protein sources such as salmon, turkey, lamb, and chicken, omega-3 fatty acid sources such as avocado and fish, and vegetables. In addition, mixed tocopherols, fruits, citric acid, and rosemary extract are added as natural antioxidant sources to pet food. This growing demand from pet owners is why many pet food companies have placed a greater focus on nutrition and health of pets. A large part of that focus has been aimed at determining the quality of fiber sources to determine what role they may play in enhancing gut health.

Dietary fiber

Definition and Plant Cell Wall Composition

Dietary fiber, as defined by Prosky (2000), is the edible part of plants or analogous carbohydrates resistant to digestion and absorption in the small intestine with complete or partial fermentation in the large intestine. This includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Fiber is indigestible to mammalian enzymes due to the unique structural and chemical aspects of the plant cell wall and some cell contents, as well as β -glycosidic bonds between monosaccharide units that make fiber indigestible to mammalian enzymes (Buxton and Redfearn, 1997). Pancreatic amylase and isomaltase only hydrolyze α -1,4 and α -1,6 glycosidic bonds found primarily in starches (Coombe and Siddons, 1973). Enzymes in the hindgut, such as β -galactosidase, α -glucosidase, β -glucosidase, and β -glucuronidase, hydrolyze polysaccharides with β -glycosidic bonds (Tan et al., 2010).

The plant cell wall is what gives plants their structural features and consists of major components such as polysaccharides, lignin, and proteins, and minor components including acetyl groups and phenolic constituents (Guillon et al., 2007). Different tissues of the plant are composed of different types of cell walls: primary, secondary, and tertiary cell walls. Primary cell wall constitutes the outermost part of the wall and is composed of cellulose microfibrils and matrix polysaccharides. Cellulose microfibrils consist of β 1,4 glycosidic linkages and have varying degrees of hydrogen bonding between the glucan chains in different tissues (Rose and Hamaker, 2011). Neighboring cells are separated by a middle lamella of amorphous pectins and associated calcium ions. The role of the primary cell wall is to provide strength and stability to the plant, and also allow for expansion during growth. Inside the primary cell wall lies the secondary cell wall, which is comprised of several layers that are oriented differently than the

primary cell wall with cellulose microfibrils (Complex Carbohydrate Research Center, 2007).

The secondary cell wall is thicker than the primary cell wall and also has the highest content of lignin (Rose and Hamaker, 2011). As the plant begins to mature and have increased upright growth, more strength and stability is required. Secondary cell wall has the highest carbohydrate content and is lignified to aid in this stability. Cellulose and hemicelluloses are present in both primary and secondary walls, but the degree of cross linking between cellulose microfibrils and hemicelluloses and lignin is greater in the secondary cell wall. Cross linking is what makes this area hydrophobic, decreasing its susceptibility to being hydrolyzed enzymatically (Complex Carbohydrate Research Center, 2007). The tertiary cell wall is a thin membranous layer that is highly lignified and is non-fermentable. Overall, the primary and secondary cell walls have higher lignin content than the tertiary cell wall, which has less, but a more concentrated, lignin. The tertiary cell wall has a predominant role in providing strength and stability to the fully grown plant. Plant cell wall is very diverse in terms of polysaccharide constituents and varies depending on the type of tissue. Fruits and vegetables are high in primary cell wall, which is less lignified, whereas certain forages and woody tissues contain secondary and tertiary cell wall which is highly lignified (Rose and Hamaker, 2011).

Categories of Fiber

Polysaccharides in the plant cell wall can be separated into two categories: soluble fiber, which may be viscous and highly fermentable, and insoluble fiber, which generally is non-viscous and partially to non-fermentable. Soluble fibers found in the plant cell wall include β -glucans, pectins, and gums. Beta-glucans, found in oats and barley, are a viscous fermentable fiber source and can slow transit time through the gastrointestinal tract. Beta-glucans are high in β -1,3 and β -1,4 glycosidic bonds (Rose and Hamaker, 2011). Pectins and gums are fermentable

carbohydrates primarily found in pomaces such as apple peel and core, citrus peel, and sugarbeet pulp. Pectins are composed of linear polymers of α -1,4 linked galacturonic acid and have varying degrees of methyl esterification. Pectin is primarily found in primary cell wall and is used as a gelling agent. The primary cell wall and the surrounding middle lamella are higher in pectins and gums than secondary and tertiary cell walls (Complex Carbohydrate Research Center, 2007). Gums are common in the middle lamella, and help to join adjacent plant cells. A common gum found in foods is guar gum, or guaran, and is found mainly in ground endosperm of guar beans. Guar has a basic chemical structure of β -1,4 – linked D-mannopyranosyl backbone with α -D-galactose side chains linked at positions 1 and 6. Physical properties of guar gum, however, differ due to degree and pattern of galactosyl branching (Daas et al., 2002). Soluble fiber sources are thought to benefit gut health due their fermentability and modulation of the microbial ecology in the hindgut.

Insoluble fibers in plant cell wall components include hemicelluloses, cellulose, lignin, and phenolics (Rose and Hamaker, 2011). Hemicelluloses are structural heteropolysaccharides found in most plants and are partially fermentable in the large bowel. They are branched polymers that are structurally weak in comparison to cellulose. Hemicelluloses are mainly composed of D-pentose monosaccharides with xylose being the main component. Other constituents of hemicelluloses are galactose and galacturonic acid as well as mannose and mannuronic acid (Complex Carbohydrate Research Center, 2007). Cellulose, unlike hemicelluloses, is a structural homopolysaccharide made up of glucose units and is the most abundant plant polysaccharide. Fermentation of cellulose is variable due to the degree of hydrogen bonding between the cellulose microfibrils. Wood cellulose is unfermentable due to its

high degree of hydrogen bonding between β -glycosidic sheets making it hydrophobic and incapable of being utilized by bacterial enzymes (Rose and Hamaker, 2011).

One of the biggest impediments to fermentation of any fiber source is lignin. Lignin is considered by most to be an insoluble fiber present in all vascular plants and non-fermentable by bacterial species. Lignin is second in abundance to cellulose (Institute of Paper Science and Technology, 2013). It is a complex polymer of phenylpropane units that are cross-linked by different chemical bonds. Along with lignin, certain phenolics are found in plants that also decrease the plant's nutritive value. Phenolics are considered an insoluble fiber component of plant cell wall. Phenolics are present in many plants, act as a defense mechanism, and have antinutritive properties and antioxidant activity (Richard, 1996). Within the plant cell wall are the plant cell contents. Plant cell contents also have indigestible carbohydrates like resistant starch and fructan oligosaccharides that can be considered as a fiber.

Health benefits of dietary fiber

Gut health

Many health benefits have been associated with ingestion of dietary fiber. In terms of digestive health, fiber has been found to increase stool weight and promote normal laxation (Grabitske and Slavin, 2008). Dietary fiber has been found to decrease diarrhea because of its ability to absorb water and add bulk to stools; this helps regulate transit rate by having appropriate amounts of water in the hindgut. Irritable Bowel Syndrome (IBS) is defined as an alteration in bowel habit and abdominal distention, discomfort, and or pain (Drossman et al., 2002). Wheat bran, a source of insoluble fiber, has been an effective treatment for IBS for more than 20 years. Nearly 70% of patients with IBS reported improvements in symptoms by adding 7 grams of wheat bran to their diet for 6 weeks (Manning et al., 1977). However, about 30% found

that their symptoms were exacerbated with the addition of wheat bran to their diet (Manning et al., 1977). Wheat bran and other insoluble fibers increase fecal bulk, increase stool weight and frequency, and reduce whole gut transit time. Fiber is also an effective treatment for mild or moderate constipation, but is ineffective for patients with severe constipation (Muller-Lissner et al., 2005). Three mechanisms may explain why fiber alleviates moderate constipation: 1) fiber serves as a bulking agent, 2) coarseness of fiber irritates the colonic epithelium, causing more motility of the gut and overall easier passage of stools, and 3) fiber relaxes the stretch receptors in the colon. Diverticulosis is caused by the development of pouches of mucus membranes projecting through the intestinal muscular layer into the surrounding fat. It occurs mostly on the underside of the sigmoid colon where the blood vessels are vulnerable to penetration. This condition primarily affects older individuals where one out of three people experience this problem by the age of 60, and almost 50% by the age of 90 (Stollman and Raskin, 2004). Coarse bran and psyllium are more effective at increasing stool weight, but less effective at reducing colonic pressure. The combination of insoluble and soluble fiber has been effective in the treatment of diverticulosis (Fisher et al., 1985).

Colorectal and other GI cancers

One of the most profound effects of fiber is its protective role against colorectal cancer. There are two FDA approved health claims that link fiber and cancer (21 CFR 101.75 and 21 CFR 101.78). A study done within a Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial showed that patients in the highest quintile of fiber intake have a 27% lower risk of developing colorectal adenoma compared to the lowest quintile of fiber intake (Peters et al., 2003). Other studies showed that an increase in total fiber intake resulted in a 14 to 25% lower risk of colorectal cancer (Bingham et al., 2003; Bingham et al., 2005; Schatzkin et al., 2007).

The reduction in risk of colorectal cancer may be caused by the dilution of carcinogenic protein catabolites by fiber in the colon, an increased transit rate that reduces exposure to these carcinogens, increased butyrate that maintains colonic health, and/or increased short-chain fatty acid (SCFA) production that stimulates bacterial growth to utilize free ammonia (Klurfeld, 1992; Macfarlane et al., 1992). Increased fiber intake also has been associated with a decreased risk of other gastrointestinal (GI) cancers including those affecting the esophagus, stomach, and small intestine (Mayne et al., 2001; Schatzkin et al., 2008; Terry et al., 2001; Wu et al., 2007). In 2007, a case-control study found that people consuming more than two servings of fiber per day versus zero servings per day had a 43% lower risk of developing pancreatic cancer (Chan et al., 2007). Overall, the potential mechanisms that could explain how dietary fiber decreases incidence of GI cancers would be its role diluting carcinogenic compounds entering the large bowel and prevent the formation of adenomatous polyps (Anderson et al., 2009). Dietary fibers having high amounts of antioxidants and phytochemicals also may make them important in the prevention of these diseases (Liu, 2007).

Type II diabetes

Dietary fiber also is effective in controlling the symptoms of diabetes. Diabetes mellitus is characterized by an increase in blood glucose concentration due to an absolute or relative loss of the hormone, insulin. There are two types of diabetes: type I, insulin-dependent diabetes mellitus, and type II, non-insulin-dependent diabetes mellitus. Type I diabetes is caused by an absolute deficiency of insulin and requires the patient to administer regular injections of insulin to maintain glycemic control. Type II diabetes is a condition in which the tissues with insulin-dependent cells (e.g., adipose, pancreas, liver, skeletal muscle, etc.) become resistant to the action of insulin. In addition, there is a decrease in insulin secretion from pancreatic β -cells. This

results in an increased concentration of glucose in the blood and energy deprivation to these tissues. The peak incidence of type II diabetes is middle age or later, whereas type I is usually observed in children from ages 10 to 12 (ADA, 2006). Additional complications associated with diabetes are an increased risk of coronary heart disease (CHD) and microvascular complications such as diabetic nephropathy and retinopathy (Cade, 2008). To control diabetes with diet, it is important to maintain blood glucose and lipid concentrations. The glycemic response to foods is defined by the rise and fall in concentrations of blood glucose over a 3 h period of time in response to a carbohydrate-containing meal (Dipietro et al., 2012). The glycemic response is a bell-shaped response curve. The initial rise in the curve represents glucose being absorbed from the gut and being sent to the peripheral blood circulation via the liver. The subsequent fall in blood glucose is due to the release of insulin from the pancreas in response to various components of the meal, such as glucose, amino acids, gut hormones, and neurotransmitters (Jenkins et al., 2008). Insulin allows glucose to be taken from the peripheral blood and into various tissues like muscle and fat. The glycemic index compares this response curve to different carbohydrate supplements. Soluble fiber decreases the spike in blood glucose concentrations normally seen after a meal (Anderson et al., 1999; Rodriguez-Moran et al., 1998; Ziai et al., 2005) whereas insoluble fibers have little effect on postprandial glycemic responses. Unmilled legumes have a low glycemic index, even when cooked, due to the cell wall structure that prevents the starch granule from swelling during gelatinization (Morris and Zemel, 1999). Guar gum is an effective food additive to blunt the glycemic response and could be effective in diabetes-preventative diets (Aro et al., 1981). Intake of fiber concentrations as high as 35 to 45 g/d have shown a small but significant improvement in both mean blood glucose concentrations and in 24 h blood glucose profiles of type II diabetic patients (Brand et al., 1991; Pick et al.,

1996). Overall, insoluble fiber has a greater effect on fasting blood glucose concentrations, while soluble fiber has a greater effect on postprandial blood glucose concentrations.

