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The use of dietary fat and cricket powder as alternatives in diets fed to healthy adult dogs

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The use of dietary fat and cricket powder as alternatives in diets fed to healthy adult dogs

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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ABSTRACT

Consumers are becoming more aware of how to nutritionally provide for their pets which is leading to the demand for new pet foods such as the use of less processed diets and sustainable protein sources. Pet foods may be formulated with decreased starch to meet consumer demands for less processed diets. Fats and oils may be added to low starch diets to meet energy requirements, but little is known about its effects on canine health. The study objective was to evaluate the effects of feeding healthy adult dogs low carbohydrate, high-fat diets on apparent total tract digestibility, fecal characteristics, and overall health status. Eight adult Beagles were enrolled in a replicated 4x4 Latin Square design feeding trial. Dogs were randomly assigned to 1 of 4 dietary fat level treatments (T) within each period: 32% (T1), 37% (T2), 42% (T3), and 47% (T4) fat on a dry matter basis. Fat levels were adjusted with inclusion of canola oil added to a commercial diet. Each dog was fed to exceed their energy requirement based on NRC (2006). Blood samples were analyzed for complete blood counts, chemistry profiles, and canine pancreatic lipase immunoreactivity levels. Apparent total tract digestibility improved ($P < 0.05$) as the fat level increased for dry matter, organic matter, fat, and gross energy. Fecal output decreased as levels of fat increased in the diet ($P = 0.002$). There was no effect of fat level on stool quality or short chain fatty acid and ammonia concentrations in fecal samples ($P \geq 0.20$). Blood urea nitrogen levels decreased with increased fat level ($P = 0.035$). No significant differences were seen in canine pancreatic lipase immunoreactivity ($P = 0.110$). All blood parameters remained within normal reference intervals. In summary, increased dietary fat improved apparent total tract digestibility, did not alter fecal characteristics, and maintained the health status of all dogs. Fecal samples were also collected from this study for microbial analysis. When comparing entire bacterial communities of treatment groups using

PERMANOVA, no significant differences were observed among treatments ($P = 0.681$). However, when comparing the 100 most abundant individual OTUs, 36 showed significant differences in abundances between treatment groups. Overall, OTUs assigned to genera related to fat digestion increased while OTUs assigned to genera involved in carbohydrate digestion decreased. In conclusion, the microbial community adapted to dietary intervention without jeopardizing the health of the animals.

Insects may meet the consumer demand for a more sustainable high-quality protein source for pet foods. However, little research has been done investigating the use of this source in pet foods. The study objective was to evaluate the apparent digestibility and possible health effects of diets containing graded levels of cricket powder fed to healthy adult dogs. Thirty-two adult Beagles were randomly assigned to 1 of 4 dietary treatments: 0%, 8%, 16%, or 24% cricket powder. Dogs were fed their respective diet for a total of 29 days with a 6-d collection phase. Fecal samples were collected to measure total fecal output as well as apparent digestibility for dry matter, organic matter, crude protein, fat, total dietary fiber, and gross energy. Blood samples were taken prior to the study and on d 29 for hematology and chemistry profiles. Total fecal output increased on both an as-is ($P = 0.030$) and dry matter basis ($P = 0.024$). The apparent digestibility of each nutrient on a dry matter basis decreased ($P < 0.001$) with the increasing level of cricket powder inclusion. All blood values remained within desired reference intervals indicating healthy dogs. Slight fluctuations in blood urea nitrogen ($P = 0.037$) and hemoglobin ($P = 0.044$) levels were observed but were not considered of biological significance. Even with the decrease in digestibility with the inclusion of cricket powder, diets remained highly digestible at greater than 80% total apparent digestibility. In conclusion, crickets were demonstrated to be an acceptable source of protein for dogs.

CHAPTER 1. INTRODUCTION

The pet food industry is unique and follows the dietary trends of humans. In recent years, the pet food industry has drastically shifted. Consumers are becoming more aware of how they are nutritionally providing for their pets (Bontempo, 2005). With this, they are demanding novel, high-quality ingredients. They have a desire for more natural products with less processing than what is currently used (Morelli et al., 2019). However, specific ingredients are needed to maintain the structure of manufactured diets, such as starch for kibble production. According to consumers, carbohydrates are a low-quality ingredient (Carter et al., 2014). In addition to the processing considerations, carbohydrates are typically added to diets to meet energy demands and provide a lower cost ingredient. Nevertheless, dogs do not have a nutrient requirement for carbohydrates meaning they may be removed from diets if other ingredients are able to meet energy and processing requirements. Fat may be added to diets to replace the energy demand, but high inclusion rates may lead to the need for new processing techniques and/or adverse health effects in animals consuming such a diet.

Consumers are also becoming environmentally aware of the protein sources used in pet food. In addition, the protein sources commonly used in pet food may be in direct competition with human grade food (Swanson et al., 2013; Meeker and Meisinger, 2015; Okin, 2017). With the increasing population, there is concern on how to feed the world. If the pet food industry can utilize an alternative protein source with a low environmental impact, that does not directly compete with human food production, this may be less of a concern. Insects may be the novel protein source the industry is looking for. Insects have the ability to provide a high-nutritive value with a lower environmental impact compared to typical protein sources (Oonincx and Boer, 2012; Bosch et al., 2014; Finke, 2015).

Consumer demand for less processed, more environmentally friendly products increases the need for novel companion animal research. Prior to commercial implementation, pet food companies require support from scientific research showing these diets can meet an animal's nutrient requirements without leading to adverse health effects. It is important that scientific research is always one step ahead of the next emerging trend in the pet food industry.

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CHAPTER 2. LITERATURE REVIEW

Part 1: Dietary Fat

Use of dietary fat in pet food

Dietary fat contains approximately 8.5 kcal of metabolizable energy per gram while protein and carbohydrate provide about 3.5 kcal/g. Most dietary fats found in high quality pet food are highly digestible with greater than 90% apparent digestibility. Due to its high energy content and digestibility, adding fat to a diet greatly increases energy density. If other nutritional requirements are met, dogs can consume a wide range of dietary fat content. When high concentration of dietary fat is present in the diet, less total food may be consumed, because most animals eat to meet their energy demands. Due to less volume consumed, a balanced diet is crucial to ensure that all nutrient requirements are met (Case et al., 1995). However, dogs typically overconsume which may cause unwanted weight gain. Therefore, feed consumption needs to be monitored to prevent obesity.

Dietary fat is necessary in dog diets to meet energy demands and provide essential fatty acids (EFA) that cannot be synthesized by the body. Linoleic acid from the n-6 series and alpha-linolenic acid from the n-3 series are fatty acids necessary for normal metabolic function in dogs. These EFAs are long-chain, polyunsaturated fatty acids. If the correct amount of dietary linoleic acid is fed, dogs can meet their nutritional requirements of n-6 fatty acids. There are two key enzymes in the conversion pathway of linoleic acid to gamma-linolenic and arachidonic acid: delta-6-desaturase and delta-5-desaturase (Case et al., 1995). This conversion allows the animal to meet the demands of additional fatty acids that may not be provided in the diet.

After domestication and evolution, dogs can still maintain normal lipid levels if fed high fat diets like those consumed by their ancestors. Conditions such as hyperlipidemia and

atherosclerosis, common concerns of high fat diets, normally develop due to genetic background or from an underlying disease and not from fat consumption in dogs. In addition, the concern of high fat diets and heart disease is not relevant in companion animals (Case et al., 1995).

Classification of dietary lipids

The terms fat, oil, and lipid are often used interchangeably. However, fats and oils are a subgroup of lipids. Lipids are organic-solvent-soluble and are primarily composed of hydrocarbon structures. Triglycerides are considered simple lipids and contain three fatty acids attached to a glycerol backbone. Fat triglycerides are primarily composed of saturated, trans fatty acids and typically contain a long chain of fatty acids making them solid at room temperature. On the other hand, oil triglycerides are primarily composed of unsaturated fatty acids which are liquid at room temperature even if they consist of long chains. Triglycerides are the main sources of fatty acids as it relates to dog nutrition (NRC, 2006).

Fatty acids are a subgroup of lipids built of hydrocarbon chains that can be saturated or unsaturated. Fatty acids are the main components of lipids and can be identified by their number of carbon chain lengths. Natural fatty acids occur in even chains, with the most common being C_{16} and C_{18}. Fatty acids can also be classified by their degree of unsaturation: saturated = no unsaturated bonds, mono-unsaturated = 1 unsaturated bond, or poly-unsaturated = 2 or greater bonds (Hennessy et al., 2016). Fatty acids may also be classified as *cis* and *trans* as it relates to its double bond orientation. *Cis* refers to functional units on the same side of the chain while *trans* refers to functional units on opposite sides of the chain. *Cis* bonds create a bend in the structure which changes the properties of the molecule. For example, *Cis* molecules tend to have lower melting points because of their inability to tightly bind due to branching. *Cis* fatty acids

are more likely to occur in nature while *trans* fatty acids are more likely to be formed during industrial hydrogenation or rumen biohydrogenation (Scrimgeour, 2005).

In order to discuss fatty acids, three main naming systems are used: delta nomenclature, omega nomenclature, and common names. The use of the omega system and common names is more popular in the field of nutrition. For the delta system, the number of carbons in the fatty acid, the number of double bonds, and the number of carbons from the carboxylic acid end to the first carbon in the double bond are needed. For example, oleic acid would be represented by 18:1 Δ9 where 18 is the number of carbons, 1 is the number of double bonds, and 9 is the double bond location. Omega system differs by counting from the methyl end instead of the carboxylic end and an omega symbol or n is used instead. Using the omega system, Oleic acid would be 18:1 ω-9 or 18:1 n-9. In contrast to the delta and omega nomenclature, common names must be memorized because they do not represent the structure of the molecule (Gropper et al., 2009). Some of the most popular common names given to fatty acids are listed in **Table 2.1**.

Lipids are primarily consumed in the diet as triglycerides. These triglycerides need to be broken down during digestion in order to be absorbed and utilized by the body. Mechanical breakdown of dietary fat begins in the mouth.

Digestion of dietary lipids

Mouth

Digestion of dietary fat will start in the mouth through the mechanical process of chewing. In addition to mechanical breakdown of fat in the mouth, some species have lingual lipase secreted from salivary glands to start enzymatic fat breakdown. These species include humans, rats, and ruminants. In contrast, lingual lipase is virtually absent in adult dogs (Iverson et al., 1991). Therefore, enzymatic digestion of fat does not begin until the stomach in dogs.

Stomach

The major enzyme in a dog's stomach is gastric lipase (NRC, 2006) which targets fatty acid linkages on the sn-3 position of triglycerides (Drackley, 2000) and breaks down triglycerides to diglycerides and free fatty acids (FFA). Carriere et al. (1991) reported that unlike other species, gastric lipase activity was found throughout the entire gastric mucosa of dogs. However, dog gastric lipase (DGL) does have a very similar composition to both human and rat gastric lipase, with similar carbohydrate and amino acid concentrations. Gastric lipase has no requirements for cofactors or bile salts to function unlike other digestive lipases (Hamosh, 1990). Nevertheless, many parameters can affect lipase function such as fatty acid chain length, interfacial tension, presence of emulsifiers, and tensio-active agents (Carriere et al., 1991). The maximum activity of DGL was found on long-chain triglycerides; 13 times more active than on short-chain triglycerides. In contrast, the maximum activity of human and rat gastric lipase was found on short chain triglycerides (Carriere et. al., 1991). Gastric lipase functions in a wide pH range but is inactivated with a pH below 1.5 or greater than 7. The process of fat digestion in the stomach results in acid chyme, a semifluid substance, which then moves into the small intestine for further break down and eventually absorption.

Small intestine

The primary site of lipid digestion is the small intestine. Acid chyme entering the duodenum from the stomach activates the release of pancreatic juices. These pancreatic juices include many enzymes but the one most important to lipid digestion is pancreatic lipase. Another major player in lipid digestion is bile. These components are required in order to form micelles which allow uptake of fat into enterocytes.

Bile

Bile, a dark green to yellowish brown fluid, is released in response to fat entering the small intestine and works as an emulsifier. Bile is produced in the liver but is stored and concentrated in the gallbladder. The release of bile from the liver is stimulated by serotonin which responds to the acidity of chyme. The release of digestive enzymes from the pancreas and bile from the gallbladder is activated by cholecystokinin (CCK) which senses the fat in chyme. CCK and serotonin are produced and secreted from the enteroendocrine cells in the duodenum. The peak release time from the gallbladder occurs 30 minutes after a meal and decreases two hours after a meal (Madrid et al., 1983, Traynor et al., 1984). Smeets-Peeters et al. (1998) have shown that bile secreted from the gallbladder has a different concentration than bile secreted directly from the liver. For example, the concentration of bile salts ranges from 79-150 to 5-24g/L for bile secreted from the gallbladder and the liver, respectively. The main components of bile are bile salts and phospholipids. Bile salts, made from cholesterol in the liver, are responsible for lipid emulsification. More than 99% of bile acids are conjugated with the amino acid, taurine. The combination of taurine forms taurocholic acid, taurodeoxycholic acid, and taurochenodeoxycholic acid (NRC, 2006). Washizu et al. (1990) found that taurocholic acid is the main component of dog bile at 97 ± 30mg/mL. The conjugation of taurine increases the water solubility and decreases the cellular toxicity of the bile salts (Drackley, 2000). The formed amphiphilic characteristic, possessing both hydrophobic and hydrophilic areas, of bile salts allow them to lie at the water-lipid interface to form monolayers and micelles (Scrimgeour, 2005).

Pancreatic colipase and lipase

Pancreatic lipase adhesion to the triglyceride droplet is prevented by bile salts and phospholipids. The addition of pancreatic colipase to the lipid substrate allows pancreatic lipase

to attach to the droplet. Pancreatic colipase is a water-soluble exocrine pancreatic protein. It exists in pancreatic juice as pro-colipase which is activated by trypsin in the intestine (Larsson and Erlanson-Albertsson, 1981). Pancreatic lipase is another water-soluble globular protein containing histidine in its active site (Chapus and Semeriva, 1976). Lipase can absorb to a lipid-water interface allowing lipid hydrolysis to occur at a higher rate comparted to a water-soluble substrate (Sarda and Desnuelle, 1958). Pancreatic lipase acts on the sn-1 and sn-3 linkages of triglycerides which results in the formation of two monoglycerides and free fatty acids (Drackley, 2000). The colipase/lipase complex is lipid dependent, meaning colipase and the lipid substrate regulate lipase activity (Erlanson-Albertsson, 1983).

Micelle Formation

For dietary fat to be absorbed it must transport across the unstirred water layer at the surface of the intestinal microvilli, this requires the formation of a micelle (Drackley, 2000). This process involves a combination of enzymes and substrates. Cholesterol esterase is dependent on the hydrolysis of triglycerides by pancreatic lipase and functions to hydrolyze cholesterol or retinyl esters. Its wide substrate specificity complements the narrow specificity of pancreatic lipase (NRC, 2006). Phospholipase A_2 is needed to hydrolyze phospholipids to free fatty acids due to the phospholipid's ability to resist pancreatic lipase. Colipase then transports the free fatty acids and the monoglycerides from the lipid droplets to form a micelle. In the presence of bile salts, the fatty acids, monoglycerides (produced from pancreatic lipase), cholesterol, cholesterol esters, and phospholipids spontaneously aggregate into mixed micelles. Bile salts arrange by locating their polar end to the direction of the lumen and their nonpolar end towards the center of the micelle (Zwicker and Agellon, 2013) creating a hydrophobic center with a hydrophilic edge.

The hydrophilic micelle allows the lipid complex to travel to the brush boarder where its fat components are absorbed into the cell (Case et al., 1995).

Absorption of dietary lipids

The concentration of lipid material into micelles creates a gradient which allows lipid constituents to passively diffuse into the enterocyte by a non-energy dependent system (Johnston and Borgstrom, 1964). Micelles are in constant contact with the epithelium, lipid droplets, and other micelles. This constant contact allows micelles to equally distribute lipid constituents. Therefore, micelle saturation limits lipid digestion in the small intestine. Shuttling of lipid constituents across the unstirred water layer require a lower cellular concentration of lipids at the enterocyte (Jones and Rideout, 2012). Intestinal fatty acid binding protein may increase fatty acid uptake by binding to the FFA and trapping them within the apical membrane (Stremmel et al., 2001). When lipid concentrations are low, absorption may be carrier dependent (Chow and Hollander, 1979). Long chain fatty acids may use intestinal fatty acid binding proteins or fatty acid translocase for absorption (Minich et al., 1997). Both passive and active transport is thought to maintain linoleic and linolenic acid when dietary intake is low (Kindel et al., 2010). Some absorption may be facilitated by specific transport proteins (i.e. cholesterol) but the main transport system of fatty acids and monoglycerides into the enterocyte is passive (Case et al., 1995). Bile salts use an active transport system to be absorbed in the ileum to recycle back to the liver to be reincorporated into bile. A small amount of bile salts does enter the large intestine instead of being returned to the liver, these bile salts end up in feces. This is the only way cholesterol is excreted from the body (Drackley, 2000).

Circulation and uptake of fatty acids by target tissue

After diffusing into the enterocyte, long-chain fatty acids are re-esterified in the endoplasmic reticulum via the glycerol-3-phosphate or the monoacylglycerol pathway (Cunningham and Leat, 1969). Re-esterified triglycerides and cholesterol esters are then packaged into chylomicrons (Sabesin and Frase, 1977). Chylomicrons are composed of 80 to 95% triglycerides, 2 to 7% cholesterol and 3 to 9% phospholipids (Jones and Rideout, 2012). Chylomicrons are surrounded by a phospholipid bi-layer and apolipoproteins which increase solubility and enzymatic recognition (Shiau, 1981). Chylomicrons are secreted from the intestinal cells and enter the lacteals of the lymphatic system. The lacteals then drain into the venous blood at the thoracic duct, appearing in the blood approximately two hours after consuming a meal (Goldberg, 1996).

Short and medium chain fatty acids can be carried in the bloodstream by albumin, a serum protein. These fatty acids can be circulated in a non-esterified form and are directed to the liver via the portal vein (Bach and Bobayan, 1982; Jones and Rideout, 2012).

Mobilized fatty acids can be transported by lipoproteins. Lipoproteins increase solubility, lipid concentration, and recognition of enzymes and receptors (Jones and Rideout, 2012). Dogs have four major classes of lipoproteins: chylomicrons, very low-density lipoproteins (VLDL), low density lipoproteins (LDL), and high-density lipoproteins (HDL) (Case et al., 1995).

Once the chylomicron is in circulation, the lipid products can be stored in adipocytes or oxidized by other cells (Jones and Rideout, 2012). If insulin is elevated, chylomicrons will be stored in adipocytes and lipoprotein lipase will be expressed in the capillary lumen of the adipocyte which will process triglyceride-rich chylomicrons and other lipoproteins (Wang and Eckel, 2009). Fatty acids will be passively diffused into the adipocyte (via fatty acid binding

proteins) and re-esterified for storage as a triglyceride in the adipocyte (Jones and Rideout, 2012).

Post-absorptive metabolism of lipids

Unlike humans and rodents, de novo lipogenesis occurs in the adipose tissue of dogs (Kersten, 2001). During glycolysis, glucose is converted to pyruvate in the cytoplasm of the cell. Pyruvate can then be converted to alanine, oxaloacetate, lactate, or acetyl-CoA (Heckler,1997). In the conversion of pyruvate to acetyl-CoA, Co-A, NAD+, and pyruvate dehydrogenase is required. This creates acetyl-CoA, NADH, and H+ (Denton et al., 1975). If energy is needed by the dog, the acetyl-CoA can enter the TCA cycle for ATP production. When there is excess acetyl Co-A (not needed for energy), lipogenesis is activated (Lawes and Gilbert, 1886).

Acetyl-CoA must be converted to citrate by the enzyme citrate synthase in order to pass to the cytosol from the mitochondria. After reaching the cytoplasm through the tricarboxylate transporter, it must be converted back to acetyl Co-A (Remington, 1992). ATP citrate lyase will be activated by the high citrate concentration in the adipocyte to reconvert acetyl Co-A by cleaving oxaloacetate from citrate.

Oxaloacetate must be converted by malate dehydrogenase to malate in order to re-enter the mitochondrial matrix. Citrate is exchanged for malate through the tricarboxylate transporter creating a concentration gradient (Danis and Farkas, 2009). If the citrate concentration is not high enough in the mitochondria to create a concentration gradient, malate can be oxidized to pyruvate. This oxidation produces an NADPH for later use in de novo lipogenesis. Pyruvate can then be exchanged with a hydrogen ion back to the mitochondria via the pyruvate transporter (Flatt, 1970).

The rate limiting step of de novo lipogenesis is acetyl Co-A carboxylase. Acetyl Co-A carboxylase transfers the carboxyl group to acetyl-CoA to form malonyl Co-A (Lane et al., 1974). Fatty acid synthase adds two carbon units to malonyl Co-A until terminal thioesterase releases the completed fatty acid. Terminal thioesterase occurs with the formation of palmitic acid, a saturated fatty acid containing 16 carbons (Clarke, 1993).

Since triglycerides are too large to exit the adipocyte, lipolysis is required to mobilize fat for use in other tissues. This process breaks down triglycerides into three non-esterified fatty acids (NEFA) and one molecule of glycerol. If energy demand is met, animals are not reliant on lipolysis to provide energy. However, lipolysis is constantly occurring in the adipocyte, so fatty acids are continuously re-esterified and stored in the adipocyte in adequate energy states (Mears and Mendel, 1974). If energy demands are not met such as in a fasting state, dogs may resort to lipolysis to provide the required energy.

For lipolysis to occur, adipose triglyceride lipase cleaves the first ester bond of the triglyceride to create an FFA and a diglyceride. Hormone sensitive lipase then cleaves the diglyceride to create two FFA and a monoglyceride. Monoglyceride lipase cleaves the final fatty acid from the glycerol backbone. These enzymatic reactions result in three NEFA and a glycerol. Protein kinase A, activated by cyclic adenosine monophosphate, activates these enzymes by phosphorylation. NEFA will now bind to fatty acid binding proteins to reach the endothelial barrier. Once reaching the barrier, they bind to albumin and are transported into circulation (Young and Zechner, 2013).

Long chain fatty acids must be bound to carnitine via palmitoyl transferase-mediated binding to transverse the mitochondrial membrane (Jones and Rideout, 2012). After entering the mitochondria, carnitine is efficiently recycled back to the cytoplasm and fatty acids are

reactivated by CoA. The two beta carbon atoms of the acyl chain undergo degradation by the removal of hydrogens, the addition or water, or by cleavage. This oxidation cycle continues until the acyl chain is completely oxidized (Jones and Rideout, 2012).

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Table 2.1: Common names of fatty acids

Common name	Shorthand nomenclature
Caprylic acid	8:0
Capric acid	10:0
Myristic acid	14:0
Palmitic acid	16:0
Oleic acid	18:1 n-9
Linoleic acid	18:2 n-6
α-Linolenic acid	18:3 n-3
EPA	20:5 n-3
DHA	22:6 n-3

Part 2: The Microbiome

Introduction

A symbiotic relationship between the gastrointestinal (GI) microbes and host is critical for host health (Mackie et al., 1999; Hooper et al., 2001). The microbiota comprises at least hundreds, perhaps thousands of interdependent and/or competing species (Eckburg et al., 2005; Ley et al., 2008; Spor et al., 2011), that are not fully characterized (Hand et al., 2013). The colon has the richest microbial population and is the main site of fermentation (Hooda et al., 2012) with most bacterial activity occurring in the proximal colon, where substrate availability is the highest. Decline of substrate and extraction of free water reduces diffusion of substrates and microbial production in the distal colon (Besten et al., 2013). The microbiota can enhance metabolic capabilities, protect against pathogens, develop the immune system, and modulate gastrointestinal development (Backhed et al., 2005). It protects against pathogens by creating a

barrier, this is known as colonization resistance (Kanauchi et al., 2005). Genetic background, age, sex, initial environmental exposure, and diet contribute to the development and maintenance of core intestinal microbiota (Louis et al., 2007; Khachatryan et al., 2008; Mariat et al., 2009). The gene pool remains relatively constant throughout life but microbes themselves are continuously replaced in response to environmental change (Hand et al., 2013). The gut microbiome is likely a result of co-evolution of host and its microbes over millions of years and shaped by selection pressure (Ley et al., 2006).

Short chain fatty acid production

Microbes contain enzymes that digest fiber and non-digestible carbohydrates to produce short chain fatty acids (SCFA) (Sunvold et al., 1995). Acetate, propionate, and butyrate are primary SCFA that are rapidly absorbed from the colon to be used as an energy source (De Filippo et al., 2010). Butyrate is the major energy source for colonocytes (Herstad et al., 2017), accounting for up to 60-70% of energy supply (Scheppach and Weiler, 2004). In addition, butyrate stimulates cell proliferation, promotes apoptosis, and prevents cancer (Wong et al., 2006). Propionate is taken up by the liver and can be a precursor for gluconeogenesis, lipogenesis, and protein synthesis (Wolever et al., 1991). Acetate enters the peripheral circulation to be metabolized by peripheral tissues and is a substrate for cholesterol synthesis (Wolever et al., 1989). Without the microbiota the host would not receive this additional energy source.

In addition, SCFA protect against gut inflammation (Scheppach and Weiler, 2004) and alter intestinal pH which affects microbe populations (Vernia et al., 2003). For example, Walker et al. (2005) showed that at a pH of 5.5, butyrate producing bacteria such as *Roseburia* and *faecalibacterium prausnitzii* of the *Firmicutes* phylum comprised 20% of the total population. However, butyrate bacteria completely disappeared while acetate and propionate producing

Bacteroides became more dominant at a pH of 6.5 in the distal large intestine (Walker et al., 2005).

SCFA supply ~10% of human caloric requirement and ~80% of maintenance energy for ruminants (Bergman, 1990). Unlike other species, dogs do not rely heavily on microbial fermentation to meet daily energy requirements due to the simplicity of their GI tract, even when fed high fiber diets (Swanson et al., 2011; Hooda et al., 2012; Deng and Swanson, 2015). However, a balanced microbiota is critical to maintain a healthy gastrointestinal tract. A disruption, or dysbiosis, of microbiota has been associated with disease in both humans and dogs including chronic diarrhea (Bell et al., 2008; Jia et al., 2010) and inflammatory bowel disease (IBD) (Nobaek et al., 2000; Janeczko et al., 2008; Xenoulis et al., 2008; Suchodolski et al., 2010). There are usually specific shifts in microbial population or a decrease in overall diversity when disease occurs, making disturbances a possible early warning sign of such (Deng and Swanson, 2015). For example, a decrease in the butyrate producer *faecalibacterium prausnitzii* has been associated with IBD (Herstad et al., 2017) and a decrease in fecal bacterial richness has been shown in humans with diarrhea (Mai et al., 2006). Even though the microbiota is redundant in metabolic functionality (Abubucker et al., 2012; Herstad et al., 2017), increased diversity may be a marker of a healthy microbiome (Li et al., 2017).

Normal for dogs

Before investigating how diet may affect the microbiota, it is important to first understand what is considered “normal” in healthy dogs. Compared to humans, knowledge of canine intestinal microbiota is much less complete (Hand et al., 2013). To date, due to ease of collection, most data regarding the canine microbiota have come from fecal samples of healthy research dogs (Handl et al., 2011; Deng and Swanson, 2015; Herstad et al., 2017). Typically,

beagle lab dogs are used in a controlled setting (Vanhoutte et al., 2005; Middelbos et al., 2010; Hang et al., 2012; Beloshapka et al., 2013; Panasevich et al., 2015) which may not be applicable to client owned dogs in various homes (Herstad et al., 2017). In addition, data from fecal samples mainly represent the microbiota from the lumen of the distal large intestine and may not accurately represent other regions of the GI tract (Eckburg et al., 2005; Suchodolski et al., 2008; Flint et al., 2012) due to each region having a specific population determined by the acidic nature of the stomach, bile salts, and enzymes present in the small intestine (Hooda et al., 2012). Bacterial populations have been found to be different between intestinal biopsy and fecal samples (Momozawa et al., 2011) questioning that samples with direct contact with the intestinal microbiota might be more relevant to gut health (Suchodolski et al., 2010).

In the canine microbiota, bacteria account for approximately 99% of total sequences (Middelbos et al., 2010; Handl et al., 2011; Swanson et al., 2011; Garcia-Mazcorro et al., 2011; Hand et al., 2013). Individual studies differ in proportion, but the predominant phyla found in healthy dogs are *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria* and *Actinobacteria* (Suchodolski et al., 2008; Middelbos et al., 2010; Swanson et al., 2011; Hooda et al., 2012; Deng and Swanson, 2015; Herstad et al., 2017; Li et al., 2017; Schauf et al., 2018). These results are similar to humans who have a microbiota dominated, greater than 75%, in *Bacteroidetes* and *Firmicutes* (Eckburg et al., 2005; Li et al., 2008; De Filippo et al., 2010; Jia et al., 2010; Middelbos et al., 2010; Deng and Swanson, 2015). The key difference between dogs and humans is the predominance of *Fusobacteria* (Deng and Swanson, 2015), which is unusual in mammals (Turnbaugh et al., 2006; Dowd et al., 2008; Ley et al., 2008; Ritchie et al., 2010). An explanation for the increased *Fusobacterium*, a proteolytic bacterium, may be due to the dog's carnivorous origin (Herstad et al., 2017). *Fusobacteria* have also been shown to ferment carbohydrates and

certain amino acids to produce SCFA (Shah et al., 2009). On the other hand, *Fusobacterium* in humans have been associated with development of colorectal cancer (Castellarin et al., 2012; Kostic et al., 2012) and ulcerative colitis (Ohkusa et al., 2002), diseases not commonly found in dogs (Schaffer, 1968; van der Gaag, 1988). Differences in mammals may be related to evolution or starting resident bacteria (Amato et al., 2015).

Wide ranges of the predominant phyla have been reported in dogs: 14-48% *Firmicutes*, 12-38% *Bacteroidetes*, 5-23% *Proteobacteria*, 7-44% *Fusobacteria*, and 0.8-1.4% *Actinobacteria* (Suchodolski et al., 2008; Middelbos et al., 2010; Swanson et al., 2011; Herstad et al., 2017; Li et al., 2017). Most studies have reported a high abundance of *Bacteroidetes*, however Jia et al. (2010), Garcia-Mazcorro et al. (2011), and Handl et al. (2011) reported unusually low levels. Additional phyla such as *Spirochaetes* and *Tenericutes* have also been reported in dogs (Middelbos et al., 2010), with *Tenericutes* being the only phyla not reported in humans. The variability among studies may be due to animals (breed, diet, age), living environment, or lab methods (Deng and Swanson, 2015).

For a more in-depth analysis, further division of taxonomy has been reported in healthy dogs (i.e. class, genera, family). **Table 2.2** comprises a list of the most commonly reported bacterial taxonomy in healthy dogs. Further classifications follow the trend seen at the phylum level with similar top classifications but differing abundances among studies. Schauf et al. (2018) reported classes *Bacteroidia* and *Fusobacteria*, including the genera *Bacteroides*, *Prevotella*, *Clostridium cluster XIX*, and *Fusobacterium*, accounted for greater than 50% of total sequences in healthy dogs. Herstad et al. (2017) reported *Fusobacterium* (28%), *Bacteroides* (14%), and *Clostridiaceae other* (14%) as the most abundant genera. The most abundant families reported by Garcia-Mazcorro et al. (2011) and Handl et al. (2011) were *Ruminococcaceae*,

Clostridiaceae, *Lachnospiraceae*, and *Erysipelotrichaceae*. Many studies have reported *Bifidobacteria* in dogs (Balish et al., 1977; Benno and Mitsuoka, 1992; Simpson et al., 2002). However, *Bifidobacteria* are also seldom or never isolated in other studies (Martineau, 1999; Greetham et al., 2002; Suchodolski et al., 2008). Non-consistent results may relate to low levels of *Bifidobacteria* in canine gut (Vanhoutte et al., 2005), absence of growth substrates in canine gut (Willard et al., 2000) or lack of growth media for isolation (Greetham et al., 2002). In addition, *clostridium difficile*, c*lostridium perfringens*, *enterocccus spp.*, *E. coli*, and *helicobacter* were reported in healthy dogs, indicating bacterial groups considered potential pathogens are part of a healthy microbiota in dogs (Jia et al., 2010; Handl et al., 2011; Goldstein et al., 2012).

Archaea are commensal organisms in ruminant intestine and have also been recently described in the intestine of humans and typically account for 1% of all sequencing in the canine microbiota (Swanson et al., 2011; Deng and Swanson, 2015), with *Methanobacteriales* most commonly reported (Eckburg et al., 2005; Zhang et al., 2009). According to a study by Swanson et al. (2011), the two distinct archaeal phyla are *Crenarchaeota* and *Euryachaeota* with methanogens being the most abundant and diverse group. Overall abundance of archaea in dogs was similar to that in humans but *Sulfolabales*, *Halobacteriales*, and *Nanoarchaeum* were not present in dogs (Swanson et al., 2011). Archaea are typically considered commensals but mutualistic interactions with other microorganisms may lead to pathogenicity (Conway de Macario and Macario, 2009). Archaea may also lead to disease. Zhang et al. (2009) reported higher numbers of methanogenic archaea in obese humans. Methanogens reduce hydrogen into methane, enhancing growth of polysaccharide fermenting bacteria and leading to a higher energy utilization of the diet which may result in obesity (Swanson et al., 2011).

There may not be a consensus view of what is considered "normal" for the canine microbiota due to variation among studies (Hand et al., 2013), but we may have enough information to see an impact with dietary intervention. We can investigate how a specific nutrient or ingredient shifts resident microbiota and then determine if this shift was beneficial or detrimental to the host by analyzing what bacteria now dominates the GI tract as well as any clinical signs presented by the host.

Dietary intervention and the microbiota

In relation to the "normal" canine microbiota, even less is known on the effects of external factors such as diet (Middelbos et al., 2010). Even though diet is one of the greatest influences that can rapidly affect the gut microbiota (Benson et al., 2010; David et al., 2014; Xu and Knight, 2015), manipulation of canine GI microbiota to improve health via diet did not begin until early 1990s (Hooda et al., 2012). Due to functionality in the gut, some nutrients have been studied more extensively than others.

Fiber and the microbiota

Due to potential health benefits, the effects of dietary fiber and non-digestible carbohydrates on the microbiome have been the primary focus in the past decades (Simpson et al., 2002; Vanhoutte et al., 2005; Middelbos et al., 2010; Swanson et al., 2011; Deng and Swanson, 2015; Schauf et al., 2018). An increase in beet pulp in canine diets has been shown to decrease *Fusobacteria* and *Actinobacteria*, increase *Firmicutes*, and leave *Bacteroidetes* unaltered (Middelbos et al., 2010). The shift from *Fusobacteria* to *Firmicutes* could possibly be due to diet selection for complex fermentative activity. Swanson et al. (2011) also increased dietary beet pulp in canine diets which increased *Bacteroidetes*/*Chlorobi* group and *Firmicutes*. On the other hand, Simpson et al. (2002) showed no difference in dogs with fiber enriched diets.

Jia et al. (2010) reported a fiber blend modified the microbiota of healthy dogs but the microbiota of dogs with chronic diarrhea remained stable. Overall, there is variation among studies regarding the effects of dietary fiber. Even with possible health benefits, large amounts of fiber are not observed in pet foods.

Protein and the microbiota

The effect of dietary protein has also been investigated due to potential health concerns resulting from undesirable end products. Bermingham et al. (2013) discovered a shift in microbiota between cats eating dry and wet food on short term. Cats on dry food (lower protein) had increased *Actinobacteria* and lower *Fusobacteria* and *Proteobacteria*. Hang et al. (2012) showed a high protein diet favored growth of *Fusobacteriales* while a high carbohydrate diet favored *Erysipelotrichales* growth. Hooda et al. (2012) reported greater *Actinobacteria* but lower *Fusobacteria* in kittens fed a moderate protein and carbohydrate diet compared to a high protein, low carbohydrate diet. Due to the large inclusion of protein in pet food, it is important to determine the optimum level which meets nutrient requirements without leading to unfavorable effects on the microbiota.

Fat and the microbiota

Compared to other nutrients, there is less information concerning the possible relationship of dietary fat and the microbial population (Deng and Swanson, 2015). Dietary fat and its effect on the microbiota have been underestimated due to the previous argument that since it is mainly digested in the small intestine little reaches the colon where most bacteria reside (Salonen and de Vos, 2014). However, Gabert et al. (2011) showed that 7% of 13C labelled dietary fatty acids were excreted in healthy subjects with 86% being free fatty acids. Free fatty acids are known to have potent antimicrobial effects even at small doses (Huang et al.,

2010; Candido et al., 2018). Therefore, the small amounts of fat reaching the colon could interact with the microbes. In addition, a higher fat content will require an increased amount of bile acids for digestion, which are also known to have an antimicrobial effect (Stacey and Webb, 1947). Specific bacteria are even known to be involved with the digestion and absorption of dietary fat. *Lactobacillus*, *Bifidobacterium*, *Enterobacter*, *Bacteroides*, and *Clostridium* are involved in bile acid metabolism and affect the absorption of dietary fats and lipid-soluble vitamins (Ridlon et al., 2006, Swann et al., 2011). *Faecalibacterium prausnitzii* and *Bifidobacterium* are associated with choline metabolism to modulate lipid metabolism and glucose homeostasis (Martin et al., 2010; Wang et al., 2011). The effects of dietary fat are pertinent due to the high inclusion level in pet foods.

Of the studies available, mainly in humans and rodents, there is a general conclusion regarding the effects of high fat diets on the microbiota: a decrease in *Bacteroidetes* with an increase in *Firmicutes* (Murphy et al., 2015), as well as a decrease in microbial diversity and overall abundance (Hildebrandt et al., 2009; Zhang et al., 2012). This shift has been proven to lead to disease, but without clear reason. The improved capacity for energy harvest and storage of the microbiota as well as enhanced gut permeability and inflammation with high fat diets may be possible explanations (Murphy et al., 2015). Enhanced gut permeability and inflammation can lead to increased intestinal lipopolysaccharide (LPS) bearing bacteria, activation of toll like receptors on immune cells, and a reduction in tight junction proteins (Murphy et al., 2015). Some studies reported that more *Bacteroidetes* than *Firmicutes* was correlated with increased metabolic benefits (Ley et al., 2005; Turnbaugh, Hamady et al., 2009; Ley, 2010) while others showed no correlation or opposite correlations (Collado et al., 2008; Duncan et al., 2008; Schwiertz et al., 2010). In addition, *Firmicutes* has been reported to possibly induce weight loss

in dogs (Li et al., 2017). Differing results may be due to fat type (Huang et al., 2013) or species. Overall, the increase of dietary fat has been linked to dysbiosis of the microbiota.