Type-II diabetes research suggests that with an increase in fiber intake, there is a 25% decreased chance of developing type –II diabetes (Salmeron et al., 1997a, b; Stevens et al., 2002). Some of the potential mechanisms of fiber in controlling blood glucose concentration are that it increases insulin sensitivity and SCFA production, as well as decreases low-density lipoprotein (LDL) blood cholesterol, triacylglycerol (TAG) concentrations, blood pressure, body weight, fibrinolysis, and C-reactive protein (Erkkila and Lichtenstein, 2006). Epidemiological evidence from two studies suggests that whole grains in particular elicit a 21 and 27% risk reduction of type-II diabetes with 3 servings a day (Liu et al., 2000; Meyer et al., 2000). Furthermore, 7 cohort studies indicated that there was an average 28% lower incidence of type II diabetes in the highest quintiles with increased whole grain intake. Whole grains, being a low glycemic food, increases insulin sensitivity (de Munter et al., 2007; Fung et al., 2002; Kochar et al., 2007; Liu et al., 2000; Meyer et al., 2000; Montonen et al., 2003; Qi et al., 2006). Liese et al. (2003) found that 0.8 servings/d of whole grains could significantly increase insulin sensitivity. A study done in overweight, hyperinsulinemic adults found that when patients were fed whole and refined grains for 6 wk, fasting insulin concentrations were reduced by 10% (Pereira et al., 2002).

Coronary Heart Disease

Patients with diabetes have an increased risk of CHD; therefore, it is important to control blood lipid concentrations. Soluble dietary fiber has been found to lower the incidence of CHD. Coronary heart disease is a multifactorial disease associated with the development of plaque in the walls of the coronary arteries caused by elevated serum lipids, low-density lipoprotein (LDL)

cholesterol, and triglycerides (Poulter, 1999). Soluble fibers have been shown to lower plasma cholesterol in diabetic, normal, and hyperlipidemic subjects (Anderson et al., 2000; Anderson et al., 1999; Glassman et al., 1990). Currently, three FDA approved health claims are associated with fiber and CHD (21 CFR 101.77, 21 CFR 101.81, 21 CFR 101.83). Fiber reduces the accumulation of plaque by lowering serum cholesterol concentrations (Anderson et al., 2000). A 10-yr prospective cohort study in the USA (1987 – 1996) with 2,909 healthy white and black young adults, aged 18 to 30 years old, found there was a 5 mg/ dL decrease in LDL cholesterol concentrations with participants with high fiber intakes (Ludwig et al., 1999). Other studies have shown a 50% reduced risk of nonfatal CHD with increased intake of whole grains (Fraser et al., 1992). Pooled analyses of several cohort studies examining dietary fiber intake and CHD risk have shown an additional 10 g/d of dietary fiber reduced CHD risk and deaths (Pereira et al., 2004). This decrease in the risk of CHD is predominantly from the intake of cereal grains and less from the intake of fruit and vegetable fibers (Morris et al., 1977; Trowell, 1972). The potential mechanisms that could explain this risk reduction are that soluble fiber reduces blood lipids and cholesterol, as well as increases intake of antioxidants, lignans, and phytoestrogens associated with both soluble and insoluble fibers. High fiber foods, such as whole grains, are associated with lower C-peptide involved in the synthesis of insulin (Wu et al., 2004).

Consumption of a whole grain diet has been associated with plasma concentrations of anti-inflammatory markers and endothelial dysfunction (Lopez-Garcia et al., 2004). Consumption of oat and oat bran also may elicit pronounced cholesterol-lowering effects (Romero et al., 1998). Oat products selectively lower LDL cholesterol and result in a more favorable ratio of LDL:HDL (Othman et al., 2011). Some fibers have been shown to decrease bile acid reabsorption and metabolism that cause a reduction in the body's total cholesterol pool. Fiber binds to bile acids

and cholesterol so they are physically inaccessible to be reabsorbed (Zhang et al., 2011).

Production of SCFA, in particular propionate, inhibits cholesterol synthesis in the liver and in peripheral tissues, which results in decreased LDL synthesis and increased LDL clearance (Cheng and Lai, 2000).

Fiber in companion animal diets

Fiber has beneficial health effects in companion animal diets. It is commonly thought that dogs are carnivores and, therefore, they should consume diets high in protein and low in fiber and grains. However, dogs are ‘opportunistic carnivores’, meaning they consume meat if readily available, but will also consume fruits, vegetables, and grains if available (Legrand-Defretin, 1994). With the domestication of the dog, more balanced and high quality diets are in high demand from both pet owners and pet food companies. Dietary fiber is a common additive to pet foods to enhance stool quality. Many of the health benefits from fiber observed in humans are relevant to dogs. Gut health, stool quality, fermentation kinetics, prebiotic effects, and systemic effects (e.g., low glycemic index, inducing satiety, etc.) noted in humans as a result of fiber intake also have been observed in dogs (Faber, 2011). When evaluating fiber sources for companion animals, desirable characteristics include a moderate to high fermentative capacity with minimal negative effects on nutrient digestibility. Beet pulp is a common fiber source used in dog foods that effectively meet these criteria. Fahey et al. (1990b) evaluated beet pulp and determined that organic matter and dry matter digestibilities decreased by an average of 6% with inclusion of beet pulp; 7.5% was determined to be an ideal inclusion level. *In vitro* and *in vivo* studies showed that fiber sources such as solka floc, xanthan gum, and oat fiber have lower total SCFA production and elicit better fecal quality scores than more fermentable fibers such as citrus peel and apple pomace (Sunvold et al., 1995).

Highly fermentable fibers in dog diets may cause GI distress, but adding them to the diet in low amounts has proven beneficial. Soluble fibers like inulin, oligofructose, and short-chain fructooligosaccharide (scFOS) are beneficial as they increase fecal quality by decreasing fecal odor components like biogenic amines, phenols, and indoles. However, high amounts of fermentable fiber in the diet may increase flatulence and diarrhea. When dogs were fed diets with 0 to 0.9% oligofructose and inulin, fecal scores averaged 2.8 out of 5. No significant differences were observed among diets, and an overall acceptable stool quality resulted (Propst et al., 2003). Oligofructose had a tendency to decrease digestibility of dry matter, organic matter, and crude protein (Propst et al., 2003). Overall, the inclusion of fiber in companion animal diets benefits gut health, but it must be included in select amounts to minimize detrimental effects on nutrient intake and digestibility and stool quality.

The gut microbiota

The gastrointestinal tract harbors the most diversified and largest microbial ecosystem in the mammalian host (Qin et al., 2010). In the adult human, the diversity of microbes is greatest in the colon (Backhed et al., 2005; Hooper et al., 2002). The density of microbes in the large intestine is high, ranging from 10^{11} to 10^{12} bacteria / mL (Kaper and Sperandio, 2005). Furthermore, analysis of the fecal microbial ecology by 16S ribosomal RNA and direct sequencing of genetic material suggests that the human gut harbors a diverse community of 100 trillion archaeal and bacterial cells distributed over more than 1,000 species (Qin et al., 2010; Tremaroli and Backhed, 2012). Bacteria that reside in the large intestine have either beneficial, harmful, or benign effects on the host. Beneficial bacteria include species such as *Bifidobacterium* spp. and *Lactobacillus* spp., and their ability to produce greater amounts of SCFA relative to other species is considered beneficial to the host (Wang and Gibson, 1993).

Harmful species include *Clostridium*, *Staphylococci*, and *Vibrionaceae* spp. because they are involved in the production of toxins and putrefactive compounds. Benign bacteria like *E. coli*, *Enterobacteria* spp., and *Eubacteria* spp. may produce some potential carcinogens, but they also help stimulate the immune system and can aid in digestion. Bacteria can use nutrients from 3 sources; 1) host exogenous sources (diet); 2) endogenous sources from spent enzymes and sloughed epithelial cells from the host; and 3) other colonic bacteria. The microbiome could be modified in numbers as well as diversity of bacteria by factors such as types of fermentable substrates, pH, availability of the substrates, transit time, and peristaltic activity of the small intestine (Roberfroid et al., 2010).

Recent research has focused on the gut microbiota and its interactions with the host, in particular, its role in health and disease of the host. Newer technologies and advanced methods have been able to depict the entire gut microbiome and how it shifts with diet and physiologic state of the host. For example, genetically obese mice were found to have higher *Firmicutes* spp. and fewer *Bacteroidetes* spp. compared with leaner wild-type mice (Ley et al., 2005). Similar changes have been seen in obese humans, where *Bacteroidetes* spp. concentrations increased when the subjects weight was reduced (Ley et al., 2006). Newer technologies have enabled more research into how novel carbohydrates may modulate the gut microbiota.

Prebiotics

Prebiotics are a common food additive in the human food industry and are increasingly popular in companion animal diets. Prebiotics are defined as non-digestible food ingredients that, when consumed in sufficient amounts, selectively stimulate the growth, activity or both of one or a limited number of microbial genera or species in the gut microbiota that ultimately benefits health of the host (Tremaroli and Backhed, 2012). Ingestion of a substrate by the host, such as a

soluble fiber, could be used by bacteria like *Bifidobacterium* and *Lactobacillus* spp. to promote growth of their numbers while simultaneously decreasing harmful bacteria like *Clostridium perfringens* and *E. coli*. Common prebiotics used in human foods also are present in pet foods and include inulin, FOS, and resistant starch (Tomasik and Tomasik, 2003). Inulin and *trans* – galactooligosaccharides both are considered bifidogenic prebiotics due to their ability to selectively stimulate the growth of *Bifidobacterium* spp. (Roberfroid et al., 2010). Chicory root, artichokes, garlic, onion, bananas, rye, wheat, oats, and soybean are other common sources of naturally occurring prebiotic oligosaccharides (Yen, 2003).

Research has shown that inclusion of certain soluble fibers like inulin and FOS has a prebiotic effect in the dog (Propst et al., 2003; Swanson and Fahey, 2006). Too much FOS and inulin in the diet may affect digestibility of the diet and cause GI distress. Recently, a study found that supplementation of galactoglucomannan oligosaccharide (GGMO) to dog diets resulted in a significant increase in fecal *Bifidobacterium* spp. (Faber et al., 2011). Chemically modified starches such as dextrans also have been found to have prebiotic effects in dogs by showing numerical increases in fecal bifidobacteria concentrations with resistant maltodextrin supplementation (Flickinger et al., 2000).

Probiotics

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit to the host (Tremaroli and Backhed, 2012). Common species of probiotics used in both human and pet foods are *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* spp. (Yen, 2003). Strompfova et al. (2004) characterized enterococci as potential probiotic additives. A study investigating the efficacy of *Enterococcus faecium* found that it modulated the hindgut microbiota, but results were inconclusive on whether it had probiotic potential in dogs (Vahjen

and Manner, 2003). Baillon et al. (2004) revealed that the probiotic strain *Lactobacillus acidophilus* can survive transit through the canine gastrointestinal tract and is associated with increased numbers of fecal lactobacilli and decreased numbers of clostridial species. Probiotics also have been linked with decreasing calcium oxalate uroliths in dogs (Weese et al., 2004). Overall, whether the use of probiotics is beneficial in petfoods has yet to be proven.