There is clear evidence that dietary fat alters the murine microbiota (Schauf et al., 2018). Hildebrandt et al. (2009) and Zhang et al. (2012) reported an increase in *Proteobacteria* and *Firmicutes* with a decrease in *Bacteroidetes* when feeding a high fat diet to rodents. Similar phylum level shifts were found following high fat and high sucrose diets (Parks et al., 2013). Daniel et al. (2014) also reported similar results when comparing a high fat diet (60%) to a high carbohydrate diet (66% carbohydrate, 11% fat). Cani and Dezenne (2011) reported a reduction in gram positive *Bifidobacterium* species with high fat diets. Carmody et al. (2015) showed a high fat and high sugar diet affected microbiota of mice regardless of genetics, with an average of only 3.5 days for each diet to create a stable microbiota. Carmody et al. (2015) also reported that changes in microbiota due to diet were reversible and abundance of certain bacteria depended on prior consumption. Turnbaugh et al. (2008) reported a decrease in bacterial abundance and diversity with an increase in *Firmicutes* and a decrease in *Bacteroidetes* in mice fed high fat diets. De La Serre et al. (2010) also showed a decrease in total bacterial density in rats fed high fat diets. In support of Everard et al. (2011), *Akkermansia muciniphila*, a beneficial mucin-degrading bacterium, was reduced with high fat diets (Belzer and de Vos, 2012; Everard et al., 2013). Studies in rodents support the consensus that dietary fat may have a negative impact on the microbiota.

Similar results have been reported in humans as in rodents. A well-known study by De Filippo et al. (2010) reported that *Firmicutes* and *Proteobacteria* were predominant in humans on Western diets (high fat and sugar) while *Bacteroidetes* and *Actinobacteria* were predominant in humans consuming primarily vegetarian diets. Proportions of *Actinobacteria* are important

due to its essential role in obesity maintenance (Turnbaugh, Hamady et al., 2009). In addition, *Prevotella*, *Xylanibacter*, and *Treponema* were only present in humans consuming the vegetarian diets which may be related to the high fiber intake (De Filippo et al., 2010). Analysis from the Western diet showed undesirable results such as less SCFA production and greater *Enterobacteriaceae* such as *Shigella* and *Escherichia*, which are known to cause diarrhea. In addition, community richness was greater in people consuming traditional diets vs Westernized diets (De Filippo et al., 2010). Reduction of microbial richness may be due to the consumption of nutrient-rich foods which may explain the increased incidence of intestinal diseases in the Western world (De Filippo et al., 2010).

The simplicity of the canine GI tract and the typical diet composition of their ancestors may lead to different dietary effects on the microbiota, possibly allowing the dog to consume a high fat diet without the previously stated concerns. Schauf et al. (2018) reported no difference in bacterial richness or diversity when comparing a high-fat-low-starch diet (43% fat, 22% starch) and a low-fat-high-starch diet (23% fat, 42% starch) in dogs. In addition, diet did not affect fecal bacteria composition at phyla or class levels and clustering of OTUs was mostly by dog rather than diet (Schauf et al., 2018). However, there was lower relative abundance of *Prevotella*, *Solobacterium*, and *Coprobacillus* on the high-fat-low-starch diet (Schauf et al., 2018). In agreement, lower relative abundance of *Prevotella* was shown in humans consuming an animal-based compared to plant-based (Wu et al., 2011; David et al., 2014). De Filippo et al. (2010) reported that *Prevotella* made up 53% of total sequences in children consuming mainly cereals but was not detected in children consuming diets low in cereals. The negative impact of dietary fat on *Prevotella* could be explained by the low starch content of the diets and/or the increase in bile acids to the hindgut (Schauf et al., 2018). Schauf et al. (2018) reported the

relative abundance of *Prevotella* was influenced by order of the diet, with a greater decrease in abundance from low-fat-high-starch diet to high-fat-low-starch diet. The effect of diet order may detect low or slow recovery time of *Prevotella*. There was also a trend to higher relative abundance of *Megamonas* in the high-fat-low-starch diet (Schauf et al., 2018). A study comparing a high minced beef diet (HMB), high in fat and protein, to a control diet in dogs found that species richness was decreased in the HMB diet but observed species were not different (Herstad et al., 2017). *Clostridiaceae*, *Clostridiaceae other*, *Dorea*, *Coriobacteriales*, *Coriobacteriaceae*, and *Slackia* were the most abundant in HMB diet. In addition, *clostridia hiranonis*, of the *Clostridiaceae* family, was increased in the HMB diet compared to the control. This species in known to dehydroxylate primary bile acids to secondary bile acids (Kitahara et al., 2001) and was also increased in humans consuming a high fat diet (Song et al., 2013). *C. hiranonis* is considered to have carcinogenic potential in humans (Ajouz et al., 2014) but has been described as a normal commensal in dogs (Mentula et al., 2005; Beloshapka et al, 2013). Herstad et al. (2017) reported higher abundance of *Faecalibacterium* in the control compared to the HMB diet. Higher proportion of Coriobacteriales and *Erysipelotrichaceae* in HMB may be explained by a high fat content which is also described in a study of hamsters and mice (Claus et al., 2011; Martinez et al., 2013). Lower relative abundance of *faecalibacterium prausnitzii* with the HMB diet may be a result of the low fiber content compared to the control. However, *Bacteroidetes*, another genus with proteolytic characteristics, did not increase in fecal samples of HMB diet (Herstad et al., 2017). This contradicts some human studies, which showed increased *Bacteroidetes* after consuming a Western diet (De Filippo et al., 2010; Wu et al., 2011; O'Keefe et al., 2015; David et al., 2014). There are inconsistent results regarding the effect of high fat diets on the abundance of *Bacteroidetes*. Murphy et al. (2010) reported that *Bacteroidetes* was

unaltered in mice fed high fat diets. However, there was an increase in *Bacteroidetes* in humans consuming high fat and protein compared to high carbohydrates (Wu et al., 2011; David et al., 2014). Wu et al. (2011) reported that *Bacteroidetes* is positively correlated with saturated fats in humans. In addition, some *Bacteroidetes* species may be tolerant to bile acids (Begley et al., 2005). The differences could be due to fat source (Schauf et al., 2018) or species. Herstad et al. (2017) showed that diet induced changes on the HMB diet were reversible when reintroduced to control diet and *Roseburia*, a butyrate producer, increased after reintroduction. Shifts in canine microbiota with high fat diets may be revealing adaptation of the microbiota with diet change which may not be damaging to host health.

Carbohydrate and the microbiota

When formulating diets, an increase in one macronutrient typically results in the decrease of another which could lead to confounding effects. Usually with an increase in dietary fat, fiber and carbohydrates are decreased in the diet (Hildebrandt et al., 2009; Turnbaugh, Ridaura et al., 2009; Murphy et al., 2010; Wu et al., 2011). Reduction of carbohydrates may stress the microbiome (Hildebrandt et al., 2009) and reduce energy substrates for beneficial bacteria growth such as *Bifidobacteria* (Cani et al., 2009) and *akkermansia muciniphila* (Everad et al., 2011). Due to a shift in proportion of multiple nutrients, it is difficult to determine exactly which dietary source lead to the changed microbiota. Therefore, it is also important to consider the effects of dietary carbohydrates on the microbiota. Li at al. (2017) reported a higher *Bacteroidetes*/*Firmicutes* ratio in dogs fed carbohydrate rich diets. In addition, carbohydrate rich diets favored the growth of *Prevotella*, *B. uniformis*, and *C. butyricum* (De Filippo et al., 2010; Wu et al., 2011; Kovatcheva-Datchary et al., 2015; Li et al., 2017). Newell et al. (2016) investigated possible effects of a ketogenic diet (75% kcal from fat) on the microbiota in a mouse

model. Overall, the ketogenic diet caused an anti-microbial effect and decreased bacterial numbers. A decrease in bacterial numbers may occur due to the microbiota's primary role of degrading undigested carbohydrates which are diminished in this diet (Janssen and Kersten, 2015). The increase in fat and decrease in carbohydrate, in combination, impact the microbiota.

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Table 2.2: Common bacterial taxonomic classifications reported in GI tract and feces of healthy dogs

Phylum	*Firmicutes*	*Bacteroidetes*	*Proteobacteria*	*Fusobacteria*	*Actinobacteria*
Class	*Erysipelotrichi* *Clostridia* *Negativicutes* *Bacilli*				
Order	*Lactobacillales* *Eysipelotrichales*	*Bacteroidia*		*Fusobacteriales*	*Coriobacteriales* *Actinomycetales*
Family	*Ruminococcaceae* *Clostridiaceae* *Lachnospiraceae* *Erysipelotrichaceae*		*Enterobacteriaceae*		*Coriobacteriaceae*
Genus	*Clostridium clusters XIX, XIVa, XI* *Fusobacterium* *Clostridiaceae* *Eubacterium* *Lactobaillus-Enterococcus group* *Clostridium* *Rumminococcus* *Dorea* *Roseburia* *Streptococcus* *Lactobacillus* *Turicibacter* *Catenibacterium* *Coprobacillus* *Enterococcus*	*Bacteroides* *Prevotella* *Megamonas*	*Sutterela*	*Fusobacterium* *Helicobacter*	*Atopobium cluster* *Bifidobacteria* *Collinsella* *Slackia*
Species	*Clostridium perfringens* *Clostridium difficile* *Clostridium perfringens*		*E. coli*		

Benno et al., 1992; Johnston et al., 2001; Mentula et al., 2005; Suchodolski et al., 2008; Jia et al., 2010; Garcia-Mazcorro et al., 2011; Handl et al., 2011; Hand et al., 2013; Herstad et al., 2017; Schauf et al., 2018

Part 3: Crickets as a Protein Source

Introduction

The world is starting to realize that conventional sources of protein will not be enough to feed the growing population and alternative sources will be required (Zielinska, 2015). Researches have recently begun to investigate the use of insects as a possible protein alternative due to their ability to serve as an environmentally friendly source and a lower impact animal species (Oonincx and Boer, 2012). In addition, insects have a nutrient profile comparable to commonly used protein sources. Most research has been focused on the practicality and safety for human consumption. Consequently, with the increased interest in human consumption, the idea of including insects in pet food has developed. Protein sources are typically included at high rates in pet foods. The protein included in pet food is commonly derived from human food production, leading to direct competition. Replacing these protein sources with insects in pet food could not only provide a high nutritive value but could also offer additional protein for human consumption.

Nutritional composition of insects

Insects have a high nutritive value, with the main component being protein. On an average dry weight basis, insects can range from 50-82% protein. For example, crickets may contain 70% protein (Zielinska et al., 2015), while chicken meal typically included in pet food comprises about 66% protein (Dozier et al., 2003). In addition, insect protein contains all the essential amino acids (Zielinska, 2015). Osimani et al. (2017) found that 47% of amino acids in various insect species were essential and the ratio of essential to nonessential amino acids was 0.9. These numbers exceed the FAO/WHO requirements of 40% essential amino acids with a 0.6

ratio. In a study done by Finke et al. (1989) cricket meal was found to be superior to soy protein when fed to weanling rats.

In contrast to other protein sources, insects contain chitin, an amino-polysaccharide with cellulose-like β 1-4 linkages. Chitin is part of the exoskeleton covering the insect's body for protection and support. In nutrient analyses chitin contributes to the fiber component. Zielinska et al. (2015) reported 3.65% of fiber in crickets. Fiber has been shown to have beneficial health effects for animals and humans with some of its main benefits related to increase of digesta passage rate and relief of constipation (Cole et al.,1989). Fiber sources, such as brown rice and bran, are currently included in dog diets with inclusion levels that are typically low, at only 1-5% dry matter. Clinical diets may contain up to 20% fiber to aid in treatment of chronic diseases such as obesity (Diez et el., 1998). However, increase in dietary fiber impacts overall digestibility due to fiber's ability to resist hydrolysis by endogenous enzymes (NRC, 2006). Most dietary fiber passes through the small intestine undigested and is fermented by microbes (NRC, 2006). Previous studies have reported a decrease in digestibility with an increase in dietary fiber in dogs (Cole et al., 1989; Fahey et al., 1990). Dietary fiber is also known to have a "bulking effect" resulting in an increase in wet fecal weight with increased consumption (Bueno et al., 1981; Cole et al., 1989; Fahey et al., 1990; McPherson-Kay, 1987). This effect appears to be most strongly associated with insoluble fiber sources which are poorly fermentable and have a good water-binding capacity (Diez et al., 1998).

Even with the fiber component insects remain highly digestible, ranging from a 75-90% digestibility (Sun-Waterhouse et el., 2016). Interestingly, Bosch et al. (2014) reported the *in vitro* OM digestibility of house crickets to be 88% which was similar when compared to poultry meat meal at 85.8%.

Overall, it is important to note that the nutritional value of edible insects is variable. The insect's species, life stage and diet can affect nutrient levels. In addition, nutrient levels are affected by processing. For example, a 7% decrease in protein digestibility was shown after toasting. Therefore, standard processing techniques will need to be established to maintain the high nutrient quality.

Sustainability of insects

Due to an increased need for sustainability, insects may serve as a viable alternative to current meat production. Conventional greenhouse gas emission is much lower for insects (2-122g/kg mass gain) than beef cattle (2850g/kg mass gain) and in the lower range for pigs (80-1130g/kg mass gain). Per kg of edible protein, insects have less global warming protentional compared to milk, chicken, pork and beef production (Oonincx and Boer, 2012). The fossil energy use of insects is greater than milk and chicken production, similar to pork production, and lower than beef production. Insects are poikilothermic, meaning they depend on suitable ambient temperatures. Therefore, they require climate-controlled rearing facilitates. The need for heat increases energy use greater than what is required for milk and chicken production (Oonincx and Boer, 2012). Due to the size of insects and their living conditions, less space is needed to raise them compared to livestock. For example, mealworms require only 10% of the land used for beef production. Since land availability is fixed and limited, slowing down the expansion of agricultural land is a critical step towards sustainability (Oonincx and Boer, 2012). Insects also have increased feed efficiency with only 1.7kg of feed required to produce 1kg of cricket protein while beef require 10kg of feed (van Huis, 2013). Unlike current species raised for protein, insects have a high reproduction rate and a short maturation period. The common cricket lays up to 1,500 eggs in about a month (Nakagaki and DeFoliart, 1991). In addition, insects are mostly

omnivorous, can live on organic waste, and can convert manure into biomass (van Huis and Oonincx, 2017). Since insects are cold blooded, they can meet their water requirement through their food source which decreases the amount of water usage (Rumpold, 2013).

Possible concerns of edible insects

Even with the proven benefits of insect consumption, there is still apprehension due to regulations, the possibility of microbial contamination, and consumer acceptance. Concrete regulations are a major concern with the inclusion of insects into diets. Some manufactures believe that there is a lack of clear legislation to regulate the product (Belluco et al., 2017). Currently, FDA does have regulations in place to ensure the safety of edible insects. The Federal Food, Drug, and Cosmetic Act considers insects as food if that is the intended use. In addition, insects must be produced, packaged, stored, and transported under sanitary conditions and be properly labeled (including scientific name of insect) if being used for human consumption or pet food. FDA (2016) has also established the following recommendations if marketed for human consumption or pet food: (1) insects must be raised specifically for human food following current good manufacturing practices, (2) insects raised for animal consumption cannot be diverted to human food, (3) insects cannot be collected in the wild and sold as food due to the potential of carrying diseases or pesticides, and (4) manufactures need to demonstrate the healthiness of the product.

Even with these regulations in place, there is still concern regarding microbial and parasite contamination, allergenicity, and bioaccumulation of minerals (Koutsos et al., 2019). In addition, fungi and mycotoxins may also be a concern. The risk of microbial contamination and fungi can be decreased with the use of an appropriate kill step during processing (Fernandez-Cassi et al., 2019). However, mycotoxins are not effectively detoxified with heat processing. Of

note, some insect species have been shown to detoxify mycotoxins (Bosch et al., 2017; Purshcke et al., 2017; Camenzuli et al., 2018), but further research is needed to characterize the safety of utilization. There has been limited work regarding the presence of parasites and viruses therefore the concern is unknown. Allergenicity may present a concern, particularly for humans allergic to crustaceans. This risk is due to similar proteins present in some insects which are known to cause allergies in crustaceans (Fernandez-Cassi et al., 2019). This affect may translate to pets as well but there is little research regarding the use of insects in pet food. In addition, some insects bioaccumulate minerals from their diet or water source which may lead to toxicity in the animals consuming them. Therefore, it is important to carefully regulate an insect's diet source (Koutsos et al., 2019).

Consumer perception is also preventing the widespread consumption of insects. The consumption of edible insects has been practiced in certain human populations for centuries (Bukkens, 1997). Insects are even considered delicacies in some parts of the world (Nonaka, 2009). In addition, 2.5 billion people supplement insects in their diet (FAO). However, most people in Western countries view entomophagy, the practice of eating insects, with disgust.

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Part 4: Conclusions

In conclusion, the evolution of the pet food industry has led to increased challenges for research and development. The rise in consumer interest in how to nutritionally provide for their pets is creating the desire for novel diets with high quality ingredients like those consumed by their canine ancestors. These novel diets may include proportional shifts in nutrients typically provided such as the use of high fat, low starch diets. In addition, there is concern of the

environmental impact of protein sources used in pet food as well as the direct competition these sources may have with human food production. To decrease this concern, the industry is investigating alternative protein sources, such as insects.

The simplicity of the dog's GI tract and descending from animals who consume diets high in fat may allow the dog to consume a wide range of dietary fat unlike other species. Previous concern of pancreatitis and diarrhea with consumption of high fat diets requires further investigation in dog models. In addition, increased dietary fat is reported to have a negative impact on the microbiota which has been characterized with decreased microbial diversity and a shift from *Bacteroides* to *Firmicutes*. However, this result may be different in the dog.

Due to the low environmental impact of insects and their high nutritive value, they may serve as a high-quality protein source. However, there is limited published work on the use of insects in pet food. Even more so, the use of crickets as an alternative protein source has never been tested in dogs. The possible concern of microbial contamination may be diminished with proper processing techniques. As the number of insects raised for consumption increases, regulations will improve just like they have with typical protein sources. In addition, as humans become educated about the use of edible insects, their opinions may shift in favor.

CHAPTER 3. APPARENT TOTAL TRACT DIGESTIBILITY, FECAL CHARACTERISTICS, AND BLOOD PARAMETERS OF ADULT DOGS FED HIGH FAT DIETS

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Abstract:

Pet foods may be formulated with decreased starch to meet consumer demands for less processed diets. Fats and oils may be added to low starch diets to meet energy requirements, but little is known about its effects on canine health. The study objective was to evaluate the effects of feeding healthy adult dogs low carbohydrate, high-fat diets on apparent total tract digestibility, fecal characteristics, and overall health status. Eight adult Beagles were enrolled in a replicated 4x4 Latin Square design feeding trial. Dogs were randomly assigned to 1 of 4 dietary fat level treatments (T) within each period: 32% (T1), 37% (T2), 42% (T3), and 47% (T4) fat on a dry matter basis. Fat levels were adjusted with inclusion of canola oil added to a commercial diet. Each dog was fed to exceed their energy requirement based on NRC (2006). Blood samples were analyzed for complete blood counts, chemistry profiles, and canine pancreatic lipase immunoreactivity levels. Apparent total tract digestibility improved ($P < 0.05$) as the fat level increased for dry matter, organic matter, fat, and gross energy. Fecal output decreased as levels of fat increased in the diet ($P = 0.002$). There was no effect of fat level on stool quality or short chain fatty acid and ammonia concentrations in fecal samples ($P \geq 0.20$). Blood urea nitrogen levels decreased with increased fat level ($P = 0.035$). No significant differences were seen in

canine pancreatic lipase immunoreactivity ($P = 0.110$). All blood parameters remained within normal reference intervals. In summary, increased dietary fat improved apparent total tract digestibility, did not alter fecal characteristics, and maintained the health status of all dogs.

Introduction

Throughout evolution, dogs have developed the ability to digest and metabolize carbohydrates but do not have a nutrient requirement for them (NRC 2006). Nonetheless, pet foods contain high amounts of carbohydrates to meet energy requirements, provide lower cost products, and for processing considerations. Pet owners have developed an interest in the nutrition and dietary ingredients present in pet foods (Buff et al., 2014). Consumers have recently developed a desire to feed their dogs less processed foods as compared to the instinctual diets eaten by their canine ancestors (Morelli et al., 2019). There has also been increased popularity in less processed products such as freeze dried and raw diets (Buff et al., 2014; Carter et al., 2014; Schlesinger and Joffe, 2011). To create these products, diets may be formulated with a decreased concentration of starches. However, with this decrease in carbohydrates it is still necessary to maintain energy requirements of the diet with additional ingredients that fulfill this energy demand. Fat is included from 8-22% on a dry matter basis (DMB) in kibble diets and 20-32% (DMB) in canned diets to increase caloric density and improve palatability. Fat is typically not included at higher levels due to difficulties in processing, health concerns, and the fact that current fat levels are already above nutrient requirements (Lin et al., 1997; NRC 2006). One commonly mentioned health concern is the increased risk of pancreatitis with consumption of high fat diets in dogs (Xenoulis et al., 2008). However, little is known about the effects of high fat diets with low levels of starch on canine health. The study objective was to evaluate the effects of feeding healthy adult dogs increasing levels of fat in low carbohydrate diets on

apparent digestibility, fecal characteristics, and overall health status. We hypothesized that increased dietary fat would improve the apparent digestibility of the diet while maintaining fecal characteristics and overall health status of each dog.

Materials and Methods

The protocol for this experiment was reviewed and approved by the Iowa State University Institutional Animal Care and Use Committee before initiation of the experiment (IACUC #9-17-8605-K).

Animals and Housing

Eight healthy spayed female Beagles, 1 year of age with an average body weight of 8.57 ± 0.93 kg and body condition score (BCS) of 4.75 ± 1.16, according to the Royal Canin body condition score chart for small dogs, were enrolled in this study. All dogs were housed in pairs at the College of Veterinary Medicine at Iowa State University in temperature-controlled rooms (20°C) on a 12:12 hour light: dark schedule. During feeding and collection periods, dogs were separated by gate closure.

Experimental Design

Dogs were randomly assigned to one of four dietary treatments using a replicated 4x4 Latin Square design consisting of 15-d periods. This design allowed each dog to receive each diet for one period during each replicate. Each period included a 10-d diet adaption phase followed by a 5-d total collection phase.

Diets and Feeding

A commercially manufactured canned canine diet (**Table 3.1**) was used as control. Increasing inclusion levels of fat (2%, 4%, or 6% canola oil, as-fed basis) were added to control diet to create three more treatments. Treatment diets contained 32% (T1), 37% (T2), 42% (T3),

and 47% (T4) total dietary fat (DMB) (**Table 3.2**). Dogs were fed twice daily (0800 h and 1700 h) to meet their daily energy requirements. Total daily energy requirements were calculated per treatment for each individual dog based on body weight at the beginning of each period. Weights and BCS were recorded weekly. If needed, feed intake was adjusted during the adaption phase to maintain an ideal BCS of a 4 or 5 according to the Royal Canin body condition score chart for small dogs. Water was provided *ad libitum* throughout the study.

Sample Collection

Before beginning of the trial, a 5mL sample of blood was collected from each dog via jugular venipuncture to assess complete blood count (CBC) and chemistry panels to determine any underlying health concerns that were present and could confound data collection. Fecal samples were also collected and evaluated before the start of the study to ensure all dogs were parasite free.

A 5mL sample of blood was also collected from each dog via jugular venipuncture on d 15 of each period. The blood samples were split into 2 collections tubes: one red-top tube and one lavender-top EDTA tube. Samples were submitted to the Clinical Pathology Laboratory at Iowa State University College of Veterinary Medicine (Ames, IA) for a CBC, chemistry panel, and canine pancreatic lipase immunoreactivity (cPLI) analysis.

Kennels were checked for feces at least every hour for 24-hrs during each collection day. Feces was weighed, scored, and stored at -20°C until laboratory analyses. Fecal output and fecal scores were recorded for each dog during each collection period. Fecal scores were determined using the following scale: 1=hard dry and crumbly feces to 5=watery diarrhea (Moxham, 2001). Fresh samples (within 15 minutes) were collected for short chain fatty acid and ammonia concentrations. pH was also determined from this sample. Two milliliters of HCl were added to

2 grams of feces and placed in -20°C for short chain fatty acid and ammonia analyses. Two grams of feces were placed into a cryovial tube and immediately stored in -80°C for microbe analysis.

Chemical Analyses

Total fecal collections and dietary treatments were analyzed for macronutrient composition and energy. All chemical analyses were conducted in the Comparative Nutrition Laboratory at Iowa State University (Ames, IA). A sub-sample (100g) of each diet was pooled and homogenized. Feces collected during the 5-d collection period were pooled and homogenized for each dog for nutrient analysis. Fecal samples and dietary sub-samples were dried at 65°C in a forced air-drying oven and ground with a coffee grinder to accommodate small sample size (model BCG11OB; KitchenAid). Diet and fecal samples were analyzed for dry matter (DM) (AOAC 934.01) and organic matter (OM) (AOAC 942.05). Crude protein (CP) was determined using a LECO Nitrogen Analyzer (AOAC 992.15; model TruMacN; LECO Corporation; St. Joseph, MI). An EDTA sample of 9.56% nitrogen was used as the standard for calibration. Crude fat was determined via acid hydrolysis and hexane extraction (AOAC 960.39). Gross energy (GE) was determined via bomb calorimetry (model 6200; Parr Instrument Co.; Moline, IL) with benzoic acid (6,318 kcal GE/kg; Parr Instrument Co.) used as the standard for calibration. Total dietary fiber (TDF) and starch content were determined with the use of assay kits (Megazyme International, Wicklow, Ireland). Metabolizable energy (ME) values were estimated using the AAFCO modified Atwater equation:

$$\text{ME} = 8.5\ \text{kcal ME/g of fat} + 3.5\ \text{kcal/g of CP} + 3.5\ \text{kcal/g of nitrogen} - \text{free extract}$$

Apparent Total Tract Digestibility and Energy Calculations

Feed intake was recorded for each dog throughout the experiment. Total fecal output collected daily during the collection phase of each period was averaged to determine daily fecal output (g as-is/d).

Apparent total tract macronutrient and energy digestibility were determined using chemical composition data from diet and fecal samples and feed intake/fecal output records. Apparent total tract macronutrient and GE digestibility was calculated using the following equation:

$$\text{Apparent digestibility (\%)} = \left(\frac{intake - fecal\ output}{intake}\right) \times 100.$$

Statistical Analysis

All data were analyzed in a linear mixed model as a replicated 4x4 Latin Square design including fixed effects of diet and room (i.e. replicate) and random effects of period and animal (PROC MIXED, Version 9.4, SAS Inst., Cary, NC). Baseline biomarker value, initial body weight, and/or initial body condition score were used as covariates in the model depending on each specific trait. Orthogonal contrasts were also performed to analyze linear, quadratic, and/or cubic relationships among treatments. A significant effect of diet and/or of orthogonal contrast was considered at $P < 0.05$.

Results and Discussion

Diet and Fecal Chemical Analyses

Nutrient concentrations ranged for DM (22.2-26.7%), OM (89.0- 91.6%), CP (46.9-38.2%), Fat (32.1-46.5%), TDF (3.41- 3.20%), total starch (1.08-1.02%), and GE (6,068-6,705kcal/kg) between T1 and T4, respectively. With each addition of 2% canola oil, the overall fat content of the diet increased by 5%, ranging from 32% to 47% total dietary fat for T1 and T4,

respectively. Diets were originally formulated based on the estimated protein to fat ratios. Protein and fat often account for most of the nutrient composition in canned diets and are an important factor to ensure a well-balanced diet during formulation. The protein to fat ratios of the final diets were 1.46 and 0.82 for T1 and T4 resulting in a shift from the primary macronutrient of protein to fat. While the addition of fat increased the DM, OM, fat, and GE in the diets, it decreased the amount of protein, TDF, and starch. Of note, canola oil is a source of pure fat; therefore, it does not contribute other nutrients. In addition, canola oil has a high DM percentage of 99% increasing the diet's DM percentage which may impact fecal output and nutrient digestibility.

Fecal DM significantly increased with inclusion of fat ($P = 0.047$) and followed a linear relationship with treatments ($P = 0.008$) (**Table 3.3**). An increase in fecal DM content can best be explained by the increase in DM percentage of the diets or the increase in digestibility as fat increased. Fahey et al. (1990) reported a decrease in fecal DM as diets became less digestible. Organic matter, CP, fat and GE of fecal samples were not different ($P \geq 0.10$).

Feed Intake and Fecal Characteristics

Feed intake and fecal characteristics are presented in **Table 3.4**. Feed intake was controlled to exceed at least 10% of each animal's energy requirement, following NRC guidelines for lab animals. Average feed intake on an as-fed basis decreased ($P = 0.001$) from 547.5 to 388.2 g/day with a negative linear relationship ($P < 0.001$) as dietary fat level increased. However, feed intake on a DM basis and GE intake were similar throughout treatments ($P \geq 0.09$). As levels of dietary fat increased from T1 to T4, feed offered in g/day were smaller for treatments with greater energy density. Nonetheless, all nutrients were offered and consumed

based on NRC (2006) nutrient requirements on a g/day basis. Dogs also maintained ideal body weight and BCS throughout the trial (**Table 3.5**).

Fecal output decreased from T1 to T4 on an as-is ($P = 0.002$) and DM basis ($P = 0.004$) with negative linear responses ($P < 0.001$) as dietary fat increased. The decreased total feed intake followed by the improved digestibly with the increased fat percentage may explain the decrease in total fecal output (Kerr et al., 2012). Fecal scores were similar among treatments with an average of 2.4 indicating a well-formed (normal) stool. The normal stool consistency was surprising due to previous concern of loose stool as a result of high fat diets (Ballaban-Gil et al., 1998; Hosain et al., 2005; Seo et al., 2007; Liu, 2008; Liu and Wang, 2013). Fecal and urine pH were all within normal limits and remained consistent among diets, suggesting that end products, such as short chain fatty acids and ammonia, were not affected by the increase of dietary fat.

Short chain fatty acids (SCFA) are end products of dietary fiber fermentation (Besten et al., 2013). Overall, there were no significant treatment differences among SCFA for the various diets ($P \geq 0.30$) (**Table 3.6**). The average percentage of acetate, butyrate, and propionate throughout treatments was 54.5%, 12.3%, and 27.1%, respectively. Of note, proportions of SCFA were similar to those previously reported by Swanson et al. (2002), Bosch et al. (2009), and Schauf et al. (2018) in dogs. Comparable treatment values may show that the slight fluctuation of fiber and starch levels with increased dietary fat was not enough to affect SCFA production. This indicates that starch fermentation in the hindgut was not altered by the diet. This is important as it is critical to maintain physiological SCFA levels for overall health status as SCFA are the main energy source for colonocytes, with butyrate accounting for up to 70% of total energy consumption (Roediger, 1980; Roediger, 1982). In addition, SCFA production has a significant role in gut homeostasis (Thorburn et al., 2014) and can limit the growth of pathogenic

species by decreasing luminal pH (Swanson et al., 2002). The production of SCFA also promotes a favorable luminal environment for protective bacterial species, including lactic acid bacteria. The maintenance of normal SCFA levels indicate that the dietary alteration in this study did not impact the production level of SCFA.

Ammonia and BCFA percentages remained similar among treatments ($P \geq 0.20$) (**Table 3.6**). The average percent ammonia among all treatments was 16.44%. The average percentage of all treatments for isobutyrate, isovalerate, and valerate was 1.9%, 3.7%, and 0.5%, respectively. Similar ammonia and BCFA levels were also found by Hesta et al. (2003), Barry et al. (2009), and Herstad et al. (2017) in dogs fed varying diets. In agreement with Schauf et el. (2018), fecal ammonia and BCFA were not affected by a high fat, low starch diet in dogs. Ammonia and BCFA are putrefactive compounds produced from unutilized amino acids (Kerr et al., 2012). If increased they may have detrimental effects on intestinal and host health (Swanson et al., 2002). Comparable results among treatments may imply that the protein content of each diet was similar enough to maintain consistent fermentation products.

Apparent Total Tract Digestibility

Changes were observed in nutrient digestibility ($P \leq 0.02$) except for CP with a linear increase among treatments for DM, OM, fat, and GE ($P \leq 0.003$) as fat increased (**Table 3.4**). There was also a cubic relationship for increasing GE ($P = 0.036$), possibly indicating a point of optimal energy digestibility. Finally, consumption of T4 led to the greatest digestibility in DM (87.6%), OM (91.4%), and fat (97.6%) compared to other diets.

Overall, the addition of dietary fat increased digestibility. Compared to extruded dry diets, the diets in the present study were similar or higher in digestibility (Castrillo et al., 2001). Specifically, the digestibility of fat exceeded 95% in each treatment. The increase in apparent

digestibility with increased levels of dietary fat was to be expected due to the high digestibility of fat. Many studies have reported an increase in digestibility with the addition of dietary fat in swine (Clawson et al., 1962; Lowrey et al., 1962; Greeley et al., 1964; Jorgensen et al., 1992). Clawson et al. (1962) and Greeley et al. (1964) reported that the addition of dietary fat did not affect protein digestibility. Due to microbial fermentation in the large intestine, apparent fecal digestibility may not be an accurate representation of crude protein digestibility (Hendriks and Sritharan, 2002), which could explain the observed similar results in protein digestibility.

Blood Panels

All blood parameters remained within the desired reference intervals indicating healthy animals with only two showing significant differences among treatments (**Table 3.7** and **Table 3.8**). Red blood cell distribution width (RDW) resulted in a difference among treatments (P = 0.030). A quadratic relationship was observed for RDW among treatments (P = 0.010) with values of 12.28%, 12.41%, 12.66%, and 12.19% for T1, T2, T3, and T4, respectively. Measurement of RDW can serve as a possible indicator for cardiovascular disease (Tonelli et al., 2008). However, RDW values remained within reference ranges and were not of clinical significance. Blood urea nitrogen (BUN) levels were impacted by diet (P = 0.035), with a linear decrease with the addition of fat (P = 0.005). Even with the decrease in BUN levels from 14.75 to 13.00 mg/dl from T1 to T4, respectively, BUN levels remained within reference intervals even at the highest fat level indicating results were not of clinal significance. As a result of protein metabolism, urea is produced by the liver and is carried by the blood to the kidney for excretion. Therefore, the decrease in total protein intake could have led to fluctuations in BUN levels (Hosten 1990). In addition to remaining within the desired reference intervals, BUN levels are

not a concern in this study because protein requirements were met (g/day) according to NRC (2006).

In addition to chemistry and complete blood count profiles, cPLI levels were analyzed due to previous concern of high fat diets contributing to the development of pancreatitis in dogs (Xenoulis et al., 2008). Pancreatitis is characterized by inflammation of the pancreas when damage to pancreatic tissue occurs as digestive enzymes are activated before release. Currently, serum pancreatic lipase immunoreactivity is the recommended assay for the diagnosis of pancreatitis in dogs since large quantities of pancreatic lipase may enter blood circulation in cases of pancreatitis (Lem et al., 2008). Levels of cPLI for T1, T2, T3, and T4 were 34.63, 44.13, 42.88, and 39.50 μg/L, respectively, with no significant treatment differences ($P = 0.110$). The normal cPLI levels obtained in this study (≤ 200 μg/L) indicate that the elevated levels of dietary fat did not result in adverse side effects in the pancreas. The concern for pancreatitis may instead be related to underlying disease such as obesity or have a genetic component which we did not analyze in this study (Hess et al., 1999). Alternatively, pancreatitis is likely a function of an acute ingestion of a high fat dose from inappropriate consumption rather than a controlled and consistent intake of fat as was fed in this study.

Conclusion

In conclusion, the increase of dietary fat improved digestibility while maintaining fecal characteristics and blood parameters in healthy adult dogs. Further research is needed regarding optimum and maximum inclusion level of dietary fat in canine diets. The practicality of high dietary fat also needs to be investigated as it pertains to pet food processing. Of note, the goal of this study was to investigate the use of high levels of dietary fat in an ideal situation, with the use of healthy adult dogs, to observe if dogs could utilize the high fat content and maintain health.

However, further research is needed to determine the effect of increased dietary fat in broader populations such as with the use of different breeds, senior, diseased, and/or overweight dogs.

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Table 3.1: Ingredient composition of control diet

Diet	Ingredients
Control	Chicken, chicken broth, chicken liver, carrots, peas, dried egg product, guar gum, carrageenan, ground flaxseed, potassium chloride, salt, cassia gum, minerals (zinc amino acid chelate, iron amino acid chelate, copper amino acid chelate, manganese amino acid chelate, sodium selenite, potassium iodine), vitamins (vitamin E supplement, thiamine mononitrate, niacin supplement, d-calcium pantothenate, vitamin A supplement, riboflavin supplement, biotin, vitamin B12 supplement, pyridoxine hydrochloride, vitamin D3 supplement, folic acid), choline chloride

Table 3.2: Analyzed chemical composition and estimated metabolizable energy (ME) of diets (DM basis)[1]

	Canola Oil			
Item	0%	2%	4%	6%
DM, %	22.15	24.85	24.94	26.74
Moisture, %	77.85	75.15	75.06	73.26
OM, %	88.96	90.74	90.63	91.60
Ash, %	11.05	9.27	9.37	8.41
CP, %	46.88	42.72	40.02	38.19
Fat, %	32.05	37.15	41.86	46.49
Total Dietary Fiber, %	3.41	3.34	3.27	3.20
Total Starch, %	1.08	1.06	1.04	1.02
GE, kcal/kg	6068.01	6361.67	6488.54	6705.12
ME[2], kcal/kg	4596.40	4916.15	5150.60	5418.15

[1] All analyses were performed using 2 replicates/diet with a coefficient of variation (CV) < 2

[2] ME = 8.5 kcal of ME/g of fat + 3.5 kcal of ME/g of CP + 3.5 kcal of ME/g of nitrogen-free extract

Table 3.3: Chemical composition of fecal samples (DM basis)

	Canola Oil					P-Value			
Item	0%	2%	4%	6%	SEM	Treatment	Linear	Quadratic	Cubic
DM, %	33.38[b]	34.98[a,b]	35.22[a,b]	36.40[a]	1.38	0.047	0.008	0.773	0.466
OM, %	62.57	63.16	63.79	63.41	0.50	0.208	0.092	0.238	0.557
Ash, %	37.43	36.84	36.21	36.59	0.50	0.208	0.092	0.238	0.557
CP, %	29.49	29.58	29.83	29.74	1.18	0.955	0.644	0.848	0.805
Fat, %	8.59	9.59	9.90	8.99	0.88	0.222	0.473	0.055	0.802
GE, kcal/kg	3506.06	3561.34	3639.02	3586.61	53.10	0.189	0.103	0.211	0.422

[a-c] Means within a row lacking a common superscript letter are different ($P < 0.05$).