Starch

Definition

Starch is a polymer of D-glucose units linked α - 1,4 and is the storage form of energy for plants (Miles et al., 1984). It can be separated into two main fractions: amylose and amylopectin. Amylose is a linear polymer of α -1,4 glycosidic linkages while amylopectin is a branched polymer with α -1,4 glycosidic linkages in the backbone and α -1,6 glycosidic branched linkages every 24 to 32 glucose units (Miles et al., 1984). Amylose has an average molecular mass of 10^5 daltons while amylopectin is 10^8 daltons (Majzoobi et al., 2003; Roger et al., 1999; Roger and Colonna, 1996). This difference in molecular mass is due to differences in degree of polymerization. Amylose could have a DP ranging from 10 to 10,000 consecutive glucose units whereas amylopectin could have a DP of 10^7 glucose units (Topping and Clifton, 2001). Both amylose and amylopectin are hydrolyzed by mammalian enzymes. Amylase is the mammalian enzyme that hydrolyzes α -1,4 glycosidic bonds that constitute the linear backbone of amylose and amylopectin (Bowen, 2006). Upon hydrolysis, free glucose can be absorbed via active or passive transport in the small intestine. The branch points of amylopectin are hydrolyzed by maltases present on the brush border before being absorbed (Bowen, 2006).

Raw starch has two crystalline forms: A and B. A-form starches are mostly found in cereals and have glucose chain lengths of 23 to 29 glucose units. B-form starches are amylose

and are abundantly found in tuber plants with a chain length of 30 to 44 glucose units. The crystalline regions of the starch granule are composed of the branch points of amylopectin, while the amorphous regions of starch are composed of amylose (Topping and Clifton, 2001). Raw starches undergo changes in conformation when heat and moisture are applied to them.

Gelatinization is the liberation of the starch granule by heat and moisture, making the granule lose its crystalline ordered structure (Topping and Clifton, 2001). Upon gelatinization, the starch granule is easily digested. Reformation of the linear sequences in the starch granule occurs upon cooling. The short sequences formed are α -1,4 linked glucose units and are insoluble crystallites that are resistant to enzymatic hydrolysis (Gidley et al., 1995; Sievert and Pomeranz, 1989). The process of gelatinization and reformation of short linear glucose sequences is referred to as retrogradation (Gidley et al., 1995; Sievert and Pomeranz, 1989). Retrogradation occurs mainly when foods are cooked and then cooled. Starches with high amylose content, such as high amylose maize and rice products, require higher temperatures and pressure to cause gelatinization compared with starches of higher amylopectin content (Colonna and Mercier, 1985).

Resistant Starch

Resistant starch is the portion of the starch granule that is resistant to hydrolytic and enzymatic digestion in the small intestine and, therefore, enters the large intestine intact. There are four classifications of resistant starch: Resistant Starch 1 (RS1), Resistant Starch 2 (RS2), Resistant Starch 3 (RS3), and Resistant Starch 4 (RS4). Resistant starch 1 is physically inaccessible to being degraded. The degree to which RS1 is inaccessible depends on the extent of ingredient milling and thus, the amount of plant cell wall left intact. The amount of chewing also could affect accessibility of RS1. Examples of RS1 would be partly milled grains and seeds.

Resistant starch 2 is composed of resistant granules and includes raw potato, green banana, some legumes, and high-amylose starches. Resistant starch 3 is retrograded starch resulting from the repeated heating and cooling cycles during processing with examples including cooked and cooled potato, bread, and cornflakes. Resistant starch 4 is a chemically-modified starch composed of etherized, esterified, or cross-bonded starches. Resistant starch 4 is found mainly in processed foods (Asp et al., 1992).

Resistant starch has many beneficial effects on gut health. Much of the research on resistant starch focuses on its protective roles in colorectal cancer. Starch is an important nutrient to many societies, and its intake varies across countries (Topping and Clifton, 2001). Westernized countries consume less than 150 g/person per day as opposed to more agrarian populations that consume greater than 350 g/person per day (Langkilde et al., 1998). People from agrarian societies have a reduced risk of colorectal cancer and higher starch intakes than those in western societies who have higher intakes of fiber (Langkilde et al., 1998). In contrast, it was proven that increased fiber intake and colorectal cancer incidence had no correlation (Cassidy et al., 1994).

Resistant starch is more protective than fiber because of its unique fermentation characteristics. Some evidence suggests that resistant starch increases butyrate production in the hindgut (Christl et al., 1997; Silvester et al., 1995; Weaver et al., 1992, 1988). Butyrate is the preferred energy source for colonocytes and promotes healthy cell turnover in the colon (Noakes et al., 1996; Phillips et al., 1995; van Munster et al., 1994). In a study of patients with colon cancer, 6 g of starch/d reached the large intestine as opposed to 10 g/d in controls (Thornton et al., 1987). This reduction in RS reaching the large intestine could explain why these patients had lower fecal butyrate concentrations. Furthermore, increased consumption of RS2 was found to

lower the incidence of colorectal cancer, partly by its ability to lower fecal secondary bile acid concentrations such as deoxycholate, which is favorable due to its cytotoxicity (van Munster et al., 1994). If lower bile acid secretion favors higher butyrate concentrations, then the effect could be additive (Topping and Clifton, 2001). Resistant starch 3 was found to lower cholic acid excretion by 42% in human ileostomists (Langkilde et al., 1998). Resistant starch is similar in action to fermentable fibers and, thus, may dilute carcinogenic compounds entering the large intestine and decrease the risk of colorectal cancer (Toden et al., 2005). Overall, RS is butyrogenic, has the ability to dilute carcinogenic compounds, and lowers the cytotoxic potential of bile acids; these are all possible explanations for its role in decreasing colorectal cancer risk.

Hindgut fermentation

Hydrolytic/Enzymatic digestion and absorption

In monogastric animals, hydrolytic and enzymatic digestion occurs before fermentation (Ganong, 2001). When nutrients are initially ingested, mastication and some lingual enzymes begin to digest food. Fats and carbohydrates are broken down in the mouth by mastication, lingual lipase, and salivary amylase. Various enzymes then are activated to help degrade these macronutrients into smaller molecules to aid in absorption. Proteins are first broken down by pepsin in the stomach and then by pancreatic trypsin and chymotrypsin in the small intestine into smaller peptides. These smaller peptides then are digested to free amino acids and di- and tri-peptides by pancreatic carboxypeptidases and brush border amino peptidases (Ganong, 2001). Dietary fats and carbohydrates are hydrolyzed to their monomeric units by pancreatic lipase and pancreatic amylase. Brush border enzymes also are able to hydrolyze peptides and saccharides into their monomeric units. Proteins, fats, and carbohydrates then can be absorbed through passive or active transport mechanisms once in monomeric form. Any substrate that is not

digested and absorbed in the small intestine will then enter the large intestine where bacteria can ferment carbohydrate and nitrogenous materials to be used as energy and for bacterial protein synthesis (Hughes et al., 2000).

Fermentation

Fermentation is an enzymatically controlled anaerobic process that breaks down an energy-containing compound (Enger et al., 2012). The hindgut is where undigested nutrients from the small intestine enter and are fermented by the microbiota to produce different fermentative end-products. Carbohydrates, protein, and fat are the main substrates that reach the large intestine. The carbon- and nitrogen- containing substrates entering the human large intestine include resistant starch (8 to 40 g/d), nonstarch polysaccharides (10 to 20 g/d), unabsorbed sugars or sugar alcohols (2 to 10 g/d), oligosaccharides (2 to 6 g/d), dietary protein (3 to 9 g/d, but varies with diet), some nitrate, and endogenous materials including GI secretions (4 to 6 g/d), urea, mucus, bacterial cell lysis, and sloughed epithelial cells (Gibson and Roberfroid, 1995; Macfarlane and Cummings, 1991; Van Loo et al., 1995a). Bacteria need a source of carbon and nitrogen from fermentable carbohydrates and protein to survive.

Carbohydrates are used as a substrate to supply energy for bacteria as well as the colonocytes indirectly (Hughes et al., 2000). Three main fermentative end-products predominantly produced from carbohydrate fermentation are the short-chain fatty acids (SCFA) acetate, propionate, and butyrate (Topping and Clifton, 2001). Ethanol, lactate, and succinate are intermediates formed during carbohydrate fermentation. Nitrogen can be assimilated for bacterial protein synthesis (Hughes et al., 2000). Fermentative end-products from protein include ammonia, phenolic and indolic compounds, amines, the branched-chain fatty acids (BCFA) isovalerate, isobutyrate, 2-methylbutyrate, and valerate, and sulfides (Hughes et al., 2000). Other

fermentative end-products include gases such as carbon dioxide (CO₂), hydrogen g (H₂), and methane (CH₄) from carbohydrate fermentation and H₂S from the reduction of dietary sulfate (SO₄), and bacterial cells from cell lysis (Hughes et al., 2000; Levitt, 1994). With increased fermentation and an increase in SCFA comes a subsequent drop in pH (Wong et al., 2006); thus, colonic pH varies due to the availability of fermentable substrates.

Short-chain fatty acids

Acetate, propionate, and butyrate are the main SCFA produced in carbohydrate fermentation. The concentration of these compounds varies through all sections of the GI tract. Generally, in all species, the proximal colon has a higher concentration of SCFA (70 to 140 millimolar) due to greater availability of carbohydrate substrates than in the distal colon (20 to 70 millimolar) where most of the carbohydrates already have been metabolized (Topping and Clifton, 2001). Short-chain fatty acid concentration also is dependent on the fermentability of the substrate. Substrates differ in their susceptibility to microbial breakdown. For example, oat fiber can produce up to 247 μmol/g of total SCFA while gum arabic can produce 9,384 μmol/g of total SCFA (Titgemeyer et al., 1991). In the typical monogastric animal, the normal ratio of these three SCFA is 60:20:20 acetate: propionate: butyrate (Cummings, 1981). This ratio can vary depending on substrate. For example, soy fiber produces 71% acetate, 21% propionate, and 8% butyrate (Titgemeyer et al., 1991). Sugarbeet fiber changes the proportion of total SCFA to 93% acetate, 7% propionate, and 1% butyrate (Titgemeyer et al., 1991). In general, resistant starch primarily shifts the balance to produce more acetate and butyrate, whereas pectin fermentation results in primarily acetate (Sunvold et al., 1995; Topping and Clifton, 2001). However, it should be noted that much of these data were generated from *in vitro* experiments that tend to have less biological variability. There are numerous factors that influence changes in SCFA proportions *in*

vivo, including different microbial populations, heterogenous substrates entering the colon, and differences in absorption rates proportions (Wong et al., 2006).

SCFA and the Gut Microbiota

Microbes in the hindgut require a source of adenosine triphosphate (ATP) for growth and replication. Anaerobic bacteria are what mainly reside in the hindgut and are able to utilize carbon-containing substrates for energy in the absence of oxygen. In an anaerobic environment, microbes are restricted by the requirement to maintain redox balance and dispose of protons generated by catabolism to prevent acidosis. Anaerobes are unable to use a free electron acceptor like oxygen (Cummings and Macfarlane, 1997). Therefore, they need to use portions of substrates or other exogenous compounds such as sulfates and nitrates for protein synthesis. Aerobic metabolism is more efficient than anaerobic metabolism at generating ATP. Glucose can generate 38 ATP/mole when it is aerobically metabolized as opposed to 2 to 6 ATP/mole when anaerobically fermented (Powers, 2005). The combustible energy that is in the original substrate is often times retained in end products of fermentation, mainly SCFA. These end products can be used for energy by the host and the microbiota. Most of the carbon-containing substrates that are being fermented are polysaccharides (Cummings and Macfarlane, 1997), and the extent of their breakdown depends on the type of polysaccharide (Cummings and Macfarlane, 1997). Soluble fibers like starch, fructans, and pectin are fermented rapidly in the large intestine. Fermentation of insoluble fibers such as hemicelluloses and cellulose is more variable, with hemicelluloses being more fermentable than cellulose (hemicelluloses can vary from 50 to 80% fermentability) (Younes, 2012). Depending on the crystallinity of cellulose, it can range from 0 (wood cellulose) to 30 - 50% fermentable as noted for very amorphous forms of cellulose (Younes, 2012).