Table 3.4: Feed intake, fecal output, fecal score, fecal pH, urine pH, apparent total tract macronutrient and energy digestibility

	Canola Oil					P-Value			
Item	0%	2%	4%	6%	SEM	Treatment	Linear	Quadratic	Cubic
Intake									
Feed intake, g AF[1]/d	547.50[a]	479.33[b]	440.70[b,c]	388.23[c]	28.54	0.001	<0.001	0.726	0.666
Feed intake, g DM/d	121.54	119.35	109.73	103.66	6.89	0.097	0.017	0.721	0.653
GE intake, kcal/d	737.54	759.28	712.02	695.03	43.73	0.566	0.263	0.574	0.520
Output									
Fecal output, g as-is/d	60.38[a]	52.78[a,b]	43.25[b,c]	35.53[c]	6.33	0.002	<0.001	0.987	0.833
Fecal output, g DM/d	19.89[a]	18.08[a,b]	14.81[b,c]	13.55[c]	1.78	0.004	<0.001	0.812	0.513
Fecal Score[2]	2.42	2.48	2.33	2.30	0.10	0.429	0.192	0.589	0.393
Fecal pH	6.83	6.93	6.93	6.80	0.10	0.629	0.846	0.206	0.915
Urine pH	7.00	ND	ND	7.25	0.35	0.194	ND	ND	ND
Apparent Digestibility									
DM, %	83.61[c]	84.69[b,c]	86.55[a,b]	87.62[a]	1.05	0.021	0.002	0.998	0.694
OM, %	88.48[c]	89.35[b,c]	90.50[a]	91.40[a]	0.76	0.019	0.002	0.983	0.852
CP, %	89.73	89.38	89.93	90.22	0.99	0.891	0.567	0.691	0.741
Fat, %	95.57[c]	96.02[b,c]	96.84[a,b]	97.62[a]	0.43	0.001	<0.001	0.613	0.788
GE, %	90.53[c]	91.43[b,c]	95.01[a]	93.35[a,b]	0.79	0.003	0.003	0.118	0.036

[a-c] Means within a row lacking a common superscript letter are different ($P < 0.05$).

[1] AF = as fed.

[2] Fecal score determined with the use of the Waltham Faeces Scoring System.

Table 3.5: Body weight and body condition score of dogs fed high fat diets

	Canola Oil					P-Value			
Item	0%	2%	4%	6%	SEM	Treatment	Linear	Quadratic	Cubic
Body weight	7.66	7.50	7.53	7.55	0.18	0.199	0.228	0.111	0.385
Body condition score	3.63	3.56	3.56	3.50	0.24	0.907	0.492	1.000	0.818

Table 3.6: Ammonia and volatile fatty acid (VFA) composition of fecal samples

	Canola Oil					P-Value			
Item	0%	2%	4%	6%	SEM	Treatment	Linear	Quadratic	Cubic
Ammonia, %	17.02	16.00	15.55	17.17	1.59	0.864	0.998	0.417	0.836
VFA concentration, mmol/g									
Acetate	30.77	30.29	30.30	29.59	2.82	0.981	0.699	0.957	0.893
Propionate	15.06	15.46	13.90	15.52	1.44	0.670	0.966	0.565	0.282
Butyrate	7.04	7.01	6.93	6.47	0.82	0.932	0.572	0.767	0.915
Isobutyrate	1.05	1.31	0.98	1.10	0.21	0.612	0.833	0.696	0.215
Isovalerate	2.03	2.19	1.91	2.12	0.28	0.883	0.996	0.935	0.433
Valerate	0.31	0.34	0.23	0.18	0.08	0.211	0.059	0.546	0.469
VFA molar proportion, %[1]									
Acetate	54.91	53.68	55.45	54.05	1.03	0.612	0.861	0.937	0.195
Propionate	26.76	27.25	26.13	28.07	0.76	0.301	0.394	0.320	0.162
Butyrate	12.43	12.41	12.51	11.73	0.66	0.827	0.513	0.573	0.738
Isobutyrate	1.75	2.23	1.85	1.94	0.28	0.564	0.858	0.444	0.243
Isovalerate	3.57	3.81	3.65	3.88	0.40	0.902	0.604	0.973	0.598
Valerate	0.58	0.62	0.40	0.33	0.15	0.205	0.060	0.569	0.407

[1]Calculated as the individual VFA concentration / total VFA concentration x 100%.

Table 3.7: Plasma complete blood count of dogs fed high fat diets

	Canola Oil					P-Value				
Item[2]	0%	2%	4%	6%	SEM	Treatment	Linear	Quadratic	Cubic	Reference Interval[1]
WBCs, $x10^3$/ul	7.20	7.62	7.38	6.41	0.48	0.275	0.201	0.135	0.980	6.0-17.0
Neutrophils, $x10^3$/ul	4.16	4.73	4.62	3.69	0.45	0.307	0.424	0.090	0.950	3.0-11.4
Lymphocytes, $x10^3$/ul	2.31	2.18	2.15	2.21	0.20	0.936	0.705	0.613	0.973	1.0-4.8
Monocytes, $x10^3$/ul	0.52	0.37	0.34	0.35	0.07	0.183	0.074	0.209	0.745	0.15-1.35
Eosinophils, $x10^3$/ul	0.18	0.26	0.26	0.10	0.05	0.075	0.278	0.018	0.743	0.00-0.75
Basophils, $x10^3$/ul	0.00	0.00	0.00	0.00	0.00	1.000	1.000	1.000	1.000	0.00-0.10
RBCs, $x10^6$/ul	6.52	6.55	6.56	6.52	0.13	0.994	0.980	0.788	0.951	5.50-8.50
Hemoglobin, gm/dL	15.16	15.16	15.23	15.48	0.34	0.830	0.427	0.655	0.920	12.0-18.0
Hematocrit, %	45.61	45.63	45.16	46.46	1.11	0.757	0.594	0.464	0.568	37.0-55.0
MCV, fl	69.98	69.69	68.93	69.71	0.69	0.617	0.557	0.366	0.445	60.0-77.0
MCH, pg	23.28	23.19	23.24	23.19	0.19	0.917	0.653	0.859	0.615	19.5-30.0
MCHC, gm/dl	33.24	33.28	33.79	33.28	0.35	0.461	0.621	0.335	0.242	32.0-36.0
RDW, %	12.28^b	$12.41^{a,b}$	12.66^a	12.19^b	0.17	0.030	0.980	0.010	0.097	11.6-14.8
Platelets, $x10^3$/uL	412.75	413.87	415.87	400.00	26.38	0.816	0.537	0.518	0.748	200.0-500.0
MPV, fl	9.95	10.50	10.68	10.83	0.43	0.383	0.106	0.593	0.834	7.00-11.00

[a-c] Means within a row lacking a common superscript letter are different ($P < 0.05$).

[1] Reference intervals are specific to Iowa State University College of Veterinary Medicine Clinical Pathology Laboratory.

[2] WBCs = white blood cells; RBCs = red blood cells; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; RDW = red blood cell distribution width; MPV = mean platelet volume.

Table 3.8: Serum metabolite and electrolyte concentration of dogs fed high fat diets

Item[2]	Canola Oil				SEM	P-Value				Reference Interval[1]
	0%	2%	4%	6%		Treatment	Linear	Quadratic	Cubic	
cPLI, µg/L	34.63	44.13	42.88	39.50	4.17	0.110	0.298	0.033	0.498	≤200
BUN, mg/dl	14.75[a]	14.38[a,b]	13.38[b,c]	13.00[c]	0.90	0.035	0.005	1.000	0.529	10.00-30.00
Creatinine, mg/dl	0.71	0.75	0.69	0.76	0.04	0.271	0.508	0.525	0.083	0.50-1.50
Glucose, mg/dl	70.63	76.13	77.38	74.00	2.29	0.179	0.260	0.058	0.970	68.00-115.00
Total Protein, gm/dl	5.96	5.80	5.89	5.80	0.10	0.436	0.280	0.646	0.252	5.20-7.10
Albumin, gm/dl	3.30	3.18	3.24	3.25	0.08	0.546	0.748	0.268	0.388	2.70-4.00
Alkaline Phosphatase, IU/L	30.38	33.75	36.50	33.63	3.64	0.570	0.366	0.314	0.715	20.00-150.00
ALT, IU/L	69.88	51.75	70.25	59.75	15.63	0.420	0.769	0.674	0.117	24.00-90.00
Total Bilirubin, mg/dl	0.20	0.14	0.17	0.13	0.04	0.360	0.173	0.782	0.266	0.10-0.60
Cholesterol, mg/dl	186.38	183.75	183.25	183.75	7.89	0.982	0.756	0.795	0.967	132.00-300.00
Triglycerides, mg/dl	29.38	30.13	27.50	28.75	1.90	0.772	0.586	0.892	0.383	24.00-115.00
Sodium, mEq/L	144.73	144.37	143.87	143.87	0.76	0.616	0.214	0.738	0.783	141.00-151.00
Potassium, mEq/L	4.83	4.78	4.90	4.90	0.08	0.423	0.222	0.691	0.292	3.90-5.3
Chloride, mEq/L	114.38	114.75	115.00	113.63	0.66	0.208	0.347	0.075	0.478	112.00-121.00
Bicarbonate, mEq/L	23.00	22.13	21.88	23.13	0.61	0.268	0.958	0.057	0.713	19.00-25.00
Calcium, mg/dl	10.39	10.28	10.40	10.31	0.10	0.468	0.730	0.847	0.132	9.70-11.30
Phosphorus, mg/dl	3.98	4.05	4.35	4.14	0.20	0.123	0.124	0.205	0.149	3.20-6.00
Magnesium, mg/dl	1.96	1.93	1.94	2.02	0.04	0.301	0.247	0.135	0.827	1.70-2.50

[a-c] Means within a row lacking a common superscript letter are different ($P < 0.05$).

[1] Reference intervals are specific to Iowa State University College of Veterinary Medicine Clinical Pathology Laboratory.

[2] cPLI = canine pancreatic lipase immunoreactivity; BUN = blood urea nitrogen; ALT = alanine aminotransferase.

CHAPTER 4. HIGH FAT DIETS LED TO MICROBIAL SHIFTS IN FECAL SAMPLES OF HEALTHY ADULT DOGS

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Abstract:

Recently, there has been increased interest in the gastrointestinal (GI) microbiota of companion animals. It is important to understand the effect of dietary intervention due to the microbiota's role in host health. Most work regarding the microbiota has investigated murine models with a lack of evidence in canine models. The variation among the GI tract and typical diet compositions of these species may lead to vastly different results. Therefore, it is important to study the effects using a canine model. In addition, there has been little research investigating the effect of dietary fat on the microbiota. Due to the large inclusion rate of dietary fat in pet food, it is critical to understand its effects. Therefore, the study objective was to report the effects of high fat, low carbohydrate diets on the fecal microbiota in healthy adult dogs. Eight adult Beagles were enrolled in a replicated 4x4 Latin Square design feeding trial. Dogs were randomly assigned to 1 of 4 dietary fat level treatments (T) within each period: 32% (T1), 37% (T2), 42% (T3), and 47% (T4). Fat levels were adjusted with inclusion of canola oil added to a commercial diet. DNA was extracted from fecal samples using the Qiagen DNeasy Powerlyzer Powesoil kit and sequenced using the Illumina MiSeq platform. Sequence analysis was performed with the use of Mothur and individual OTUs were analyzed using the GLIMMIX procedure of SAS with fixed effects of diet and room, and the random effects of period and animal. When comparing

entire bacterial communities of treatment groups using PERMANOVA, no significant differences were observed among treatments (P = 0.681). However, when comparing the 100 most abundant individual OTUs, 36 showed significant differences in abundances between treatment groups. Overall, OTUs assigned to genera related to fat digestion increased while OTUs assigned to genera involved in carbohydrate digestion decreased. In conclusion, the microbial community adapted to dietary intervention without jeopardizing the health of the animals.

Introduction

The increased interest in the gastrointestinal (GI) microbiota in humans has extended to companion animals. A symbiotic relationship between the gastrointestinal microbes and host is critical for host health (Mackie et al., 1999; Hooper et al., 2001). The gastrointestinal tract microbiota comprises at least hundreds, perhaps thousands of interdependent and/or competing species (Eckburg et al., 2005; Ley et al., 2008; Spor et al., 2011), that are not fully characterized (Hand et al., 2013). The microbiota benefits the host in many ways, it can enhance metabolic capabilities, protect against pathogens, develop the immune system, and modulate gastrointestinal development (Backhed et al., 2005). In addition, microbes contain enzymes that digest fiber and carbohydrates that cannot be digested by the host to produce short chain fatty acids (SCFA) (Sunvold et al., 1995), which are used as an additional energy source for the host. SCFA account for ~10% of human caloric requirement and ~80% of maintenance energy for ruminants (Bergman, 1990). Unlike other species, dogs do not rely heavily on microbial fermentation to meet daily energy requirements, even when fed high fiber diets (Swanson et al., 2011; Hooda et al., 2012; Deng and Swanson, 2015). However, a balanced microbiota is critical to maintain a healthy gastrointestinal tract. A disruption, or dysbiosis, of the microbiota has been

associated with disease in both humans and dogs including chronic diarrhea (Bell et al., 2008; Jia et al., 2010) and inflammatory bowel disease (IBD) (Nobaek et al., 2000; Janeczko et al., 2008; Xenoulis et al., 2008; Suchodolski et al., 2010) With disease, there is usually specific shifts in microbial population or a decrease in overall diversity, making disturbances a possible early warning sign (Deng and Swanson, 2015).

To date, most of the research investigating the dog microbiota has analyzed fecal samples from healthy beagle dogs in controlled lab settings (Vanhoutte et al., 2005; Middelbos et al., 2010; Handl et al., 2011; Hang et al., 2012; Beloshapka et al., 2013; Deng and Swanson, 2015; Panasevich et al., 2015; Herstad et al., 2017). These studies have shown that bacteria dominant the canine gut microbiota accounting for approximately 99% of total sequences with archaea accounting for the remaining 1% (Middelbos et al., 2010; Handl et al., 2011; Swanson et al., 2011; Garcia-Mazcorro et al., 2011; Hand et al., 2013). The predominant phyla found in healthy dogs are *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria* and *Actinobacteria* (Suchodolski et al., 2008; Middelbos et al., 2010; Swanson et al., 2011; Hooda et al., 2012; Deng and Swanson, 2015; Herstad et al., 2017; Li et al., 2017; Schauf et al., 2018). In addition, bacterial groups typically indicated as pathogens in most species such as *Clostridium difficile*, C*lostridium perfringens*, *Enterocccus spp.*, *E. coli*, and *Helicobacter* are considered part of a dog's healthy microbiota (Jia et al., 2010; Handl et al., 2011; Goldstein et al., 2012). The fecal microbiome of the dog reflects the high concentrations of protein and fat in their diets (Moon et al., 2018).

Compared to other nutrients, dietary fat and its effect on the microbiota have been underestimated due to the argument that little dietary fat reaches the colon where the highest density of bacteria reside. However, Gabert et. al. (2011) showed that free fatty acids were being

excreted in healthy people. Free fatty acids are known to have potent antimicrobial effects even at small doses (Huang et al., 2011; Candido et al., 2018). Therefore, the small amounts of fat reaching the colon could interact with the resident microbiota. In addition, a higher fat content will require an increased amount of bile acids for digestion, which are also known to have an antimicrobial effect (Stacey and Webb, 1947). Specific bacteria are even known to be involved with the digestion and absorption of dietary fat. *Lactobacillus*, *Bifidobacterium*, *Enterobacter*, *Bacteroides*, and *Clostridium* are involved in bile acid metabolism and affect the absorption of dietary fats and lipid-soluble vitamins (Ridlon et al., 2006, Swann et al., 2011). *Faecalibacterium prausnitzii* and *Bifidobacterium* are associated with choline metabolism to modulate lipid metabolism and glucose homeostasis (Martin et al., 2010; Wang et al., 2011).

In recent studies, high fat diets are typically associated with a decrease in overall microbial abundance and diversity with a shift from *Bacteroidetes* to *Firmicutes* (Hildebrandt et al., 2009; Zhang et al., 2012; Murphy et al., 2015). This shift may lead to increased gut permeability, inflammation, and disease (Murphy et al., 2015). Studies have predominantly focused on murine models with a lack of evidence in canine or other large animal models. Due to the large inclusion rate of dietary fat in pet foods and the demand for less processed diets (decreased carbohydrates), it is important to understand the role of dietary fat on the microbiota. The study objective was to evaluate the effects of feeding adult dogs increasing levels of fat in low carbohydrate diets on the fecal microbiome. The hypothesis of this study was that microbial shifts would occur based on dietary intervention, but dogs would maintain overall health.

Materials and Methods

The protocol for this experiment was reviewed and approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC).

Animals and Housing

Eight spayed female Beagles, 1 year of age with an average body weight of 8.57 ± 0.93kg were enrolled in this study. All dogs were housed in pairs at the College of Veterinary Medicine at Iowa State University in temperature-controlled rooms (20°C) on a 12:12 hour light: dark schedule. During feeding and collection periods, dogs were separated by gate closure. Before beginning of trial, complete blood count (CBC) and chemistry panels were performed on all dogs to determine any underlying health concerns that may confound data. Fecal samples were also collected prior to the study to ensure all dogs were parasite free.

Experimental Design and Sample Collection

Dogs were randomly assigned to one of four dietary treatments in a replicated 4x4 Latin Square design consisting of 15-d periods. This design allowed each dog to receive each diet for one period during each replicate. Each period included a 10-d diet adaption phase followed by a 5-d total collection phase.

During the collection phase, two grams of fresh feces (defecated within 15 minutes) were placed into a cryovial tube and immediately stored in -80°C for microbe analysis for each dog per treatment.

Diets and Feeding

Dietary compositions are presented in **Table 4.1**. One commercially manufactured canned canine diet (**Table 4.2)** was used as a control. Increasing inclusion levels of fat (2%, 4%, or 6% canola oil) were added to the control diet to create three more treatments. Treatment diets contained 32% (T1), 37% (T2), 42% (T3), and 47% (T4) total dietary fat. Total dietary fiber and starch levels remained similar among treatments.

Dogs were fed twice daily (0800 and 1700) to meet their daily energy requirements. Total daily energy requirements were calculated per treatment for each individual dog based on body weight at the beginning of each period. Weight and body condition score (BCS) were recorded weekly. If needed, feed intake was adjusted during the adaption phase to maintain ideal BCS. Water was provided *ad libitum* throughout the study.

Chemical Analyses of Diets

Dietary treatments were analyzed for macronutrient composition and energy. A sub-sample (100g) of each diet was pooled and homogenized. All chemical analyses were conducted in the Comparative Nutrition Laboratory at Iowa State University (Ames, IA). Dietary sub-samples were dried at 65°C in a forced air-drying oven and ground with a coffee grinder to accommodate small sample size (model BCG11OB; KitchenAid). Diet samples were analyzed for dry matter (DM) (AOAC 934.01) and organic matter (OM) (AOAC 942.05). Crude protein (CP) was determined using a LECO Nitrogen Analyzer (AOAC 992.15; model TruMacN; LECO Corporation; St. Joseph, MI). An EDTA sample of 9.56% nitrogen was used as the standard for calibration. Crude fat was determined via acid hydrolysis and hexane extraction (AOAC 960.39). Gross energy (GE) was determined via bomb calorimetry (model 6200; Parr Instrument Co.; Moline, IL) with benzoic acid (6,318 kcal GE/kg; Parr Instrument Co.) used as the standard for calibration. Total dietary fiber (TDF) and starch content were determined with the use of assay kits (Megazyme International, Wicklow, Ireland).

Fecal DNA Extraction

Fecal samples were thawed, and DNA was extracted from approximately 0.25g of feces using the Qiagen DNeasy Powerlyzer Powersoil kit following manufacturer's instructions.

Mechanical cell lysis was performed using a Fischer Scientific Beadmill 24. DNA concentrations were verified using a spectrophotometer (ND-100; NanoDrop Technologies, Inc., Rockland, DE)

After extraction, DNA was sent to the ISU DNA facility for sequencing using the Illumina MiSeq platform. Briefly, the genomic DNA from each sample was amplified using Platinum™ Taq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA) with one replicate per sample using universal 16S rRNA gene bacterial primers [515F (5′ GTGYCAGCMGCCGCGGTAA-3′; (Parada et al., 2016)) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′; (Apprill et al., 2015))] amplifying the variable region V4. All samples underwent PCR (QIAquick 96 PCR Purification Kit; Qiagen Sciences Inc, Germantown, MD) with an initial denaturation step at 94°C for 3 min, followed by 45 s of denaturing at 94°C, 20 s of annealing at 50°C, and 90 s of extension at 72°C. This was repeated for 35 total PCR cycles and finished with a 10 min extension at 72°C. PCR bar-coded amplicons were mixed at equal molar ratios and used for Illumina MiSeq paired-end sequencing with 150 bp read length and cluster generation with 10% PhiX control DNA on an Illumina MiSeq platform (Illumina Inc., San Diego, CA).

Sequence Analysis

Sequence analysis was done with Mothur V1.40.5 following the Mothur MiSeq SOP. Barcode sequences, primer and low-quality sequences were trimmed using a minimum average quality score of 35, with a sliding window size of 50 bp. Chimeric sequences were removed with the “Chimera.uchime” command. For alignment and taxonomic classification of operational taxonomic units (OTU), the SILVA SSU NR reference database v132 provided by the Mothur website was used. The sequences were clustered into OTUs with a cutoff of 99% 16S DNA gene similarity (=0.01 distance).

Entire microbial communities of each sample were assigned Bray-Curtis dissimilarity coefficients to perform statistical comparisons between treatment groups. After dissimilarity coefficients were assigned to each sample, treatment groups were compared using the Analysis Of SIMilarity (ANOSIM) package provided by Mothur.

Microbial communities were visualized by plotting (ggplot2 v2_3.1.1 graphing package in R 3.6.0 (Wickham, 2009; Team, 2019)) principle coordinate analysis (PCoA) generated with the Phyloseq (v1.28.0, Mcmurdie and Holmes, 2013) and Vegan (v2.5-5) packages using the shared and taxonomy file generated in MOTHUR. Bray-Curtis Dissimilarity measures were used to generate distances between samples for the PCoA plot.

Individual OTUs were analyzed using the GLIMMIX procedure of SAS (Version 9.4, SAS Inst., Cary, NC) with fixed effects of diet and room, and the random effects of period and animal. A negative binomial was used to determine the distribution and an offset of log library size was used. P-values were then transformed to Q-values to result in fewer false positives. Q-values were used to determine significance ($Q < 0.05$).

Body Weight and Body Condition Score Analysis

Body weight and BCS were analyzed using the MIXED procedure of SAS (Version 9.4, SAS Inst., Cary, NC) with fixed effects of diet and room, and the random effects of period and animal. Initial body weight or initial body condition score were used as a covariate for their respective analysis. Differences between diets were determined using least squared means. A probability of $P < 0.05$ was considered statistically significant and standard error of the means (SEM) were determined.

Results and Discussion

Body Weight and Body Condition Score

Body weight and BCS are presented in **Table 4.3**. Mean body weight (P = 0.199) and body condition score (P = 0.907) of dogs were maintained throughout treatments. It is important that dogs maintained ideal body weight and BCS due to known changes in the microbiota with obesity which may confound diet effects (de La Serre et al., 2010; Candido et al., 2018). In addition, high fat diets and obesity have been shown to have similar effects on the microbiota making it difficult to determine which caused the microbiota to change if modeled together (Ley et al., 2005; Turnbaugh et al., 2006; Turnbaugh et., 2008; Candido et al., 2018). The maintenance of ideal BCS allowed this study to measure high fat diets independently of obesity.

Fecal Microbial Communities

Overall, 2,438 OTUs were generated after quality control and removal of OTUs representing less than 10 sequences. The average number of sequences per samples was 59,783 with a standard deviation of 25,370. 99.93% of the reads were bacterial while only 0.07% were archaeal. From the 2,438 OTUs, 19 phyla were identified with *Firmicutes* (40%), *Bacteroidetes* (34%), *Fusobacteria* (17%), *Proteobacteria* (7%), and *Actinobacteria* (1%) being the most abundant. Previous studies have shown a range of abundance for the dominant phyla in healthy dogs with 14-48% *Firmicutes*, 12-38% *Bacteroidetes*, 7-44% *Fusobacteria*, 5-23% *Proteobacteria*, and 0.8-1.4% *Actinobacteria* (Suchodolski et al., 2008; Middelbos et al., 2010; Swanson et al., 2011; Herstad et al., 2017; Li et al., 2017). The total abundance of each phyla found in this study fall within those ranges indicating normal values. The most abundant phyla per treatment are presented in *Fig. 3.1*. The families *Prevotellaceae, Peptostreptococcaceae, Fusobacteriaceae,* and *Bacteroidaceae* accounted for 23%, 17%, 17%, and 10% of total reads,

respectively. Additionally, the *Fusobacterium* genus accounted for 18% of all reads. OTU 1 was classified into the *Peptoclostridium* genus which accounted for 14% of total reads. Several OTUs were classified within the genera *Bacteroides* and *Alloprevotella*, with each accounting for 10% of all reads. In addition, the genus *Allobaculum* accounted for 3% of the total reads. The assigned classifications of the 50 most abundant OTUs are presented in **Table 4.4**.

When comparing entire bacterial communities of treatment groups using PERMANOVA, no significant differences were observed ($P = 0.681$). This result was supported with the lack of apparent clustering of treatment types seen in the PCoA (*Fig. 3.2*). In agreement with the PERMANOVA and PCoA in this study, Schauf et al. (2018) also found no difference in bacterial richness or diversity resulting from a high-fat, low-starch diet in dogs. This is contradictory to other studies showing a decrease in microbial abundance in mice fed high fat diets (Hildebrandt et al., 2009; Zhang et al., 2012). In addition, a study comparing a high minced beef diet (HMB), high in fat and protein, to a control diet in dogs found that species richness was decreased in the HMB diet but observed species were not different (Herstad et al., 2017). The decrease in species richness may be caused by the antimicrobial effect of fatty acids and/or bile acids (Stacey and Webb, 1947; Huang et al., 2011; Candido et al., 2018). The varying results may be due to the differences in physiology and typical diet composition of the species used in the studies.

However, when comparing the top 100 individual OTUs, 36 showed significant differences in abundances between treatment groups (**Table 4.5**) with 15 of the significant OTUs belonging to the phylum *Firmicutes*. There was a significant increase in the genera *Allobaculum* ($Q < 0.001$), *Paenicostridium* ($Q = 0.010$), and *Lactobacillus* ($Q < 0.001$) and a significant decrease in *Catenibacterium* ($Q = 0.037$), *Romboutsia* ($Q = 0.024$), *Blautia* ($Q = 0.024$), *Allobaculum* ($Q < 0.001$), and *Lachnoclostridium* ($Q = 0.037$) of the *Firmicutes* phylum from T1

to T4. The *Alloprevotella* ($Q < 0.001$) and *Bacteroides* ($Q = 0.018$) genera in the *Bacteroidetes* phylum significantly decreased from T1 to T4. A visual representation of shifts in significant OTU abundance per treatment among the top 50 OTUs is presented in *Fig. 3.3*.

The observed shifts in OTUs represent how the microbiome can adapt to dietary intervention. Overall, the increased OTUs from T1 to T4 were assigned to genera related to fat digestion while the OTUs that decreased in abundance were assigned to genera with roles in carbohydrate digestion. For example, *Parasutterella* (OTU 31) is known to have a role in bile acid maintenance and cholesterol metabolism (Ju et al., 2019). The increase in dietary fat may explain the increase in *Parasutterella. Blautia* (OTU 44) can utilize many types of carbohydrates to produce acetic, lactic acids, and ethanol (Liu et al., 2008). This genus was decreased with the increase in dietary fat which may be due to the decreased carbohydrates. The increase in fat in T4 perhaps led to less glucose in the diet which could explain the decrease in the genus *Catenibacterium* (OTU 9), which utilizes glucose to produce acetic, lactic, butyric, and iso-butyric acids (Kageyama and Benno, 2000). Yan et al. (2013) suggested an increase in *Catenibacterium* led to increased VFA production. In this study, the decreased *Catenibacterium* and *Blautia* did not affect short chain fatty acid production among treatments. *Allobaculum* primarily increased with the increase in dietary fat in three significant OTUs (OTU 12, 32 and 60) which has been suggested to have beneficial effects and contribute to mucus formation (Everard 2014). However, OTU 58 showed a decrease in *Allobaculum*. The presence of *Allobaculum* and its effect on the host differ among studies. Martinez et al. (2009) reported an increase in *Allobaculum* in mice fed grain sorghum lipid extract. In contrast, Everard et al. (2014) and Ravussin et al. (2012) found an increase in *Allobaculum* in lower fat diets compared to high fat diets. Jakobsson et al. (2015) questioned the beneficial role of *Allobaculum* by

showing that mice had increased mucus penetrability with increased abundance. The increase in dietary fat with low carbohydrate levels shifted the microbiota based on what functional roles were needed for nutrient digestion.

Conclusion

In conclusion, the canine microbiota can adapt to dietary intervention which was shown with the increased and decreased genera involved in fat and carbohydrate digestion. In addition, this study showed that dogs remained healthy even with the shift in microbiota resulting from high fat, low carbohydrate diets. Therefore, compared to other species, dogs may be able to utilize a diet higher in fat content. Further research is needed to investigate long term effects of high fat diet consumption on the microbiota and host health.

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Table 4.2: Ingredient composition of control diet

Diet	Ingredients
Control	Chicken, chicken broth, chicken liver, carrots, peas, dried egg product, guar gum, carrageenan, ground flaxseed, potassium chloride, salt, cassia gum, minerals (zinc amino acid chelate, iron amino acid chelate, copper amino acid chelate, manganese amino acid chelate, sodium selenite, potassium iodine), vitamins (vitamin E supplement, thiamine mononitrate, niacin supplement, d-calcium pantothenate, vitamin A supplement, riboflavin supplement, biotin, vitamin B12 supplement, pyridoxine hydrochloride, vitamin D3 supplement, folic acid), choline chloride

Table 4.3: Body weight and body condition score of dogs

	Diet					
Item	T1	T2	T3	T4	SEM	P-value
Body Weight, kg	7.66	7.50	7.53	7.55	0.18	0.199
Body Condition Score	3.63	3.56	3.56	3.50	0.24	0.907

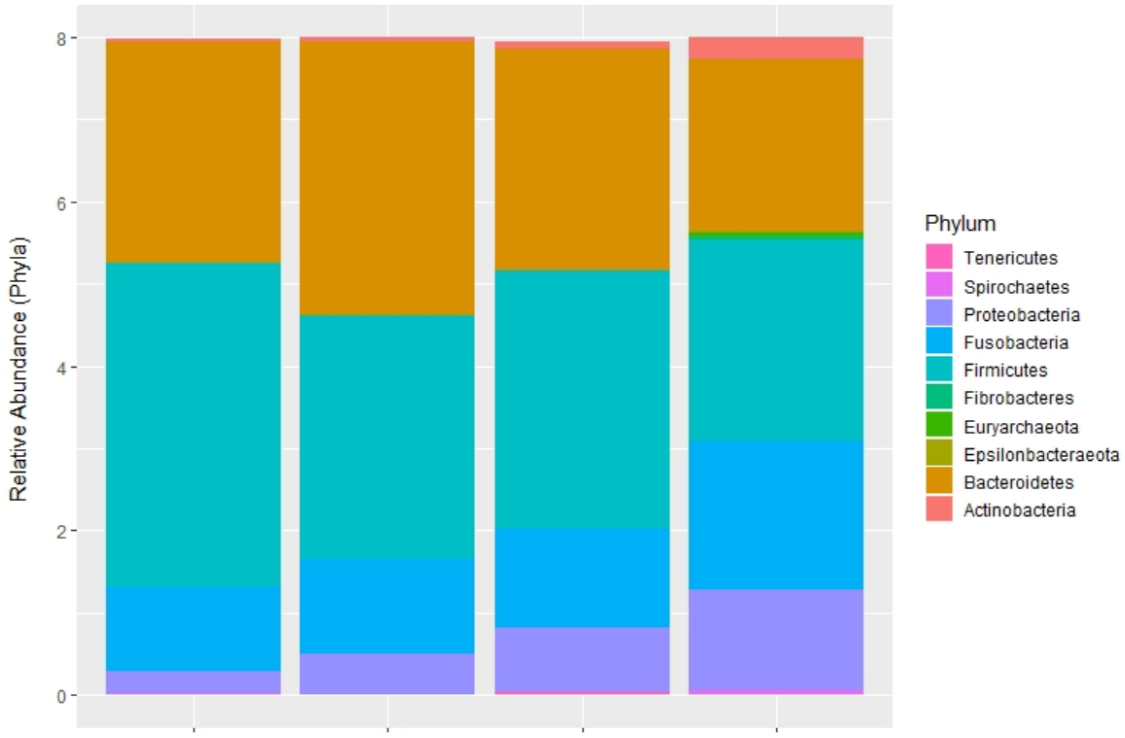

Fig. 4.1. Figure 4.1 presents the relative abundance of phyla per treatment.

Table 4.4: Assigned classifications of the 50 most abundant OTUs among fecal samples

OTU	Phylum	Class	Order	Family	Genus
1	*Firmicutes*	*Clostridia*	*Clostridiales*	*Peptostreptococcaceae*	*Peptoclostridium*
2	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotellaceae_Ga6A1_group*
3	*Fusobacteria*	*Fusobacteriia*	*Fusobacteriales*	*Fusobacteriaceae*	*Fusobacterium*
4	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Alloprevotella*
5	*Fusobacteria*	*Fusobacteriia*	*Fusobacteriales*	*Fusobacteriaceae*	*Fusobacterium*
6	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*
7	*Fusobacteria*	*Fusobacteriia*	*Fusobacteriales*	*Fusobacteriaceae*	*Fusobacterium*
8	*Firmicutes*	*Clostridia*	*Clostridiales*	*Clostridiaceae_1*	*Clostridium_sensu_stricto_1*
9	*Firmicutes*	*Erysipelotrichia*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Catenibacterium*
10	*Fusobacteria*	*Fusobacteriia*	*Fusobacteriales*	*Fusobacteriaceae*	*Fusobacterium*
11	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella_9*
12	*Firmicutes*	*Erysipelotrichia*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Allobaculum*
13	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella_9*
14	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Lachnospiraceae_ge*
15	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingomonadaceae_unclassified*
16	*Firmicutes*	*Clostridia*	*Clostridiales*	*Peptostreptococcaceae*	*Romboutsia*
17	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Alloprevotella*
18	*Firmicutes*	*Clostridia*	*Clostridiales*	*Clostridiaceae_1*	*Clostridium_sensu_stricto_1*
19	*Proteobacteria*	*Gammaproteobacteria*	*Betaproteobacteriales*	*Burkholderiaceae*	*Sutterella*
20	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Escherichia-Shigella*
21	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Alloprevotella*
22	*Firmicutes*	*Negativicutes*	*Selenomonadales*	*Veillonellaceae*	*Megamonas*
23	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*
24	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*
25	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*
26	*Firmicutes*	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*
27	*Firmicutes*	*Negativicutes*	*Selenomonadales*	*Acidaminococcaceae*	*Phascolarctobacterium*
28	*Firmicutes*	*Clostridia*	*Clostridiales*	*Family_XIII*	*Family_XIII_ge*
29	*Bacteroidetes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Lachnospiraceae_unclassified*
30	*Proteobacteria*	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*
31	*Firmicutes*	*Gammaproteobacteria*	*Betaproteobacteriales*	*Burkholderiaceae*	*Parasutterella*

Table 4.4 (Continued)

OTU	Phylum	Class	Order	Family	Genus
32	*Firmicutes*	*Erysipelotrichia*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Allobaculum*
33	*Firmicutes*	*Clostridia*	*Clostridiales*	*Peptococcaceae*	*Peptococcus*
34	*Firmicutes*	*Negativicutes*	*Selenomonadales*	*Veillonellaceae*	*Megasphaera*
35	*Fusobacteria*	*Clostridia*	*Clostridiales*	*Peptostreptococcaceae*	*Paeniclostridium*
36	*Bacteroidetes*	*Fusobacteriia*	*Fusobacteriales*	*Leptotrichiaceae*	*Leptotrichiaceae_unclassified*
37	*Firmicutes*	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*
38	*Firmicutes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Blautia*
39	*Bacteroidetes*	*Clostridia*	*Clostridiales*	*Peptostreptococcaceae*	*Peptostreptococcaceae_unclassified*
40	*Proteobacteria*	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*
41	*Proteobacteria*	*Gammaproteobacteria*	*Aeromonadales*	*Succinivibrionaceae*	*Anaerobiospirillum*
42	*Firmicutes*	*Gammaproteobacteria*	*Betaproteobacteriales*	*Burkholderiaceae*	*Burkholderiaceae_unclassified*
43	*Firmicutes*	*Clostridia*	*Clostridiales*	*Peptostreptococcaceae*	*Romboutsia*
44	*Bacteroidetes*	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Blautia*
45	*Firmicutes*	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*
46	*Firmicutes*	*Erysipelotrichia*	*Erysipelotrichales*	*Erysipelotrichaceae*	*uncultured*
47	*Proteobacteria*	*Clostridia*	*Clostridiales*	*Clostridiaceae_1*	*Clostridium_sensu_stricto_1*
48	*Actinobacteria*	*Gammaproteobacteria*	*Pasteurellales*	*Pasteurellaceae*	*Histophilus*
49	*Firmicutes*	*Actinobacteria*	*Actinomycetales*	*Actinomycetaceae*	*Trueperella*
50	*Bacteroidetes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Faecalibacterium*

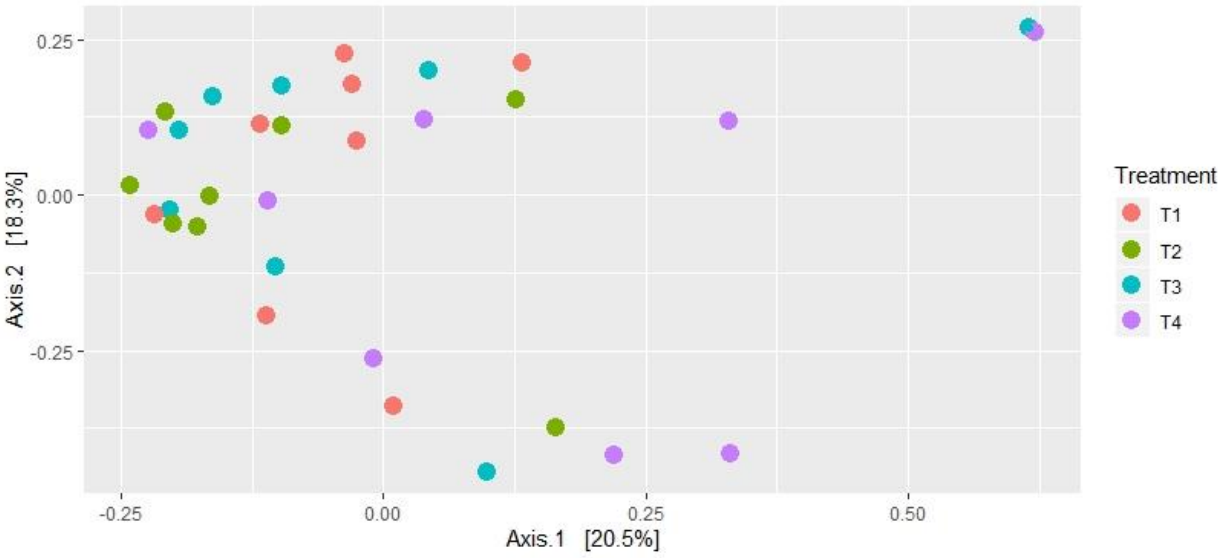

Fig. 4.2. Figure 4.2 presents a PCoA based on Bray-Curtis dissimilarities of the overall abundance of microbial communities among treatment groups. The two components that explain the most variation between samples accounted for 38.8% of variation. Each point corresponds to a community from a single dog. Colors represent each treatment.