Absorption of SCFA

Fermentative end products are utilized by both the microbiota and the host. Most fibers are thought to have energy values of 1.5 to 2.0 kcal/g, depending on the fermentability of the substrate (Venema, 2011). Non-fermentable substrates have a metabolizable energy value close to zero, while a fully fermented substrate could have a metabolizable energy value of 2 kcal/g. In a mixed diet, metabolizable energy is normally 1.5 kcal/g. Absorption of SCFA in the large intestine is very efficient, with 95 to 99% of SCFA being absorbed and utilized by humans (Ruppin et al., 1980). There are three main mechanisms to explain SCFA absorption by the colon: passive diffusion, carrier-mediated, and epithelial transporters (Gill et al., 2005). Passive diffusion requires the SCFA to be in the nonionic form. The lumen of the gut must also be acidic for absorption of SCFA via passive diffusion. When the colon is pH 4.8 to 5.8, SCFA are absorbed at about 60% passively (Fleming et al., 1991). Carrier-mediated mechanisms include SCFA/HCO₃⁻ exchange and a protonated SCFA plus a Cl⁻ ion in the enterocyte. Two of the most recent developments into transport mechanisms are the monocarboxylate transporters (MCT) and sodium monocarboxylate transporters (SMCT) (Iwanaga et al., 2006). Lactate, pyruvate, SCFA, and ketone bodies normally cross the colonic epithelium via MCT transporters. Short-chain fatty acids pass from the enterocyte to the portal circulation via MCT 4 and MCT 5 and continue to be metabolized once they reach the liver (Iwanaga et al., 2006). There are 15 known isoforms of MCTs currently known and found throughout the body (especially in the gastrointestinal tract (GIT)). Concentrations of MCTs are dependent on SCFA production. In ruminants, the highest concentrations of MCTs are in the rumen where the majority of fermentation happens (Kirat et al., 2006). In humans, the highest concentration of MCTs are in the distal part of the GI tract (Gill et al., 2005). In the distal colon, some isoforms are on the apical membrane and others are

on the basolateral membrane. Another SCFA transporter found in the colon is SMCTs, which are sodium-coupled electrogenic transporters. Sodium-coupled electrogenic transporters have a higher affinity for the absorption of butyrate than other SCFA. There are segmental differences in the GI tract as regards SCFA absorption (Scheppach, 1994). In the proximal colon, absorption is similar for all SCFA. Distal colon permeability differs for acetate and butyrate. Acetate has a lower permeability and butyrate has a higher permeability in the distal colon (Topping and Clifton, 2001). These segmental differences may be due to a number of factors such as differing chain length, lipid solubility, pH-dependent mechanisms, paracellular transport, and changing affinities for MCT or SMCT.

SCFA metabolism

Although SCFA meet 60 to 70% of the energy needs of colonocytes, they also have systemic effects. Estimates suggest 20 to 75% of absorbed acetate is utilized by the liver for fatty acid synthesis (Wong et al., 2006). Another metabolic fate of acetate includes its role in oxidation within the TCA cycle. Acetate can be converted to acetyl-CoA, which is a major intermediate in the TCA cycle and fatty acid synthesis, via the enzyme, acetyl-CoA synthetase. Nearly 100% of the propionate is taken up by the liver and used as a gluconeogenic precursor. In ruminants, 25 to 90% of glucose is derived from propionate absorption and 7% in horses (Hooper et al., 2002). In humans, the importance is unknown and likely does not have an impact on blood glucose concentrations. Propionate has been linked to a role in lipid and cholesterol metabolism in humans and animals (Wong et al., 2006). Evidence suggests that propionate decreases serum cholesterol in rats and pigs, downregulates cholesterol synthesis in rat hepatocytes, and may redistribute cholesterol from plasma back to liver or adipose tissue (Beaulieu and McBurney, 1992; Chen et al., 1984). Further studies are needed to support these

findings because human *in vivo* studies have produced less convincing results. Butyrate is the preferred energy source for colonocytes; therefore very little reaches the liver. Butyrate spares colonic oxidation of glucose and glutamine that can be used for energy in other tissues (Topping and Clifton, 2001). Butyrate accounts for up to 70% of oxygen consumption during *in vitro* testing and is used as a fatty acid precursor in other tissues such as the liver and other lipogenic tissues (Wong et al., 2006). Overall, SCFA are utilized locally by colonocytes and also help in cellular function in other parts of the body.

SCFA are important for gut health and reduce the risk of diseases like diabetes, heart disease, and obesity. Although all SCFA increase colonic cell proliferation in a dose-dependent manner, butyrate has been found to have the most profound impact (Topping and Clifton, 2001). Butyrate has protective effects against colon cancer because it increases cell proliferation in normal colonocytes and decreases cell proliferation in colonic adenocarcinoma cells (Wong et al., 2006). Short-chain fatty acids also modulate colonic and intracellular pH, which can be achieved directly by the acidic nature of SCFA or indirectly by modulating the microbiota, and subsequently decreasing bile acid solubility, increasing mineral absorption, and decreasing ammonia absorption. Short-chain fatty acids also have been found to increase mucosal functionality by increasing expression of the GLUT-2 gene. Tappenden et al. (1998) found that after SCFA were intravenously administered by total parental nutrition, there were increases in GLUT-2 mRNA in the basolateral membrane and an increase in ileal uptake of glucose. This means that increased gene expression of ileal proglucagon and glucose transporter expression occurred in response to increased SCFA. Short-chain fatty acids increase gene expression of proglucagon gene products such as glucagon-like peptide 1 (GLP1), which is produced by enteroendocrine L cells, predominantly in the ileum (Pratley and Gilbert, 2008). The primary

functions of the GLP -1 receptor include decreasing gastric emptying and acid secretion. Other systemic effects of GLP-1 include increasing neuroprotection, cardioprotection, cardiac output, and insulin secretion and sensitivity in the liver. Glucagon like peptide -1 also decreases appetite, gluconeogenesis, and β -glycosidic cell apoptosis in the stomach (Drucker, 2005). Glucagon-like peptide 2 (GLP-2) is also produced by enteroendocrine L cells, predominantly in distal small intestine (Mayo et al., 2003), and like GLP-1, GLP-2 decreases gut motility and gastric acid secretion. It also increases cell proliferation and hexose transport (via an increase in the SGLT-1 transporter) (Drucker, 2005). Overall, SCFA regulate cell proliferation, differentiation, and gene expression.

Systemic lipid metabolism also is affected by SCFA. Acetate and propionate inhibit lipolysis and release of glycerol (Robertson, 2007). This is mediated by the expression of G-protein coupled receptors (GPR). G-protein coupled receptor 41 and GPR43 are expressed on adipocytes and have a similar response to that of insulin. When cyclic AMP is decreased, hormone sensitive lipase is deactivated. In lipogenesis, SCFA stimulate fat accumulation in adipocytes by increasing peroxisome proliferator-activated receptor gamma (PPAR γ), increasing acetyl CoA carboxylase and increasing fatty acid synthase. New adipocytes have decreased insulin sensitivity and increased lipolytic activity. Fat that accumulates in tissues such as muscle, pancreas, and liver, is less metabolically active, meaning these adipose cells undergo less lipolysis and more lipogenesis. Furthermore, fatty acids decrease insulin sensitivity in these tissues that decreases the uptake of glucose from the blood. Short-chain fatty acids also affect expression of leptin, a hormone secreted by adipose tissue that has a role in controlling satiety. Xiong et al. (2004) applied SCFA to mouse adipose cell lines and primary cultures and found that propionate and acetate increased leptin expression. Insulin sensitivity also was increased

when obese dogs were fed short-chain fructooligosaccharide (scFOS), a fermentable carbohydrate (Respondek et al., 2007). Short-chain fatty acids are important in maintaining gut integrity, as well as facilitating other systemic effects that are important in health and disease.

Protein fermentation

Protein fermentation often is associated with poor gut health. Carbon is provided by the SCFA, acetate, propionate, and butyrate, while nitrogen is provided by undigested dietary protein and endogenous sources such as spent enzymes and sloughed epithelial cells (Gibson et al., 1996). On average, about 12 g of proteinaceous material enters the human large intestine each day (Hughes et al., 2000). Of this proteinaceous material, 48-51% are intact proteins, 20-30% are peptides, and a small percentage comes from amino acids (Hughes et al., 2000). On average, 50% of protein entering the proximal colon comes from the diet and 50% from endogenous material. This distribution can change depending on the amount and concentration of protein in the diet (Chacko and Cummings, 1988; Silvester and Cummings, 1995). In humans, the main health implications associated with a proteolytic fermentation are increased risk of colon cancer, putrefactive compounds, and damaging agents to the colonic epithelium. Undigested protein can be metabolized by different bacteria into four catabolites in addition to organic acids. These catabolites include ammonia, phenolic and indolic compounds, amines, and sulfides.

Ammonia

Ammonia is produced from amino acid deamination and is needed in minor amounts for bacterial protein synthesis (Hughes et al., 2000; Wrong et al., 1985). Bacteria assimilate ammonia for protein synthesis when energy-containing substrates are present in high concentrations. The concentration of ammonia is dependent on the balance between amino acid deamination and bacterial protein synthesis (Hughes et al., 2000). Studies on ammonia and gut

health are conflicting. Ammonia was found to alter morphology and intermediary metabolism of intestinal cells and affect DNA synthesis, and ammonia reduced the lifespan of colonic cells (Visek, 1978). Furthermore, ammonia was found to result in carcinogenesis in rat colon (Lin and Visek, 1991). However, Ichikawa and Sakata (1998) determined that SCFA and ammonia stimulated epithelial cell turnover. This suggested a significant interaction between ammonia and SCFA that helps to stimulate colonic cell turnover. However, these studies were conducted with direct infusions of ammonia into rat colon. This means that the effect of SCFA production and the variability of fermentable substrate entering into the large intestine was not taken into account when conducting this research.

Phenols and Indoles

Phenolic and indolic compounds are formed when the aromatic amino acids, phenylalanine, tyrosine, and tryptophan, get α or β eliminated (Hughes et al., 2000). The main products of these amino acids are phenylacetate, p-cresol + phenylpropionate, and indole propionate + indole acetate. The relationship between phenol and indole production and disease is unclear. P-cresol has been found to be mutagenic, and *in vitro* studies further determined that phenols and nitrites produce a mutagenic compound called diazoquinone (Bone et al., 1976; Kikugawa and Kato, 1987). Smith and Macfarlane (1996) observed a 60% decrease in the net production of phenolic compounds with the supplementation of a fermentable carbohydrate. In post-cholecystectomy patients, there were almost three times greater fecal 3-methyl indole concentrations than healthy adults (93.1 $\mu\text{g/g}$ vs. 38.4 $\mu\text{g/g}$). Patients also had an increased ratio of fecal 3-methyl indole to indole concentrations than in healthy adults, which is consistent to what has been found in colon cancer patients (Zuccato et al., 1993).

Biogenic Amines

Amines are formed from the decarboxylation of amino acids. Upon decarboxylation, agmatine, tyramine, pyrrolidine, histamine, piperidine, cadaverine, putresine, and 5-hydroxytryptamine are formed (Drasar and Hill, 1974). The main bacterial species that form these compounds are *Clostridium*, *Bifidobacterium*, and *Bacteriodes* spp. (Allison and Macfarlane, 1989). In healthy individuals, these compounds are normally detoxified by monoamine and diamine oxidases. However, amines have been linked to migraine headaches, hypertension, and hepatic coma (Macfarlane and Macfarlane, 1997). Tyramine in food has been linked to heart failure (Smith, 1981). In terms of gut health, cancer patients excrete higher levels of putrescine and cadaverine derivatives than do healthy individuals (Murray et al., 1993). However, putrescine has been found to regulate colonic cell growth, meaning that it may be needed in low concentrations (Murray et al., 1993; Seidel et al., 1984). Spermidine is also a biogenic amine that has beneficial effects in low concentrations due to its favorable effects on normal cell turnover (Linsalata and Russo, 2008). There is great interest in amines concerning the function they have as precursors to N-nitroso compounds. N-nitrosocompounds (NOC) are known to exert carcinogenic and mutagenic effects in cell turnover (Hughes et al., 2000). N-nitrosocompounds are formed as a result of the reaction between a nitrosating agent from nitrate metabolism and a nitrosatable substrate such as amino acids or peptides. Silvester et al. (1997) investigated the effect of high meat intakes and fecal NOC concentrations. They determined that subjects consuming diets high in meat had 3 time greater concentration of fecal NOC than subjects consuming diets low in meat (Silvester et al., 1997).