Table 4.5: Significant OTUs out of the 100 most abundant OTUs among fecal samples

		%					
OTU	Genus	T1	T2	T3	T4	SEM	Q-Value
2	*Prevotellaceae_Ga6A1_group*	7.06	8.29	9.06	4.00	2.23	0.0195
3	*Fusobacterium*	12.20	6.46	8.00	0.40	4.08	<.0001
7	*Fusobacterium*	1.59	2.92	2.72	1.46	0.91	0.0235
8	*Clostridium_sensu_stricto_1*	1.56	2.75	2.06	1.02	0.77	0.0184
9	*Catenibacterium*	7.15	0.78	0.22	0.38	1.72	0.0373
12	*Allobaculum*	0.65	1.12	0.69	2.75	1.00	<.0001
15	*Sphingomonadaceae_unclassified*	0.42	0.11	4.40	5.57	2.40	0.0004
17	*Alloprevotella*	1.69	1.84	0.94	0.50	0.72	0.0036
18	*Clostridium_sensu_stricto_1*	0.87	1.63	1.47	0.80	0.50	0.0122
21	*Alloprevotella*	0.85	1.64	1.40	0.67	0.79	<.0001
29	*Lachnospiraceae_unclassified*	0.87	0.68	0.83	0.20	0.25	0.0047
31	*Parasutterella*	0.13	0.52	0.45	0.92	0.40	<.0001
32	*Allobaculum*	0.33	0.09	0.36	0.88	0.32	<.0001
34	*Megasphaera*	1.10	0.28	0.01	0.73	0.50	0.0005
35	*Paeniclostridium*	0.03	1.00	0.77	0.47	0.30	0.0095
36	*Leptotrichiaceae_unclassified*	0.00	0.01	0.01	3.11	0.78	<.0001
37	*Bacteroides*	0.48	0.88	0.10	0.06	0.29	0.0184
41	*Anaerobiospirillum*	0.37	0.68	0.08	0.13	0.12	0.0128
42	*Burkholderiaceae_unclassified*	0.38	0.49	0.33	0.22	0.18	0.0282
43	*Romboutsia*	0.53	0.29	0.67	0.14	0.15	0.0242
44	*Blautia*	0.67	0.30	0.27	0.10	0.11	0.0241
45	*Bacteroides*	0.21	0.66	0.79	0.07	0.30	0.0002
46	*uncultured*	0.31	0.13	0.12	0.59	0.20	0.0028
48	*Histophilus*	0.01	0.01	0.00	2.07	0.52	0.0014
57	*Erysipelotrichaceae_unclassified*	0.10	0.00	0.18	0.49	0.14	<.0001
58	*Allobaculum*	0.34	0.21	0.23	0.06	0.09	0.0007
60	*Allobaculum*	0.06	0.00	0.08	0.46	0.10	<.0001
62	*Succinivibrionaceae_UCG-001*	0.01	0.01	0.87	0.49	0.27	0.0002
66	*Lachnoclostridium*	0.30	0.15	0.14	0.10	0.09	0.0369
69	*Prevotella_9*	0.14	0.12	0.00	0.66	0.17	0.0377
72	*Leptotrichiaceae_unclassified*	0.00	0.00	0.00	0.94	0.23	<.0001
86	*Lachnospiraceae_ge*	0.13	0.27	0.03	0.02	0.09	0.0019
90	*Bifidobacterium*	0.05	0.01	0.07	0.14	0.05	<.0001
96	*Alloprevotella*	0.10	0.09	0.05	0.02	0.03	0.0431
97	*Family_XIII_unclassified*	0.07	0.07	0.08	0.03	0.05	0.005
99	*Lactobacillus*	0.00	0.00	0.16	0.34	0.12	0.0002

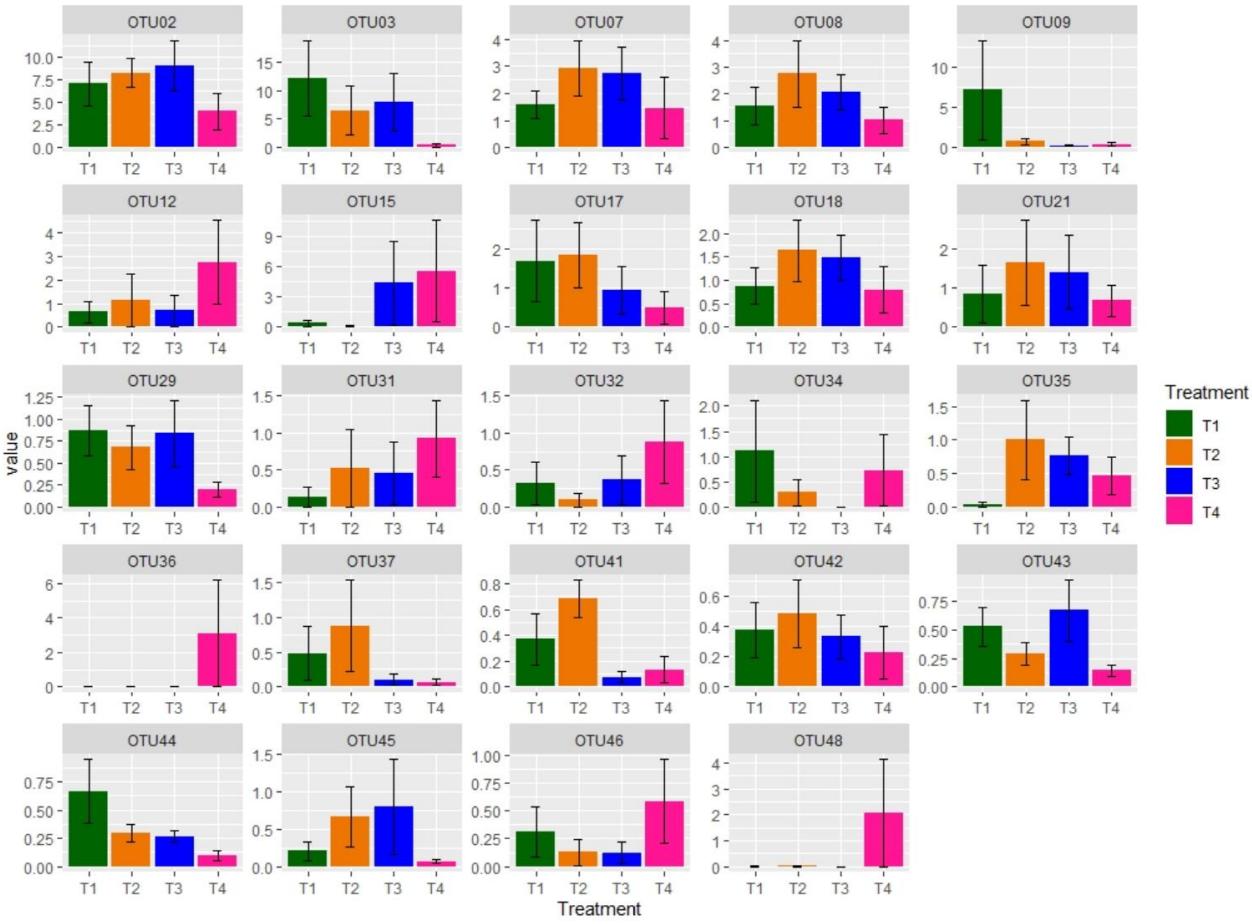

Fig. 4.3. Figure 4.3 presents a visual representation of the shifts in significant OTUs per treatment among the 50 most abundant OTUs of fecal samples.

CHAPTER 5. UTILIZATION OF CRICKETS AS A PROTEIN SOURCE IN DIETS FED TO HEALTHY ADULT DOGS: EFFECTS ON GENERAL HEALTH AND APPARENT TOTAL TRACT NUTRIENT DIGESTIBILITY

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Abstract:

Insects can serve as a sustainable high-quality protein source for pet foods. However, there is an absence of research investigating the use of insects in pet food. The study objective was to evaluate the apparent digestibility and possible health effects of diets containing graded levels of cricket powder fed to healthy adult dogs. Thirty-two adult Beagles were randomly assigned to 1 of 4 dietary treatments: 0%, 8%, 16%, or 24% cricket powder. Dogs were fed their respective diet for a total of 29 days with a 6-d collection phase. Fecal samples were collected to measure total fecal output as well as apparent digestibility for dry matter, organic matter, crude protein, fat, total dietary fiber, and gross energy. Blood samples were taken prior to the study and on d 29 for hematology and chemistry profiles. Total fecal output increased on both an as-is (P = 0.030) and dry matter basis (P = 0.024). The apparent digestibility of each nutrient on a dry matter basis decreased (P < 0.001) with the increasing level of cricket powder inclusion. All blood values remained within desired reference intervals indicating healthy dogs. Slight fluctuations in blood urea nitrogen (P = 0.037) and hemoglobin (P = 0.044) levels were observed but were not considered of biological significance. Even with the decrease in digestibility with the inclusion of cricket powder, diets remained highly digestible at greater than 80% total

apparent digestibility. In conclusion, crickets were demonstrated to be an acceptable source of protein for dogs.

Introduction

The pet industry is continuing to grow in the United States, with dog ownership estimated to be 36.5%, equivalent to 43.3 million households and 69.9 million dogs (AVMA, 2012; APPA, 2019). Considering the size of the industry, the choice of ingredients used in the production of commercial dog food can potentially have a significant environmental impact (Swanson et al., 2013; Okin, 2017). Arguably, the most important ingredient in commercial dog food from a sustainability perspective is the protein source. The American Association of Feed Control Officials (AAFCO) recommends a crude protein (CP) content of 18% dry matter (DM) for adult dogs but analyses of commercial canned and dry pet foods by Hill et al. (2009) indicated that diets on average contained significantly higher amounts. In addition, Swanson et al. (2013) estimated typical CP values in pet foods to be around 40% DM.

While by-products of meat production for human consumption, such as bone meal are often utilized as protein sources in commercial dog food, there is direct competition with both the livestock and human food industries for other common ingredients (Swanson et al., 2013; Meeker and Meisinger, 2015; Okin, 2017). The use of insects as an alternative protein source may be a solution. In addition, insects can provide a sustainable protein source for pet food (Van Huis et al., 2013; Bosch et al., 2014 and 2016; McCusker et al., 2014; Lei et al., 2019), requiring less resources and emitting fewer greenhouse gas emissions compared to livestock raised for food production (Oonincx and Boer, 2012).

Furthermore, nutritional analyses of various edible insects indicate that they are high in protein and have the potential to be excellent sources of amino acids, fatty acids, vitamins, and

minerals (Rumpold & Schlüter, 2013; Bosch et al., 2014; Finke, 2015). Studies in livestock support their suitability to partially or completely replace conventional protein sources such as fishmeal and soybean meal (Makkar et al., 2014). An *in vitro* study indicated the potential of black soldier fly larvae (*Hermetia illucens*), housefly (*Musca domesticus*) and yellow mealworm (*Tenebrio molitor*) to serve as high quality protein sources (Bosch et al., 2016). Nevertheless, it is important to support this potential in animal models. Additionally, the inclusion of black solder fly larvae (BSFL) in diets positively impacted DM and CP digestibility as well as anti-inflammatory and anti-oxidation capacity in dogs (Lei et al., 2019). However, inclusion levels of BSFL in this study were low at only 1-3% of the diet.

The house cricket (*Acheta domesticus*) and banded cricket (*Gryllodes sigillatus*) are two of the insect species reported to have the greatest potential use for food production (EFSA, 2015). While the composition of insects varies depending on an insect's species, life stage, diet and origin, crickets are reported to typically comprise of 60-70% protein and 13-18% fat on a dry matter basis (Rumpold & Schlüter, 2013; Zielinska et al., 2015). It is important to note that insects, unlike conventional protein sources, contain chitin. Chitin is found in the exoskeleton covering the insect's body for protection and support. As it relates to nutritional composition, chitin, an amino polysaccharide with cellulose-like β 1-4 linkages, creates an indigestible component. Therefore, the inclusion of insects may decrease a diet's overall digestibility compared to other protein sources. In contrast, consumption of chitin may provide beneficial roles such as supporting gut health. Burni et al. (2018) reported the chitin in black soldier fly larvae meal functioned as a prebiotic when fed to rainbow trout. In addition, chitin may have the potential to simulate the immune system in fish, birds, and mammals (Elieh Ali Komi et al., 2018; Lee et al., 2008). Khempaka et al. (2011) and Islam and Yang (2017) reported that the

inclusion of chitin in broiler diets inhibited the growth of foodborne pathogens and increased IgG and IgA levels.

Despite the highly anticipated nutritional value of crickets in feed, the published data in animals is limited to only a few studies in poultry (Nakagaki et al., 1987; Wang et al., 2005), pigs (Miech et al., 2017), and rats (Poelaert et al., 2018; Finke et al., 1989) with no studies reported in dogs. To our knowledge, the potential of crickets to replace conventional protein sources in dog food remains to be assessed. Therefore, the objective of this study was to determine the apparent digestibility and possible health effects of diets containing graded levels of cricket powder fed to healthy adult dogs.

Materials and Methods

The study was conducted at Summit Ridge Farms in Susquehanna, PA and was approved by the Summit Ridge Farms' Institutional Animal Care and Use Committee.

Animals and Housing

Thirty-two Beagles (16 males and 16 females), 4.75 ± 2.5 years old with an initial body weight of 9.69 ± 1.9 kg, were enrolled in this study. All animals were healthy, passing a veterinary physical examination and baseline hematology and clinical chemistry screening prior to the start of the study. Dogs were also of optimal weight and body condition. Dogs were housed in individual runs in a temperature-controlled facility (15-24°C) kept on a 12-hour light/12-hour dark cycle.

Diets and Feeding

A total of four diets were used containing increasing levels of cricket powder: 0% (control), 8%, 16%, or 24% cricket powder (**Table 5.1**). Diets were formulated to meet current AAFCO guidelines for dogs and processed using a X115 single screw extruder and dried using a

Wenger Enhanced Sanitary Dryer. Raw ingredients were purchased from and ground by Fairview Mills (Seneca, KS). A 3/64-inch screen was used for grinding. The cricket powder added to the diets was produced from banded crickets (*Gryllodes sigillatus*) raised on a modified chicken feed. Reared crickets were frozen before being washed, roasted at 93°C for 6 hours, and milled into a fine powder. Nutrient composition of the cricket powder, provided by the supplier, is presented in **Table 5.2**. Dry matter, CP, crude fat, and ash analyses of the cricket powder were performed using AOAC methods 950.46A, 990.03, 922.06, and 923.03, respectively. Fiber was analyzed using the AOCS Ba 6a-05 method and amino acid compositions were analyzed using the AA USDA MSS2 (1993) method.

Dogs were randomly assigned to one of four dietary treatments in a complete randomized design with 8 dogs per treatment (4 males and 4 females). Each treatment was fed for a total of 29 days, using a 23-d adaption phase followed by a 6-d collection phase. Dogs were individually fed their respective diet once a day. Feeding amounts were adjusted weekly to maintain body weight but were not adjusted during the collection period. Water was provided *ad libitum* using an automatic watering system throughout the study.

Sample Collection

Feed intake was recorded for each dog throughout the experiment. Total fecal output was collected daily during the collection phase and averaged to determine daily fecal output (g as-is/d). Feces collected during the 6-d collection period were pooled, homogenized, and stored at 4°C for each dog before nutrient analysis. Additional fecal collections were performed on d 14 and d 28 for microbial analysis (as reported in: Jarrett et al., 2019). Fecal scores were record at least three times a day during the collection phase. Blood samples were taken on d 29 for hematology and chemistry profiles.

Chemical Analyses

Total fecal collections and dietary treatments were analyzed for DM, organic matter (OM), CP, crude fat, total dietary fiber (TDF), and gross energy (GE). All chemical analyses were conducted in the Comparative Nutrition Laboratory at Iowa State University (Ames, IA). Fecal samples and dietary sub-samples were dried at 65°C in a forced air-drying oven and ground in order to pass through a 1.0 mm screen in a Wiley grinder (Model ED-5, Thomas Scientific Inc., Swedesboro, NJ). Diet and fecal samples were analyzed for DM (AOAC 934.01) and OM (AOAC 942.05). Crude protein was determined using a LECO Nitrogen Analyzer (AOAC 992.15; model TruMacN; LECO Corporation; St. Joseph, MI). An EDTA sample of 9.56% nitrogen was used as the standard for calibration. Crude fat was determined via acid hydrolysis and hexane extraction (AOAC 960.39). Gross energy was determined via bomb calorimetry (model 6200; Parr Instrument Co.; Moline, IL) with benzoic acid (6,318 kcal GE/kg; Parr Instrument Co.) used as the standard for calibration. Total dietary fiber was analyzed at Midwest Laboratories (Omaha, NE).

Apparent Total Tract Digestibility and Energy Calculations

Apparent total tract macronutrient and energy digestibility were determined using chemical composition data from diet and fecal samples and feed intake/fecal output records. Apparent total tract macronutrient and GE digestibility were calculated using the following equation:

$$\text{Apparent digestibility (\%)} = \left(\frac{intake - fecal\ output}{intake}\right) \times 100.$$

Blood Panels

A 5mL blood sample was collected from each dog via jugular venipuncture at baseline and on d 29 of the study. The sample was split into 2 collections tubes: one red-top tube and one

lavender-top EDTA tube. Red-top tubes were spun in a refrigerated centrifuge for 15 minutes at 3000 RPM after being allowed to clot. Lavender-top tubes were placed on a rocker to allow the blood to adequately mix with the anticoagulant. Blood samples were packaged and sent priority-overnight for analysis to Antech Diagnostics (Memphis, TN) for hematology (Siemens Advia 120) and clinical chemistry (Beckman Coulter AU5800).