Sulfides

Bacterial metabolism of sulfur-containing amino acids generate compounds known as sulfides (Hughes et al., 2000). Sulfur-containing amino acids include methionine, cysteine,

cystine, and taurine. The main putrefactive compound produced from the bacterial metabolism of these amino acids is hydrogen sulfide. Dietary sources of sulfur come from both sulfate / sulfites and dietary protein. In humans, the estimated daily intake is between 1.5 to 16 mmol of sulfides per day (Florin, 1991). Many studies have focused on hydrogen sulfide and its relation to health and disease. Hydrogen sulfide was found to increase mucosal apoptosis and goblet cell depletion at concentrations of 0.2 to 1.0 mmol/L for 4 h in rats (Aslam et al., 1992). A possible explanation for this effect is that hydrogen sulfide can inhibit colonic cells from butyrate oxidation at a concentration as low as 2 mmol/L (Jorgensen and Mortensen, 2001; Roediger et al., 1993a, b). Increased concentrations of hydrogen sulfide in the large intestine have been connected with diseases such as ulcerative colitis and inflammatory bowel disease. Sulfides at concentrations as low as 1 mmol/L significantly increased cell proliferation rates by 19% compared to NaCl controls, and decreased butyrate oxidation rates that are associated with ulcerative colitis (Christl et al., 1994). Furthermore, humans consuming high protein diets had fecal sulfide concentrations as high as 3.4 mmol/L (Magee et al., 2000). Many of these studies, however, report free fecal hydrogen sulfide. Fecal material contains various metallic ions such as Fe^{3+} , Cu, Zn, etc. that enter the large intestine because they are not completely absorbed by the small intestine. Metallic ions bind to H_2S , which negates the negative effects of H_2S on butyrate oxidation and colonic cell turnover (Jorgensen and Mortensen, 2001).

Overall, protein catabolites are harmful to the colonic epithelium when they are high in concentration. The majority of research done in this area investigated protein catabolites being infused into rat colon or studies used an extremely high protein diet. Providing a balanced diet with fermentable fiber and digestible protein should minimize the concentrations of protein catabolites in the hindgut and will lessen their impact overall on the colonic epithelium.

Potatoes and potato fractions as dietary ingredients

Potatoes (*Solanum tuberosum*) are a starchy plant tuber frequently present in human diets. Potatoes are the world's top non-grain food commodity and are a staple food in many countries (Gao et al., 2012). The nutrient content of a whole potato varies due to many factors, including production area, cultivar, soil, climate, agricultural practice, preparation, and cooking (International Year of the Potato, 2008). Raw potatoes are a good source of protein, water-soluble vitamins (ascorbic acid, niacin, thiamin, riboflavin, folic acid, and vitamin B₆) and minerals (Augustin et al., 1978), but starch is by far the most abundant nutrient in whole potatoes. Potato skin also is a good source of phytochemicals like carotenoids and polyphenolic compounds. Most of the carotenoids found in potato are xanthophylls, which are lutein, zexanthin, and violaxanthin (Brown, 2005). The predominant phenolic compound found in potatoes is chlorogenic acid. Potatoes also are a source of flavonoids, in particular, catechins and epicatechins (Brown, 2005).

Grains often are not as safe to eat due to irritants like gluten, but potatoes are different in that they have no gluten. Potatoes also contain notable amounts of resistant starch. Raw potato starch is considered an RS2. Upon cooking, this resistant starch undergoes gelatinization, which involves the liberation of the starch granule and its breakdown into glucose units (Topping and Clifton, 2001). Once gelatinized, the starch is 100% digestible in the mammalian small intestine. Upon cooling, however, the glucose will re-synthesize digestible amylose and amylopectin, but also recover some of the resistant starch. The process of gelatinization and subsequent cooling to re-synthesize the starch granule is referred to as retrogradation. Resistant starch that is formed from retrogradation is referred to as RS3. Having high amounts of RS2 in the raw starch form and RS3 from retrogradation makes potato starch somewhat unique.

Health Benefits

Phenolic compounds have been shown to possess antimutagenic, anticarcinogenic, antiglycemic, and antioxidant benefits (Friedman, 1997). Kahkonen et al. (1999) analyzed the antioxidant potential of 92 phenolic extracts from edible plant material and classified potato peel as having strong antioxidant activity. Other components of potatoes that deliver antioxidant potential are vitamin C, flavonoids, and carotenoids. Diets high in flavonoids and carotenoids have been associated with lower incidences of heart disease, macular degeneration, cataracts, and certain cancers (Brown, 2005; Cao et al., 1998; Hertog et al., 1993; Knekt et al., 1996; Kruezer, 2001; Wang et al., 1999). Chlorogenic acid and other polyphenols have been found to exhibit antioxidant effects on LDL resulting in less susceptibility to plaque formation. Lazarov and Werman (1996) found that consumption of potato peel lowered plasma cholesterol concentrations by 40% and a 30% reduction in hepatic cholesterol content compared to rats fed cellulose. The authors concluded that the fiber portion of the potato is responsible for this reduction, but it is likely that polyphenols play a role as well (Friedman, 1997).

Consumption of resistant potato starch provides several benefits to gut health. Resistant starch 2 and RS3 are very fermentable (Topping and Clifton, 2001). Cummings et al. (1996) observed that the consumption of potato RS2 by human subjects resulted in a 24% increase in fecal total SCFA concentrations and a 21% increase in fecal butyrate concentrations. Other studies have suggested that rats fed potato starch exhibited a 6-fold increase in butyrate production in the cecum and proximal colon, and a 3-fold increase of butyrate in the distal colon at 6 mo compared to levels at 0.5 mo (Le Blay et al., 1999). This suggests that prolonged consumption of potato resistant starch increases butyrate production throughout the entire hindgut. Furthermore, pigs consuming 15 g of potato starch per day had the highest proportion of

fecal butyrate concentrations (13% of total SCFA) when compared to pigs consuming high amylose- or retrograded high amylose-containing maize starches (7 and 6 % of total fecal SCFA concentrations, respectively) (Martin et al., 1998). An *in vitro* fermentation study with pig fecal inoculum found that potato starch resulted in a 31% increase in butyrate production compared to the control (Wang et al., 2004). Having increased amounts of butyrate suggests potatoes may be beneficial to healthy colonic cell turnover. Research on potato-specific RS3 is limited, but RS3 from other sources has been found to have similar beneficial effects in the prevention of colon cancer. Bauer-Marinovic et al. (2006) found that hydrothermally-treated RS3 prevented colon carcinogenesis by increasing apoptosis of damaged cells as well as favorable changes in differentiation of colonocytes. Overall, RS2 and RS3 have been found to have induced favorable fermentation characteristics that can aid in gut health.

Browning and Anti-Nutritive Properties

Although phenolic compounds have beneficial effects, they could have some anti-nutritive properties depending on growing and harvest conditions. Polyphenols are secondary metabolites and are involved in the defense of the plant against bacterial and fungal pathogens as well as viruses (Friedman, 1997). Potatoes may undergo browning and other discolorations during growth and harvest (Friedman, 1997). This internal browning is caused by polyphenol oxidase (PPO), which causes the oxidation of phenolic compounds to quinones. Quinones will then bind with amino acids and cause dark pigmentation in the potato (Friedman, 1997; Hurrell and Finot, 1984; Whitaker and Lee, 1995). Besides having off pigmentation, shrinkage and rotting could cause major economic losses to producers. Spot formation also could induce bruising after harvest and synthesize harmful glycoalkaloids (Friedman and Dao, 1992; Friedman and McDonald, 1997). Sulfhydryl compounds like cysteine, *N*-acetyl-L-cysteine, reduced

glutathione, as well as ascorbic and citric acids have been found to decrease the activity of PPO and reduce the amount of browning (Friedman, 1997).

Semiquinones and quinones formed from the oxidation of polyphenols also may have adverse effects on protein quality. One example of this would be with chlorogenic acid quinone with the NH₂ group on lysine, SH groups of cysteine, and OH groups of serine and tyrosine (Friedman, 1997). This binding could occur in the gut and have an effect on protein digestion and absorption. Rat feeding studies suggested that protein quality is affected by polyphenol-amino acid complexes. Griffiths (1986) found that tannins and polyphenolic compounds had negative effects on protein and starch digestibility by inhibiting the actions of α -amylase and trypsin.

Growth and harvest conditions are two main factors that could affect the nutritional quality of potatoes. Storage and shipment of potatoes also is of interest to growers and producers of potato products. Changes in potato nutrient composition often are due to improper storage temperatures and lighting (Friedman, 1997). More studies need to be done on optimal storage conditions. According to Woodell et al. (2009), the optimal home storage conditions are that the unit should be cool, ventilated, dark, and humid. Specifically examining different storage conditions on polyphenol and glycoalkaloid concentrations would provide a indication of what storage conditions should be implemented to minimize nutrient damage from browning. Research on downregulating PPO expression has been successful in decreasing browning. By knocking out the PPO gene, polyphenol-amino acid and enzymatic interactions will be minimized and, thus, nutrient content will be enhanced. Recent studies have examined decreased PPO expression and found that it decreased oxidative damage, but there could be an enhanced susceptibility to pathogens (Thipyapong et al., 2004). Overall, it is important that potatoes are

exposed to adequate conditions to preserve their nutritive value (from growth and harvest to storage).

Potatoes in Pet Foods

Many of the potato products on the market focus on alternatives to protein and grain sources. Because of an increasing demand for ingredients that support gut health, pet food companies are seeking novel substrates to incorporate into their products. Many of these novel sources come from co- and byproducts of the human food industries. Potatoes have become increasingly popular in pet foods, and there are a variety of potato products currently on the market. Examples include dried potato products (AAFCO 60.28), potato starch, potato protein, potato buds, and potato flour (Aldrich, 2005). Potato starch comes in two different forms. One form is larger pieces of potatoes in a gravy, and the other form is in kibble diets to help improve expansion and texture of the kibble. Furthermore, potato peels have been found to reduce the hydration capacity and increase the fiber content of the extrudate (Camire and Flint, 1991).

Potatoes also are a good alternative protein source, especially with the high demand for hypoallergenic pet products. Potato protein has been an important ingredient in the pet food market. Potato protein can be included as an alternative to corn gluten meal and rice protein in pet foods (Aldrich, 2005). In addition to potato protein, potato bud, a co-product of the french fry industry, is an important ingredient in pet foods. The amount of peel present in the potato bud can affect the color and expansion of extruded kibble. Potato bud is currently incorporated into high-end pet foods and can be purchased at specialty pet food stores. Potato flour, like potato buds, can be used as a functional starch substitute for wheat due to its similar consistency. However, potato flour lacks some of the primary elasticity and binding characteristics provided by wheat gluten.

Another co-product of potato processing that may have potential as a novel fiber source is potato pulp. Potato pulp is a co-product of potato starch isolate, and is widely used in the beef industry (Nelson, 2010). Potato starch processing involves rupturing the starch granules and subsequent gelatinization. The rupturing process disrupts the cells to free the starch granule (Nigam and Singh, 1995). This mash then is diluted and placed into a centrifuge to separate the skin and other cell contents from the starch. The skin and cell fragments are referred to as the pulp (Klingspohn et al., 1993). Potato pulp carbohydrates are pectins, resistant and digestible starch, cellulose, and hemicelluloses. The fiber of potato pulp is very digestible by ruminant animals, making it an excellent source of energy. Potato pulp has the potential to be a high quality fiber source in pet foods due to its considerable amount of insoluble and soluble fiber, as well as its resistant starch content. The soluble fiber fraction of potato pulp has been found to be rich in galactan-branched rhamnogalacturonan I type pectin (Thomassen, 2011). Typically, a higher amount of fiber in companion animal diets decreases nutrient digestibility (Burrows et al., 1982), so the ratio of insoluble to soluble fiber is particularly important when considering fiber sources. Insoluble fiber will affect stool quality and provide adequate bulk, while soluble fiber will provide benefits via SCFA production from fermentation and possible prebiotic effects (Swanson et al., 2001). Potatoes are used in pet foods mainly for carbohydrate or protein sources.

Very little research has been conducted on the nutritional value of potato pulp. Laerke et al. (2007) found that the soluble fiber fraction of potato pulp resulted in higher rat cecal SCFA concentrations of 173.5 μmole compared to 83.0 μmole in cellulose controls ($P < 0.05$). Furthermore, solubilized potato pulp decreased ($P < 0.05$) postprandial plasma triacylglycerol from 2.79 mmol/L in the cellulose control diet to 1.82 mmol/L in the solubilized potato pulp diet. Human data showed that consumption of enzymatically-solubilized potato fibers induced a 200%

increase in breath H₂ analysis compared to oat bran, which was interpreted as increased fermentation (Olesen et al. 1998). Thomassen et al. (2010) solubilized potato pulp and conducted an *in vitro* fermentation with human fecal inoculum to evaluate prebiotic effects. Solubilized potato pulp was 3 times more bifidogenic than FOS, by showing a 300% increase in fecal *Bifidobacterium* spp. DNA concentrations from FOS. Furthermore, it was concluded that soluble fiber fractions of greater than 100 kDa had the greatest effect. These studies focus on the soluble fiber fraction of potato pulp, but they do not evaluate potato pulp in its native form that contains significant amounts of both insoluble and soluble fibers. Furthermore, the application of potato pulp as a dietary fiber source in high quality dog foods has not been investigated.

Thesis objective

Fiber is important in both human and animal nutrition due to its beneficial effects on gut and systemic health. The overall objective of this thesis was to determine whether potato pulp may serve as a high quality fiber source in dog foods. From the nutritional perspective, fiber quality is typically determined by its ability to maintain optimal food intake and nutrient digestibility; result in an optimal stool score; and its ability to enhance gut health biomarkers. Also, with novel carbohydrates, it is important to understand substrate fermentability and how this property affects utilization of other nutrients in the diet. In this thesis, potato pulp will be investigated to determine all of these characteristics. Data will be presented on compositional analyses, *in vitro* hydrolytic digestion and fermentation, and *in vivo* utilization of potato pulp – containing diets. Gut health biomarkers include concentrations of fermentation end-products, protein catabolites, and fecal score and pH. Information from this research will enhance our understanding of potato pulp as a novel ingredient in high quality dog foods.

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CHAPTER 3

POTATO PULP AS A DIETARY FIBER SOURCE IN HIGH QUALITY DOG FOODS

Abstract

Dietary fiber is important in companion animal diets because of its positive effects on gut and systemic health. Potato pulp (PP), a co-product of potato starch processing, was evaluated as a potential novel fiber source in dog foods. The PP substrate was evaluated for chemical composition, *in vitro* digestion and fermentation characteristics, and *in vivo* responses. For the *in vitro* experiment, raw and cooked PP were fermented using dog fecal inoculum, and fermentation characteristics were measured at 0, 3, 6, 9, and 12 h. For the *in vivo* experiment, 10 female dogs with hound bloodlines (5.4 ± 0.0 yr; 22 ± 2.1 kg) were each provided 5 diets with graded levels (0, 1.5, 3, 4.5, or 6%) of PP in a replicated 5 x 5 Latin square design. Fresh fecal samples were collected to measure fecal pH and fermentation end-products. Chemical composition results revealed that raw and cooked PP contained 55% total dietary fiber (TDF), with 32% insoluble fiber and 23% soluble fiber, as well as 4% crude protein (CP) and 2% acid-hydrolyzed fat (AHF). *In vitro* digestion and fermentation revealed that raw and cooked PP are 32.3 and 27.9% enzymatically digested and are fermentable through 9 h of fermentation. Raw PP had higher ($P < 0.05$) acetate, propionate, and total SCFA at the 12 h time point compared to cooked PP. In the *in vivo* experiment, no differences were observed in dry matter (DM), organic matter (OM), CP, AHF or energy digestibilities of diets containing graded levels of PP. Total dietary fiber (TDF) digestibility increased ($P < 0.01$) in dogs fed the 3, 4.5, and 6% PP diets compared to dogs fed the 0% PP diet. Fecal pH decreased ($P < 0.01$) when dogs were fed the 4.5 and 6% PP diets compared to the 0% PP diet. Fecal acetate and total SCFA were higher ($P < 0.01$) when dogs were fed the 3, 4.5, and 6% PP diets compared to the 0% PP diet. Overall,

linear increases ($P < 0.01$) were observed for all SCFA, with a concomitant linear decrease ($P < 0.01$) in fecal pH. These findings suggest that increased inclusion of PP elicited favorable fermentation characteristics without negatively affecting nutrient digestibility. Fecal protein catabolites were physiologically low or undetectable, with the exception of spermidine, which had increased concentrations ($P < 0.05$) when dogs were fed the 6% PP diet compared to the 0% PP diet. Collectively, these data suggest that PP could be a functional dietary fiber source in high quality dog foods.

Introduction

Pet food companies are constantly searching for novel ingredients to improve their product lines and provide health benefits to pets. Including dietary fibers in pet foods has become increasingly popular due to their positive effects on intestinal health, as well as their favorable systemic effects. However, it is important to define the optimal dietary concentration of each fiber included as many digestive physiological outcomes (e.g., stool quality, nutrient digestibility, fecal SCFA and BCFA concentrations, fecal protein catabolite concentrations, etc.) will be impacted by amount fed.

High quality pet foods often include sources of moderately fermentable fiber because these carbohydrates tend to increase SCFA production without decreasing nutrient digestibility. Beet pulp and oat fiber are common sources of moderately fermentable fiber used in pet foods. Research using these dietary fiber sources has determined that an inclusion level of 7.5% maximizes hindgut fermentation without sacrificing nutrient digestibility (Fahey et al., 1992). Furthermore, these fiber sources are thought to be fermented throughout the distal GI tract, which may optimize GI health from proximal to distal colon. Overall, a fiber source that has

minimal negative effects on nutrient digestibility and is fermented throughout the entire distal GI tract would be considered a high quality fermentable fiber.

Potato pulp is a co-product of potato starch processing and has potential to be a novel fiber source for the dog. Most PP is considered agricultural waste and is typically used in cattle feeds. Cattle producers have taken advantage of feeding PP because it has a favorable fiber content and is also palatable for ruminant animals (Meyer et al., 2009). The chemical composition of PP includes fiber components such as hemicelluloses, cellulose, and pectin, and non-fiber components such as starch, oligopeptides, and free amino acids (Mayer, 1998). Furthermore, potatoes are grown worldwide and are easy to obtain, which makes PP a cost-effective option as a novel fiber ingredient. Potato pulp ostensibly could serve as a novel dietary fiber source in pet foods due, in part, to its favorable chemical composition. However, PP has not been investigated as a fiber source in dog foods. Therefore, the objective of this study was to characterize PP for its nutrient composition, *in vitro* digestion and fermentability characteristics, and *in vivo* responses.

Materials and Methods

Chemical analyses

A 500 g subsample of PP was suspended in 6 liters of distilled water and autoclaved at 121°C for 1 h with a pressure of 18 pounds per square inch (PSI). The non-autoclaved PP (i.e., raw PP) and autoclaved PP (i.e., cooked PP), as well as experimental diets and fecal samples were analyzed for dry matter (DM), organic matter (OM), and ash according to AOAC (2006) methods. Crude protein was calculated from Leco (FP2000 and TruMac) total nitrogen (N) values (AOAC, 2006). Total starch concentration of raw and cooked PP was determined using AOAC (2006) methods. Free glucose and digestible starch were determined according to Muir

and O’Dea (1993). Resistant starch was determined by subtracting digestible starch and free glucose from total starch. Total lipid content was determined by acid hydrolysis followed by ether extraction according to the American Association of Cereal Chemists (1983) and Budde (1952). Gross energy was measured using an oxygen bomb calorimeter (model 1261, Parr Instruments, Moline, IL). Free monosaccharide concentrations were determined according to Smiricky et al. (2002). Total dietary fiber (TDF), insoluble dietary fiber, and soluble dietary fiber concentrations were determined according to Prosky et al. (1992).

Fecal SCFA and branched-chain fatty acid (BCFA) concentrations were determined by gas chromatography according to Erwin et al. (1961) using a gas chromatograph (Hewlett-Packard 5890A series II, Palo Alto, CA) and a glass column (180 cm x 4 mm i.d.) packed with 10% SP-1200/1% H₃PO₄ on 80/100+ mesh Chromosorb WAW (Supelco Inc., Bellefonte, PA). Nitrogen was the carrier with a flow rate of 75 mL/min. Oven, detector, and injector temperatures were 125, 175, and 180°C, respectively. Fecal ammonia concentrations were determined according to the method of Chaney and Marbach (1962). Fecal phenol and indole concentrations were determined using gas chromatography according to the methods described by Flickinger et al. (2003). Biogenic amines concentrations were quantified using HPLC according to methods described by Flickinger et al. (2003).

In vitro hydrolytic digestion/fermentation study

Approximately 500 mg of raw and cooked PP were weighed in triplicate and incubated with 12.5 mL phosphate buffer and 5 mL of a pepsin/hydrochloric acid solution at 39°C to simulate gastric digestion. After 6 h, the pH was adjusted to 6.8 and 5 mL pancreatin solution was added to each tube. Incubation continued at 39°C for 18 h to simulate small intestinal

digestion (Boisen and Eggum, 1991). One set of samples was prepared for each end point, one set for enzymatic digestion and five sets for each fermentation pull time.

After incubation, a subset of samples was used to analyze digestible material. The PP samples were precipitated by addition of 4 volumes of 95% ethanol. These were allowed to stand for 1 h prior to filtering through Whatman 541 filter paper. Residues were dried to a constant weight and ashed to determine OM disappearance. All remaining samples were freeze dried in an FTS Tray Dryer (Stone Ridge, NY) in preparation for the fermentation portion of the *in vitro* analysis.

In vitro fermentation was performed as a modification of the method of Bourquin et al. (1993). Samples were hydrated overnight in 26 mL of anaerobic media. Canine fecal samples were collected within 10 min of defecation and maintained at 39°C until inoculum was prepared by blending 10 g of fecal material with 90 mL anaerobic diluting solution for 15 s in a Waring blender under a stream of CO₂. The resulting solution was filtered through 4 layers of cheesecloth and sealed in 125 mL serum bottles.

Samples, blanks, and standards were inoculated with 4 mL of diluted feces. Solka floc and pectin high-methoxyl (HM) were used as negative and positive fermentation controls, respectively. Tubes were incubated at 39°C with periodic mixing. A subset of tubes was removed from the incubator at 0, 3, 6, 9, and 12 h post-inoculation and processed immediately for analyses. A 2 mL subsample of the fluid was removed and acidified for SCFA and BCFA analysis. As for enzymatic digestion, the PP samples were precipitated by addition of 4 volumes of 95% ethanol. These were allowed to stand for 1 h prior to filtering through Whatman 541 filter paper. Concentrations of SCFA were determined by chromatography as described previously.