Statistical Analysis

Data were analyzed in a mixed model including the fixed effects of diet and sex (PROC MIXED, Version 9.4, SAS Inst., Cary, NC). Initial body weight was used as a covariate for analysis of body weights recorded during the duration of the study. Baseline blood values were used as a covariate for final blood parameters. Differences between diets were determined using least squared means. A probability of $P < 0.05$ was considered statistically significant and standard error of the means (SEM) were determined. Orthogonal contrasts to determine linear, quadratic, or cubic relationships were also analyzed.

Results and Discussion

Diet and Fecal Chemical Analyses

Nutrient concentrations of the diets ranged for DM (92.0-93.4%), OM (92.9-93.6%), CP (26.1-28.0%), fat (13.1-14.2%), and GE (4891-4932kcal/kg) (**Table 5.3**). Total dietary fiber ranged from 1.92-3.86% when comparing the control to the 24% cricket powder diet. Replacement of chicken meal with cricket powder increased DM, OM, CP, fat, GE and TDF in the diets. Comparing the control with the 24% diet, the fiber content was approximately 2x greater. The increased fiber content of the diets may be explained by chitin, which is recovered in fiber analyses (Koutsos et al., 2019). The level of cricket powder inclusion in canine diets might be dictated by the higher concentration of TDF in the diet.

Feed Intake and Fecal Characteristics

Feed intake and fecal characteristics are presented in **Table 5.4**. There were no significant differences for as fed ($P = 0.385$) or DM ($P = 0.380$) intake or mean body weight ($P = 0.827$) among treatments. However, there were significant differences for fecal output on both an as-is ($P = 0.030$) and DM ($P = 0.024$) basis when comparing treatments. In addition, fecal output followed a linear relationship with cricket inclusion ($P \leq 0.009$). The increased fecal output may be explained by the increase in dietary fiber. Previous studies have shown an increase in wet fecal weight with the increase in dietary fiber (Bueno et al., 1981; Cole et al., 1989; Fahey et al., 1990; McPherson-Kay, 1987). This result may be due to the "bulking effect" of fiber and appears to be most strongly associated with insoluble fiber sources which are poorly fermentable and have a good water-binding capacity (Diez et al., 1998). Typically, with the increase in wet fecal weight the DM output is not altered, meaning the main contributor is increased water content in the stool. However, in this study, the DM output was also significantly impacted. Therefore, other mechanisms may be involved such as greater microbial and short chain fatty acid production with the increase in dietary fiber (Sunvold et al., 1995). In addition, the decreased digestibility with the increase in fiber may have led to the increased DM fecal output (Diez et al., 1998). Fecal scores were maintained at ideal levels with an average of 3.4 or 3.5 for each treatment according to the following scale: 0 = none, 1 = watery diarrhea, 1.5 = diarrhea, 2 = moist, no form, 2.5 = moist, some form, 3 = moist, formed, 3.5 = well-formed, sticky, 4 = well-formed, 4.5 = hard, dry, and 5 = hard, dry, crumbly. Fecal pH also did not differ among treatments ($P = 0.232$). Although fecal output was altered, other fecal characteristics were maintained at ideal as levels of cricket powder increased.

Apparent Total Tract Digestibility

Apparent digestibility ranged for DM (88.9-83.9%), OM (91.5-86.8%), CP (88.2-82.1%), fat (96.4-94.8%), and GE (92.4-88.3%) from the control to the 24% cricket powder diet (**Table 5.4**). The apparent digestibility for fiber was much lower ranging from 57.5-46.3%. The low level of fiber digestibility is to be expected due to its ability to resist hydrolysis by endogenous enzymes. Most dietary fiber passes to the large intestine undigested where it can then be fermented by microbes (NRC 2006). Each nutrient digestibility had significant differences among treatments ($P < 0.001$). Linear ($P < 0.001$) and cubic ($P < 0.05$) relationships were observed in DM, fat, GE, OM, and CP digestibility with the increase in cricket powder. Fiber digestibility only presented a cubic relationship ($P < 0.001$). Fahey et al. (1990) showed a similar range for fiber digestibility as well as a cubic relationship when testing diets containing increasing levels of beet pulp in diets containing 5 to 14% total dietary fiber in dogs. Cubic relationships could indicate an optimum inclusion level. Cole et al. (1989) reported a linear decrease in DM, OM, and GE digestibility with an increase in soybean hulls in dog diets containing 3 to 9% total dietary fiber. Likewise, chitin has previously been implicated as a factor in reduced digestibility of insects in livestock and aquaculture (Newton et al., 2005; Dumas et al., 2018). Concerns regarding chitin and the negative impact on digestibility are complicated by a lack of analytical methods (Koutsos et al., 2019). Interestingly, Bosch et al. (2014) reported the *in vitro* OM digestibility of house crickets to be 88% which was similar when compared to poultry meat meal at 85.8%. Nonetheless, the digestibility of each treatment in this study is still greater than 80%, which is comparable to commercially manufactured dog foods (Castrillo et al., 2001).

Blood Panels

Blood results and reference intervals for healthy dogs are presented in **Table 5.5** and **Table 5.6**. Blood samples were analyzed to determine any fluctuations among treatments and to monitor health status. Blood urea nitrogen (BUN; $P = 0.037$) and hemoglobin ($P = 0.044$) levels were the only blood parameters with significant results among treatments. BUN presented a significant cubic ($P = 0.020$) relationship with the increase in cricket inclusion. As a result of protein metabolism, urea is produced by the liver and is carried by the blood to the kidney for excretion. Even though diets were formulated to be isonitrogenous, protein levels of the diets numerically increased with increased cricket powder. Therefore, the increase in dietary protein could have led to fluctuations in BUN levels (Hosten, 1990). Hemoglobin presented a linear decrease with the increase of cricket powder ($P = 0.006$). The treatment differences among BUN and hemoglobin are not of clinical concern due to all blood parameters remaining within the desired reference intervals for healthy dogs. Overall, blood parameters were consistent throughout treatments indicating no impact on health status with dietary treatment.

Conclusion

The study described, for the first time, the effect of graded levels of cricket powder in diets fed to adult dogs. In summary, inclusion of cricket powder in canine diets can serve as an acceptable source of protein when compared to a control diet with chicken meal as a protein source. The maintenance of ideal fecal characteristics and blood parameters throughout the duration of the study indicates that overall health status was upheld while animals were fed dietary treatment. Differences in apparent digestibility, likely resulting from the increase in fiber, may drive decision on optimal inclusion level of cricket powder fed to adult dogs. Future

research is needed to investigate potential functionality of the chitin component in cricket powder.

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Table 5.1: Ingredient composition of diets

	Cricket Powder			
Ingredient, %	0%	8%	16%	24%
Corn	37.57	37.57	37.57	37.57
Chicken Meal	21.69	14.46	7.22	0.00
Cricket Powder	0.00	8.00	16.00	24.00
Brewers Rice	15.00	15.00	15.00	15.00
Chicken Fat	7.69	7.06	6.43	5.80
Corn Gluten Meal	6.00	6.00	6.00	6.00
Dried Beet Pulp	3.50	3.50	3.50	3.50
Corn Starch	2.58	1.73	0.92	0.09
Natural Flavor	2.00	2.00	2.00	2.00
Dicalcium Phosphate	1.80	2.16	2.47	2.83
Calcium Carbonate	0.69	1.05	1.42	1.74
Salt	0.50	0.50	0.50	0.50
Potassium Chloride	0.40	0.40	0.40	0.40
Fish Oil	0.25	0.25	0.25	0.25
Choline Chloride 60%	0.10	0.10	0.10	0.10
LANI Vitamin Premix[1]	0.10	0.10	0.10	0.10
LANI Trace Mineral Premix[2]	0.05	0.05	0.05	0.05
LANI Organic Trace Mineral Premix[3]	0.01	0.01	0.01	0.01
LANI Naturox Plus[4]	0.04	0.04	0.04	0.04

[1]LANI Vitamin Premix (pea fiber, calcium carbonate, vitamin E, niacin, thiamine mononitrate, D-calcium pantothenate, vitamin A, sunflower oil, pyridoxine hydrochloride, riboflavin, vitamin D3, biotin, vitamin B12, folic acid).
[2]LANI Trace Mineral Premix (calcium carbonate, zinc sulfate, ferrous sulfate, copper sulfate, mineral oil, manganous oxide, sodium selenite, calcium iodate).
[3]LANI Organic Trace Mineral Premix (zinc methionine complex, calcium carbonate, zinc sulfate, iron proteinate, ferrous sulfate, copper proteinate, copper sulfate, manganese proteinate, sunflower oil, manganous oxide, sodium selenite, calcium iodate, ethylene diamine dihydroidodide).
[4]LANI Naturox Plus (amorphous silicon dioxide, citric acid, natural mixed tocopherols, vegetable oil, rosemary extract)

Table 5.2: Nutrient composition of the cricket powder included in diets (provided by supplier)

Nutrient	% DM
Dry matter	98.23
Crude protein	67.76
Crude fat	21.64
Ash	4.79
Crude fiber	7.51
Alanine	5.40
Arginine	4.12
Aspartic acid	6.67
Cystine	ND[1]
Glutamic acid	8.73
Glycine	3.13
Histidine	1.58
Isoleucine	2.80
Leucine	4.96
Lysine	3.35
Methionine	1.16
Phenylalanine	3.48
Serine	3.48
Taurine	ND
Threonine	2.76
Tryptophan	ND
Tyrosine	3.47
Valine	3.99
Amino Acid Recovery[2]	87.19

[1] Not Determined

[2] Amino Acid Recovery = sum of amino acids/ % crude protein

Table 5.3: Analyzed chemical composition of diets (DM basis)

	Cricket Powder			
Item	0%	8%	16%	24%
Dry Matter, %	91.96	92.44	93.00	93.45
Moisture, %	8.04	7.56	7.00	6.55
Organic Matter, %	93.22	92.87	93.49	93.55
Ash, %	6.78	7.14	6.51	6.45
Crude Protein, %	26.13	26.39	27.80	27.98
Fat, %	13.44	13.10	14.22	13.74
Total Dietary Fiber, %	1.92	2.44	3.48	3.86
Gross Energy, kcal/kg	4901.13	4891.42	4930.30	4932.09

Table 5.4: Average feed intake, fecal output, fecal score, fecal pH, apparent total tract macronutrient and energy digestibility

	Cricket Powder					P-Value			
Item	0%	8%	16%	24%	SEM	Treatment	Linear	Quadradic	Cubic
Intake									
Feed intake, g AF/d	231.45	193.03	226.58	222.05	16.81	0.385	0.944	0.322	0.155
Feed intake, g DM/d	212.85	178.43	210.71	207.50	15.61	0.380	0.818	0.327	0.155
GE intake, kcal/d	1043.19	872.78	1038.88	1023.39	76.78	0.354	0.758	0.322	0.143
Output									
Fecal output, g as-is/d	64.80[a]	66.18[a]	70.30[a,b]	93.35[c]	7.16	0.030	0.009	0.142	0.618
Fecal output, g DM/d	23.41[a]	24.00[a]	26.35[a,b]	33.64[c]	2.44	0.024	0.005	0.181	0.773
Fecal score	3.40	3.44	3.47	3.43	0.03	0.336	0.324	0.136	0.682
Fecal pH	6.53	6.36	6.19	6.18	0.14	0.232	0.053	0.545	0.792
Apparent Digestibility									
Dry Matter, %	88.90[a]	86.52[b]	87.27[a,b]	83.89[c]	0.68	<0.001	<0.001	0.475	0.025
Organic Matter, %	91.51[a]	89.41[b]	89.98[a.b]	86.78[c]	0.54	<0.001	<0.001	0.320	0.013
Crude Protein, %	88.21[a]	84.83[b]	86.01[b]	82.08[c]	0.74	<0.001	<0.001	0.715	0.007
Fat, %	96.43[a]	95.66[b]	95.99[a,b]	94.82[c]	0.22	<0.001	<0.001	0.360	0.013
Total Dietary Fiber, %	57.46[a]	43.66[b]	61.29[a]	46.25[b]	2.81	<0.001	0.214	0.828	<0.001
Gross Energy, %	92.40[a]	90.39[b]	90.77[b]	88.28[c]	0.49	<0.001	<0.001	0.628	0.024

[a-c]Means within a row lacking a common superscript letter are different (P<0.05).

Table 5.5: Serum chemistry analysis of dogs fed diets containing graded levels of cricket powder

Item	Cricket Powder				SEM	P-Value				Reference Interval[1]
	0%	8%	16%	24%		Treatment	Linear	Quadratic	Cubic	
BUN, mg/dL	11.66[a]	11.30[a]	13.24[b]	12.17[a]	0.47	0.037	0.118	0.464	0.020	6.0-31.0
Creatinine, mg/dL	0.56	0.60	0.60	0.58	0.02	0.605	0.582	0.212	0.979	0.5-1.6
BUN/Creat Ratio	21.11	19.16	22.00	21.61	1.08	0.273	0.379	0.487	0.108	4.0-27.0
Glucose, mg/dL	81.49	84.65	84.16	81.32	2.43	0.758	0.922	0.300	0.902	70.0-138.0
Total Protein, g/dL	6.26	6.17	6.29	6.21	0.09	0.811	0.973	0.927	0.339	5.0-7.4
Albumin, g/dL	3.25	3.29	3.26	3.27	0.05	0.953	0.815	0.754	0.689	2.7-4.4
Globulin, g/dL	3.00	2.89	2.99	2.98	0.08	0.688	0.968	0.448	0.352	1.6-3.6
A/G Ratio	1.13	1.17	1.11	1.12	0.04	0.699	0.617	0.611	0.348	0.8-2.0
Alk Phos, U/L	72.57	59.04	49.51	60.75	7.42	0.203	0.188	0.105	0.622	5.0-131.0
AST, U/L	26.35	24.47	27.05	24.88	1.31	0.470	0.764	0.915	0.126	15.0-66.0
ALT, U/L	43.81	40.47	37.35	33.12	3.28	0.148	0.024	0.894	0.929	12.0-118.0
GGTP, U/L	5.29	4.50	5.09	4.37	0.29	0.109	0.109	0.902	0.052	1.0-12.0
CPK, U/L	104.91	100.61	142.33	122.91	15.35	0.228	0.178	0.625	0.130	59.0-895.0
Bilirubin, mg/dL	0.16	0.17	0.14	0.15	0.02	0.840	0.596	0.839	0.493	0.1-0.3
Cholesterol, mg/dL	171.52	174.09	167.01	167.75	7.16	0.878	0.573	0.901	0.593	93.0-324.0
Triglycerides, mg/dL	62.19	55.04	60.40	56.75	4.18	0.620	0.575	0.678	0.253	29.0-291.0
Sodium, mEq/L	146.75	145.70	146.22	146.46	0.37	0.231	0.831	0.093	0.281	139.0-154.0
Potassium, mEq/L	4.38	4.19	4.31	4.17	0.09	0.399	0.254	0.798	0.199	3.6-5.5
Chloride, mEq/L	112.27	112.81	113.50	112.92	0.57	0.489	0.342	0.309	0.575	102.0-120.0
Phosphorus, mg/dL	3.79	3.41	3.76	3.42	0.19	0.315	0.423	0.934	0.092	2.5-6.0
Magnesium, mEq/L	1.48	1.55	1.56	1.58	0.03	0.131	0.030	0.466	0.654	1.5-2.5

[a-c]Means within a row lacking a common superscript letter are different ($P<0.05$).

[1]Reference intervals are laboratory specific.

Table 5.6: Hematology profile of dogs fed diets containing graded levels of cricket powder

Item	Cricket Powder				SEM	P-Value				Reference Interval[1]
	0%	8%	16%	24%		Treatment	Linear	Quadratic	Cubic	
WBC, 10^3/mm3	7.64	7.36	7.76	7.68	0.57	0.958	0.841	0.863	0.637	4.0-15.5
RBC, 10^6/mm3	6.83	6.77	6.61	6.51	0.11	0.197	0.036	0.901	0.769	4.8-9.3
Hemoglobin, g/dL	15.74[a]	15.53[a,b]	15.06[b,c]	14.94[c]	0.22	0.044	0.006	0.856	0.540	12.1-20.3
Hematocrit, %	51.06	49.59	48.44	48.03	0.81	0.060	0.009	0.533	0.908	36.0-60.0
MCV, um^3	74.36	73.59	73.14	74.03	1.05	0.856	0.762	0.441	0.833	58.0-79.0
MCH, uug	22.98	23.03	22.79	23.02	0.22	0.845	0.899	0.693	0.432	19.0-28.0
MCHC, g/dL	30.75	31.13	31.36	31.27	0.25	0.350	0.124	0.362	0.872	30.0-38.0
Platelets, 10^3/mm3	272.17	264.45	316.95	285.05	14.42	0.078	0.179	0.410	0.032	170.0-400.0
Absolute Monos	444.01	341.17	384.11	391.95	64.45	0.772	0.712	0.418	0.517	0.0-840.0
Absolute Eos	198.64	263.80	305.71	311.35	33.96	0.119	0.023	0.390	0.931	0.0-1200.0
Absolute Basos	0.00	0.00	0.00	0.00	0.00	1.000	1.000	1.000	1.000	0.0-150.0
Absolute Bands	0.00	0.00	0.00	0.00	0.00	1.000	1.000	1.000	1.000	0.0-300.0
Absolute Polys	5176.62	4654.40	4974.54	5147.68	451.14	0.843	0.910	0.471	0.617	2060.0-10600.0
Absolute Lymphs	1902.95	2009.76	2058.33	1884.96	102.97	0.578	0.991	0.182	0.723	690.0-4500.0

[a-c]Means within a row lacking a common superscript letter are different (P<0.05).

[1]Reference intervals are laboratory specific.

CHAPTER 6. CONCLUSIONS

The results of each study supported our hypotheses. The dogs enrolled in the first study were able to effectively handle the consumption of high dietary fat levels. The shift in microbiota resulting from increased levels of dietary fat may prove that the microbiota adapts to what nutrients are being consumed. The high-fat, low carbohydrate diets increased microbes with roles in fat digestion and decreased those with roles in carbohydrate digestion. Even with these shifts, dogs maintained health status. This study contradicts previous studies in humans and mice showing that high dietary fat leads to detrimental effects on the microbiota. This may indicate that the dog microbiota reacts differently to a high fat diet. The dog's short digestive tract and the ability to efficiently digest fat may contribute to these results. In addition, typical diets consumed by dogs are higher in fat level compared to other species. In conclusion, the utilization of higher fat diets may be implemented to create a more natural, less processed diet that is currently demanded by consumers.

The study investigating the use of cricket powder showed that it may serve as a high-quality protein source for dogs. Even with the decrease in digestibility with the increased levels of cricket inclusion, diets remained highly digestible and did not alter the health status of each dog. In addition, the use of cricket powder as an alternative to typical protein sources may provide environmental benefits.

The provided results are in favor for the creation of new products to meet consumer demand. The use of high dietary fat inclusion can decrease the amount of processing and use of ingredients seen as low quality by consumers. The use of cricket powder as a protein source provides a novel protein source with a lower environmental impact. Further research is needed in order to access optimum levels of both fat and cricket powder inclusion in canine diets.