In vivo digestibility study

Animals and diets

Ten female dogs with hound bloodlines (5.4 ± 0.0 yr; 22 ± 2.1 kg) were utilized. Dogs were housed in individual kennels (2.4 x 1.2 m) in two temperature-controlled rooms with a 16 h light:8 h dark cycle. A replicated 5 x 5 Latin square design experiment with 5 diets and 10 dogs in two different rooms for five, 14 d periods was conducted. The first 10 d was an adaptation period, followed by 4 d of total fecal collection. Five diets containing PP were formulated to contain approximately 26% CP and 15% crude fat (as-is basis). Each diet contained graded concentrations of PP (0, 1.5, 3, 4.5, or 6%) that replaced cellulose (Solka floc) in the diet. Low ash poultry by-product meal, poultry fat, brewer's rice, ground corn, and vitamin and mineral premixes made up the remainder of the dry, extruded, kibble diet. All diets were formulated to exceed NRC (2006) requirements for an adult maintenance diet. Since PP contains approximately 4% CP, brewers rice was substituted to maintain isonitrogenous diets. Diets were mixed and extruded at the Kansas State University Bioprocessing and Industrial Value-Added Program facility (Manhattan, KS) under the supervision of Pet Food and Ingredient Technology, Inc., Topeka, KS. Dogs were offered 155 g of the diet twice daily (0800 and 1700 h) to meet the required energy needs based on the estimated ME content of the diet. Fresh water was provided to the dogs *ad libitum*.

Sample handling and processing

Total feces excreted during the collection phase of each period were taken from the pen floor, weighed, and frozen at -20°C pending analysis. All fecal samples during the collection period were subjected to a consistency score according to the following scale: 1 = hard, dry pellets, small hard mass; 2 = hard, formed, dry stool; remains firm and soft; 3 = soft, formed, and

moist stool, retains shape; 4 = soft, unformed stool, assumes shape of container; and 5 = watery, liquid that can be poured. Seven individuals were trained on the scoring system prior to trial initiation.

Fecal samples were dried at 55°C in a forced-air oven and ground in a Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ) through a 2-mm screen. On d 11 of each period, fresh fecal samples were collected within 15 min of defecation. Aliquots for analysis of phenols, indoles, and biogenic amines were frozen at -20°C immediately after collection. One aliquot was collected and placed in approximately 2 mL of 2N hydrochloric acid for ammonia and SCFA analyses. Additional aliquots were used for pH measurement and fresh fecal DM determination.

Statistical analysis

Data were analyzed as a completely randomized design using the Mixed procedure of SAS (SAS Institute, Inc., Cary, NC). The UNIVARIATE procedure was used to assure equal variance, normal distribution, and to identify outliers. Any observation that was more than 3 standard deviations away from the mean was considered an outlier, but no outliers were identified. Data were transformed by log or square root if the normality assumption was not met. Diet was considered a fixed effect, while random effects included animal and period. Differences among dietary treatments were determined using the least significant difference method. A probability of $P < 0.05$ was accepted as being statistically significant.

Results

Substrate chemical analysis

Table 3.1 presents the chemical composition of cooked and raw PP. Both substrates were similar in DM, OM, gross energy, CP, and AHF. Total dietary fiber for both substrates was 55% with a 32% insoluble and a 23% soluble fiber fraction. Cooked PP had 4.61% resistant starch and

24.06% digestible starch, and raw PP had 6.16% resistant starch and 22.36% digestible starch.

After autoclaving, the amount of free sugars increased for cooked PP but, overall, very little free sugar was detected for either substrate.

In vitro hydrolytic digestion/fermentation

Raw and cooked PP went through an *in vitro* hydrolytic enzymatic digestion followed by a fermentation digestion to simulate transit through the entire gastrointestinal tract. Subsets of samples were pulled at 0, 3, 6, 9, and 12 h to measure SCFA, BCFA, pH change, and OM disappearance. Figure 3.1 presents pH change and SCFA concentration data over time for raw and cooked PP. Raw and cooked PP were 32 and 28% enzymatically digested, respectively (data not shown). Both raw and cooked PP had consistent decreases in pH through 9 h of fermentation. Raw PP had a continued numerical decrease in pH from the 9 to 12 h time point, while cooked PP had a slight numerical increase in pH. Acetate, propionate, butyrate, and total SCFA all increased over time for the raw PP samples. Cooked PP samples had increased ($P < 0.05$) acetate, propionate, butyrate, and total SCFA concentrations through 9 h of fermentation, then exhibited a significant decrease ($P < 0.05$) in acetate and total SCFA concentration at the 12 h time point. Raw PP had higher ($P < 0.05$) acetate, propionate, and total SCFA concentrations at 12 h than did cooked PP. Valerate was the only BCFA detected throughout fermentation, and its concentration was low (highest concentration was 970.4 $\mu\text{g/g DM}$).

In vivo experiment

Table 3.2 presents the ingredient and chemical composition of each experimental diet. All diets had similar DM, OM, CP, AHF, TDF and GE concentrations. Nutrient intake values were similar among treatments. Dogs ate all of their food with only minor feed refusals in one period.

Nutrient digestibility values were similar for all experimental treatments (Table 3.3). Total dietary fiber (TDF) digestibility was different ($P < 0.05$) among diets, with dogs fed 3, 4.5, and 6% PP having higher ($P < 0.05$) TDF digestibilities than dogs fed the 0% PP diet. A linear increase in TDF digestibility was observed with increasing levels of dietary PP ($P < 0.01$). Fecal scores were ideal across treatments.

Fecal pH and SCFA, BCFA, and ammonia concentrations are presented in Table 3.4. Dogs fed the 3, 4.5, and 6% PP diets had higher ($P \leq 0.05$) fecal acetate, propionate, and total SCFA than did dogs fed the 0% PP diet. Fecal butyrate concentrations were higher ($P \leq 0.05$) when dogs were fed 4.5 and 6% PP compared with dogs fed 0% PP. Fecal acetate and total SCFA concentrations were higher ($P < 0.05$) when dogs were fed 3% PP compared to dogs fed 6% PP. Dogs fed 1.5% PP had no significant differences in any of the SCFA compared to the control. Fecal pH data showed that the dogs fed 3 and 6% PP diets had 7 and 12% decreases in fecal pH ($P < 0.01$), respectively, compared with the control. Linear increases ($P < 0.01$) were observed for all SCFA, with a concomitant linear decrease ($P < 0.01$) in fecal pH.

Fecal BCFA and ammonia concentrations were low and showed few significant changes among diets. Fecal valerate concentrations were higher ($P < 0.01$) when dogs were fed 4.5% and 6% PP diets compared to dogs fed the control; a linear increase ($P < 0.05$) also was noted for fecal valerate concentrations.

Table 3.5 presents the fecal biogenic amine, phenol, and indole concentrations. Few differences were observed among treatments. Fecal spermidine was higher ($P < 0.05$) for dogs fed the 6% PP diet compared to the 0% PP diet, but no other treatment differences were noted.

Discussion

A wide variety of dietary fibers are found in pet foods ranging from those of low fermentability (e.g., cellulose, peanut hulls) to those whose fermentability is more moderate (e.g., beet pulp, soybean hulls). Most moderately fermentable fibers have a medium to high TDF content and have select amounts of insoluble and soluble fibers, with a preponderance of insoluble fibers. Beet pulp is one of the most commonly used moderately fermentable fibers and has a TDF content of 68.4% (Sunvold et al., 1995), although values can range from 57 - 77%. Furthermore, beet pulp contains 55.1% insoluble fiber and 13.3% soluble fiber (Sunvold et al., 1995). Because it contains considerable amounts of both insoluble and soluble fiber, this property has translated into favorable results in terms of nutrient digestibility and fermentability *in vivo* (Fahey et al., 1990). Other moderately fermentable fiber sources such as oat fiber and soybean hulls also have a high TDF value and an adequate ratio of insoluble to soluble fiber (Burkhalter et al., 2001; Fahey et al., 1992), but they have been tested less than beet pulp.

In our study, PP contained 55% TDF with 32% insoluble fiber and 23% soluble fiber. Compared to the beet pulp used by Sunvold et al. (1995), PP has a lower TDF, but a higher portion of soluble fiber. Previous research suggested that diets with an insoluble to soluble fiber ratio of less than 2 had little to no negative effects on nutrient digestibility (Burkhalter et al., 2001). Potato pulp has a I:S ratio of 1.4, which is within those limits. Potato pulp also elicited no large effects on nutrient digestibility and resulted in overall increases in fecal SCFA concentrations at the higher levels of addition. These data are in agreement with those collected on beet pulp; decreased nutrient digestibility was observed at dietary additions above 7.5% (Diez et al., 1997; Fahey et al., 1990).

Along with having relatively high amounts of TDF, PP also contains digestible and resistant starch fractions, along with low amounts of CP and AHF. Digestible starch would contribute to the maintenance high *in vivo* nutrient digestibility whereas resistant starch would behave as a moderately to highly fermentable dietary fiber. Since PP is a novel ingredient on which there exists little data as regards inclusion in dog diets, and since this ingredient would be included in the food matrix from which a kibble is prepared by the process of extrusion, it was of interest to simulate the effects of extrusion of PP and determine its effect on chemical composition, particularly its starch and sugar components. Tran et al. (2011) showed that extrusion results in chemical and physical changes in ingredients that can alter their nutrient composition. To simulate extrusion, PP was autoclaved for 1 h at 121°C and 18 psi pressure. This is more time but less pressure than exists in the actual process of extrusion. Autoclaving increased the digestible starch fraction of PP by 1.7 percentage units and reduced the resistant starch fraction from 6.2 to 4.6%, a 26% reduction. This is in agreement with the research of Murray et al. (2001) who showed increases in the digestible starch fraction of select substrates as a result of extrusion. Total starch concentration varied by only 0.6 percentage units between PP substrates. Total dietary fiber values were virtually unchanged by processing, but the insoluble dietary fiber fraction increased by 2.7% and the soluble dietary fiber decreased by 4.5% as a result of the autoclaving process. Whether similar responses would occur for PP that is a part of a dietary matrix and then conditioned, extruded, and cooled remains unknown.

An *in vitro* hydrolytic digestion/fermentation assay was used to compare raw and cooked PP for outcomes relevant to *in vivo* digestion. The substrates were first exposed to an acidic environment to simulate gastric digestion and a subsequent neutral pH to simulate the small intestinal environment. After going through these steps, raw and cooked PP had 32 and 28% OM

disappearance values. This suggests that nutrients in PP in the raw and cooked forms are partially digested by the host. It was expected that there would be an increase in enzymatic digestion for the cooked PP since the starch portion would be more digestible after gelatinization. The compositional analysis showed some increase in free sugars and digestible starch after autoclaving. Instead, a decrease in enzymatic digestion was observed for the cooked PP substrate, which may be partly explained by the interaction of amylose with protein and fat constituents in PP. Starches containing large amounts of amylose form complexes with protein and fat that makes them indigestible (Holm et al., 1983; Thorne et al., 1983). Complex formation could explain the decrease in enzymatic digestion in cooked PP (Holm et al., 1983; Thorne et al., 1983). However, raw and cooked PP were enzymatically digested, in part, due to their digestible starch content.

Following digestion, SCFA production during fermentation was consistent with the pH decline over the 12 h fermentation period for both raw and cooked PP substrates. Raw PP resulted in an increased SCFA production throughout the entire 12 h fermentation, while cooked PP resulted in an increase in SCFA through 9 h, followed by a decrease in SCFA at 12 h. This finding suggests that cooked PP is depleted of fermentable components by 9 h of fermentation, while raw PP remains fermentable at 12 h. Furthermore, these data suggest that raw and cooked PP are moderately fermentable and are likely fermented throughout the hindgut. Bosch et al. (2008) found that sugar beet fiber had 1,990, 560, 330, and 2,880 $\mu\text{moles/g DM}$ concentrations of acetate, propionate, butyrate, and total SCFA at 8 h of fermentation. These values are comparable to what was seen in cooked and raw PP.

The differences observed between raw and cooked PP could be attributed to the effect of processing. Research has shown that processing affects the composition and fermentability of

fibrous substrates (Guillon and Champ, 2000). For example, resistant starch may be depleted after extrusion (Murray et al., 2001). Our compositional analysis revealed that resistant starch decreased from 6.2% to 4.6%, which may explain why fermentability of cooked PP decreased after 9 h. Most of the fermentation of PP is due to both its soluble dietary fiber fraction as well as its resistant starch content. Potato pulp is high in cellulose and hemicelluloses, which represent insoluble fibers that are poorly to moderately fermented, and pectin, which is soluble and more fermentable (Mayer, 1998). Research suggests that soluble fiber increases, and insoluble hemicelluloses decrease, as extrusion conditions become harsher (Dust et al., 2004). Cooked PP may have a more available soluble fiber fraction, making it more fermentable earlier in the 12 h fermentation. Having more fermentable substrate available earlier in fermentation also may help explain why cooked PP was fermentable through 9 h.

The type of resistant starch present in the PP substrates is important as regards fermentability. Martin et al. (1998) reported that 87% of RS2 and 57% of RS3 from potato starch were fermented. Furthermore, RS2 potato starch yielded substantially more butyrate (25% of total SCFA) than from RS3 (14% of total SCFA) (Martin et al., 1998; Topping and Clifton, 2001). Raw PP, with all of its resistant starch content as RS2 and with less available fermentable fiber early in fermentation, could ferment longer than cooked PP. Cooked PP contains less fermentable RS3 and more available soluble fiber for microbes to ferment since it was autoclaved.

As regards the *in vivo* experiment, typically, increasing dietary fiber will decrease nutrient digestibility by non-ruminant species (Fahey et al., 1990). However, in the present experiment, only TDF digestibility was modified by graded additions of PP to the diet. Total dietary fiber digestibility increased as dietary PP concentration increased commensurate with an

increase in fecal SCFA concentrations. This occurred because PP replaced cellulose as PP dietary concentrations increased in the iso-TDF diets. Cellulose from wood in this experiment is essentially 0% fermentable, whereas PP is moderately fermentable.

Commensurate with TDF digestibility results, fecal SCFA concentrations increased while fecal pH decreased. Surprisingly, fecal consistency did not change with treatment. It is common that highly fermentable soluble fibers negatively affect fecal consistency (Wakshlag et al., 2011). Despite major compositional differences, PP behaves very similarly to beet pulp (Fahey et al., 1990) in terms of physiological responses.

Fermentation of PP is largely affected by its soluble fiber fraction, which is mostly composed of pectin (Lesiecki et al., 2012). The pectin portion of PP would have a strong effect on all fermentative and physiological responses. Although resistant starch was present in both the raw and cooked forms of PP, its presence is unlikely to have a marked effect on responses due to the low concentration provided. Indeed, Murray et al. (2001) determined that resistant starch was not recovered after low and high temperature extrusion of potato.

Other physiological outcomes measured in the *in vivo* experiment (fecal BCFA, ammonia, biogenic amine, phenol, and indole concentrations) were mostly unaffected by graded level addition of PP to diets. The one exception was spermidine whose concentration was increased significantly when PP was fed at the 6% level. Spermidine has been found to have favorable effects in low concentrations on cell turnover (Linsalata and Russo, 2008).

Overall, PP demonstrated favorable fermentative characteristics *in vitro* and favorable physiological effects *in vivo*. The chemical composition and *in vitro* assays both showed that PP was abundant in fermentable TDF with a favorable proportion of insoluble to soluble fiber. Compared with other fiber sources previously studied, PP appears to exhibit fermentation

characteristics, including increased fecal SCFA concentrations and excellent stool consistency without eliciting negative effects on nutrient digestibility or increasing concentrations of unfavorable protein catabolites. In conclusion, this study suggests that PP has the potential to be a high quality fiber source in dog foods and would compare favorably or better than most dietary fibers found in commercial foods.

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Table 3.1: Chemical composition of raw and cooked potato pulp

Item	Raw potato pulp	Cooked potato pulp ¹
Dry matter (%)	88.4	90.2
-----Dry matter basis-----		
Organic matter (%)	97.1	97.2
Gross energy (kcal/g)	4.1	4.2
Crude protein (%)	4.4	4.8
AHF fat (%)	2.1	2.1
Total dietary fiber (%)	55.2	55.0
Insoluble dietary fiber	31.9	32.7
Soluble dietary fiber	23.3	22.3
Starch, %		
Free glucose	0.0	0.1
Digestible starch	22.4	24.1
Resistant starch	6.2	4.6
Total starch	28.7	29.3
Free sugars, µg/g		
Arabinose	18.6	29.3
Galactose	19.5	29.5
Glucose	176.4	2089.7
Sucrose	872.9	628.8
Mannose	0.0	0.0
Fructose	3.6	45.8
Total	1091.0	2823.1

¹Potato pulp (500 g) was suspended in distilled water (6 liters) and autoclaved at 121 °C for 1 h.

Table 3.2: Ingredient and chemical composition of experimental diets fed to dogs.

Item	% potato pulp (raw)				
	0	1.5	3	4.5	6
Ingredient,%					
Brewer's rice	46.55	45.90	45.25	44.60	43.95
Poultry byproduct meal-low ash	25.50	25.50	25.50	25.50	25.50
Corn, yellow, ground	12.00	12.00	12.00	12.00	12.00
Poultry fat	8.00	8.00	8.00	8.00	8.00
Potato pulp ^a	0.00	1.50	3.00	4.50	6.00
Cellulose ^b	6.00	5.15	4.30	3.45	2.60
Salt	0.70	0.70	0.70	0.70	0.70
Potassium chloride	0.56	0.56	0.56	0.56	0.56
Chromic oxide	0.20	0.20	0.20	0.20	0.20
Mineral mix ^c	0.18	0.18	0.18	0.18	0.18
Vitamin mix ^d	0.18	0.18	0.18	0.18	0.18
Choline chloride-50%	0.13	0.13	0.13	0.13	0.13
Chemical composition,%					
Dry matter	95.8	95.7	95.5	95.5	95.3
Organic matter	94.2	94.4	94.2	94.1	94.2
Crude protein	25.1	24.8	24.9	25.2	25.1
Acid hydrolyzed fat	13.5	14.5	14.5	14.2	13.1
Total dietary fiber	10.8	11.2	11.4	11.2	11.4
Gross energy, kcal•g ⁻¹	4.97	5.01	5.00	5.01	4.94

^aRoquette Inc. Lestrem, France.

^bCellulose: Solka-Floc, International Fiber Corp., North Tonawanda, NY.

^cProvided per kilogram of diet: Mn (as MnSO₄), 66.00 mg; Fe (as FeSO₄), 120 mg; Cu (as CuSO₄), 18 mg; Co (as CoSO₄), 1.20 mg; Zn (as ZnSO₄), 240 mg; I (as KI), 1.8 mg; Se (as Na₂SeO₃), 0.24 mg.

^dProvided per kilogram of diet: vitamin A, 5.28 mg; vitamin D₃, 0.04 mg; vitamin E, 120 mg; vitamin K, 0.88 mg; thiamine, 4.40 mg; riboflavin, 5.72 mg; pantothenic acid, 22.00 mg; niacin, 39.60 mg; pyridoxine, 3.52 mg; biotin, 0.13 mg; folic acid, 0.44 mg; vitamin B₁₂, 0.11 mg.

Table 3.3: Nutrient digestibility and fecal scores from dogs fed diets containing graded levels of potato pulp¹.

Item	% potato pulp					SEM	<i>P</i> -value	<i>P</i> -value	
	0	1.5	3	4.5	6			Linear	Quadratic
Apparent total tract digestibility, %									
Dry matter	81.3	81.8	81.9	82.3	82.7	0.60	0.51	0.12	0.98
Organic matter	84.1	84.1	84.7	85.1	85.5	0.56	0.58	0.10	0.85
Crude protein	81.7	81.3	80.7	80.2	80.2	0.84	0.31	0.22	0.76
Acid hydrolyzed fat	94.1	94.6	94.6	94.4	94.1	0.26	0.31	0.96	0.05
Total dietary fiber	16.3 ^a	23.2 ^{ab}	27.4 ^b	32.0 ^b	37.1 ^c	2.48	0.01	0.01	0.73
Digestible energy									
%	84.9	85.3	85.5	85.6	85.7	0.51	0.79	0.26	0.66
kcal·d ⁻¹	1251	1267	1266	1218	1251	24.90	0.63		
Fecal score	2.6	2.6	2.7	2.8	2.7	0.08	0.23		

^{abcd} Mean values in the same row with unlike superscript letters differ ($P < 0.05$).

¹Values expressed on a dry matter basis.

Table 3.4: Short-chain fatty acid (SCFA), branched-chain fatty acid (BCFA), and ammonia concentrations, and fecal pH values for dogs fed potato pulp¹.

Item	% potato pulp					SEM	P-value	P -value	
	0	1.5	3	4.5	6			Linear	Quadratic
pH	6.71 ^a	6.45 ^{ab}	6.27 ^{ab}	6.13 ^b	6.00 ^b	0.13	0.01	0.01	0.54
SCFA, μ moles/g DM									
Acetate	322.4 ^a	317.8 ^{ab}	410.9 ^{bc}	448.9 ^{cd}	547.7 ^d	29.4	0.01	0.01	0.51
Propionate	102.2 ^a	117.0 ^{ab}	145.6 ^{bc}	160.9 ^c	178.6 ^c	10.1	0.01	0.01	0.85
Butyrate	55.5 ^a	52.2 ^a	64.2 ^{ab}	79.8 ^b	81.6 ^b	7.9	0.01	0.01	0.97
Total	480.1 ^a	487.0 ^a	620.7 ^b	689.5 ^{bc}	807.9 ^c	41.5	0.01	0.01	0.53
BCFA, μ moles/g DM									
Isobutyrate	0.77	0.67	0.71	0.70	0.70	0.07	0.75	0.47	0.60
Isovalerate	1.39	1.20	1.24	1.09	1.15	0.15	0.10	0.22	0.77
Valerate	0.11 ^a	0.10 ^a	0.13 ^a	0.17 ^b	0.17 ^b	0.02	0.02	0.03	0.95
Total	2.27	1.97	2.08	2.10	2.01	0.23	0.68	0.39	0.69
Ammonia, μ moles/g DM	2.10	2.32	2.49	2.48	2.38	0.34	0.78	0.65	0.45

abcd Mean values within a row with unlike superscript letters differ ($P < 0.05$).

¹Values expressed on a dry matter basis.

Table 3.5: Biogenic amine, phenol, and indole concentrations in fecal samples from dogs fed diets containing graded levels of potato pulp¹

Item	% potato pulp					SEM	P-value	P-value	
	0	1.5	3	4.5	6			Linear	Quadratic
Biogenic amines, μ moles/g DM									
Agmatine	0	0	0	0	0.36	0.17	0.14	0.04	0.11
Tryptamine	0.77	0.69	0.59	0.35	0.40	0.18	0.19	0.14	0.86
Phenylethylamine	0	0.10	0.05	0.03	0	0.05	0.55	1.00	0.25
Putresine	3.66	4.36	5.24	4.90	4.66	0.52	0.10	0.18	0.11
Cadaverine	1.60	1.63	1.64	1.84	1.73	0.18	0.88	0.62	0.91
Histamine	0	0	0	0	0				
Tyramine	0	0.32	0	0	0	0.15	0.42	1.00	0.55
Spermidine	1.55 ^a	1.63 ^a	1.43 ^a	1.58 ^a	2.10 ^b	0.19	0.02	0.04	0.09
Spermine	0.34	0.55	0.59	0.76	0.68	0.19	0.14	0.19	0.50
Total biogenic amines	7.92	9.28	9.54	9.46	9.92	0.73	0.17	0.06	0.43
Phenols and indoles, μ moles/g DM ²									
Phenol	1.90	2.20	0.83	1.26	0.86	0.68	0.26	0.25	0.86
Indole	2.22	2.29	2.16	2.40	2.17	0.21	0.95	0.86	0.82
Total phenols and indoles	4.13	4.49	3.00	3.63	3.03	0.78	0.31	0.28	0.93

^{ab} Means in the same row with unlike superscript letters differ ($P < 0.05$).

¹ Values expressed on a dry matter basis.

² Other phenolic and indolic compounds were measured but were below detection limits and assigned a value of zero.

Figure 3.1 pH and concentrations of acetate, propionate, butyrate, and total short-chain fatty acid (SCFA) during a 12 h *in vitro* fermentation. * Represents differences between treatments ($P < 0.05$).

