Extrusion process to retain resistant starch in a pet food for the purpose of altering colonic fermentation end products that benefit dog health

by

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B.S., University of São Paulo, 2012
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science & Industry
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Abstract

Starch that escapes enzymatic digestion in the small intestine is considered resistant (RS). This RS can be fermented by saccharolytic bacteria in the colon and may benefit gut health. Raw crystalline starch granules contain RS type II. Mild extrusion of pet foods may retain some RS II or develop RS type III (retrograded) that may indirectly benefit animal health. The objectives of the present work were to determine the amount of RS present in typical commercial pet foods, develop a response surface model that predicts RS to maximize RS in corn based extruded kibble, produce dog kibbles with three levels of RS based on model criteria, and determine the effects of these diets on dog colonic health, markers of satiety, metabolomics of serum and feces, and fecal microbiome. In commercial dog and cat foods which were either grain-free or grain based (20 total diets) from a sampling frame of 654 pet foods had low RS (average < 0.6% of the kibble) and did not differ among groups. For the second part of the experiment, a single nutritionally complete diet for adult dogs at maintenance (AAFCO, 2019) was formulated with corn as the sole starch ingredient (65%). Experimental diets were produced on a small-scale intermeshing co-rotating twin-screw extruder following a central composite design with 6 central points (replicates) and 20 total samples per variable. There were three factors at three levels: corn particle size (PS), extruder shaft speed (SS) and in-barrel moisture (IBM). Starch transformations were determined as RS, starch cook, and by rapid-visco analysis (RVA). Results indicated that an increased IBM and decreased SS during extrusion process favored RS retention in the kibble, with a final model of RS= 0.018*PS + 0.00161*SS + 0.0601*IBM – 0.000013*SS*PS – 3.78. Experimental foods to test *in vivo* with dogs were produced on a single screw extruder in a completely randomized design with 3 replicates per treatment. Starch transformations were measured as starch cook (glucoamylase procedure), RS, rapidly (RDS), slowly (SDS) and total

digestible starch (TDS), and RVA. Resistant starch, starch cook and raw:cooked starch RVA AUC increased linearly from high (HS) to medium (MS) and low shear (LS) foods, while SDS was greater in the LS treatment. These foods were fed to 24 dogs in a 3x3 William's Latin square design with blood and fecal samples collected at the end of each period (28 d). Microbiome was determined by high throughput targeted sequencing of 16S bacterial rRNA. Short-Chain fatty acids, serum metabolomics, and fecal microbiome were determined at a commercial laboratory (Metabolon, Morrisville, NC). Satiety hormones were measured on plasma by ELISA. Immunological markers were measured on feces and serum by a commercial laboratory (MD Biosciences; Oakdale, MN). Data were analyzed as a mixed model with diet, period, treatment sequence and carryover as fixed effects. Fecal quality was determined on a subjective consistency scale. Microbiome alpha diversity was unchanged ($P > 0.05$) by dietary treatments, but dogs fed the MS and LS foods had more indication of saccharolysis, promoted by a higher fecal glucose and oligosaccharides concentration ($P < 0.05$, $q < 0.1$). Fecal butyrate concentration increased in dogs fed the LS diet relative to HS, and MS was similar to both extremes ($P < 0.05$). Diets had no effect on satiety hormones or local immunity, which might suggest the need for a longer feeding period and(or) a diet produced with lower thermomechanical energy for a dietary effect. In conclusion, we suggest a low to mild shear extrusion process to produce corn-based kibbles that may promote gut health.

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Dedication

I would like to dedicate this dissertation to my parents Lais Betty F. Corsato Alvarenga, Ed de Souza, and Antonio Alvarenga Neto. This achievement would not have been possible if not for their teachings, example of work ethics, and opportunities provided.

Chapter 1 - **Factors affecting digestibility of starches and their implications on adult dog health**

Abstract

Pet food represents a large share of the U.S. economy, and the majority of it is produced through extrusion. Starches comprise between 30-60% of extruded dog foods and sources include cereals, tubers, legumes, and co-products from the human food chain. Starches are well digested and metabolized by dogs, with variations according to food processing parameters, starch source, and dog breed. Dogs have a higher expression of enzymes related to starch digestion, glucose absorption and metabolism compared to the wolf. Several studies have reported that starch total tract apparent digestibility (ATTD) by dogs is well above 95%. Although starches are not required by dogs, they are an important source of energy and provide structure and binding of kibbles. The degree of starch digestion depends on factors like granular structure, amylose: amylopectin ratio, degree of gelatinization, and other nutrients present in the food matrix such as lipids and proteins. Tubers and legumes are well utilized by dogs, but most times require heat treatment to deactivate anti-nutritional factors and improve digestibility, while cereal starches seem to be well digested even in their raw form. Slowly digestible or resistant starches may contribute to satiety, lower glucose and insulin responses and improved colonic microbiota. In conclusion, there is enough evidence supporting that properly processed cereals and tubers included in dog foods are digestible, palatable and do not present health concerns, but the scientific literature lacks information on pulses, ancient grains, and food processes beyond extrusion.

Keywords: Dog, starch, pet food, processing, nutrition.

List of Abbreviations: ACSM2A, acyl-CoA synthetase medium chain family member 2A; AMY2B, alpha-amylase 2B; ATTD, apparent total tract digestibility; DM, dry matter; GI, gastro-intestinal; GLP-1, glucagon-like peptide 1; HA, high-amylose; MGAM, maltase-glucoamylase; NFE, nitrogen-free extract; OM, organic matter; RVA, rapid visco analyzer; RDS, rapidly digested starch; RS, resistant starch; SCFA, short-chain fatty acids; SDS, slowly digested starch; SGLT1, sodium-glucose transport protein 1; SME, specific mechanical energy; TDF, total dietary fiber.

Introduction

It is estimated that 60.2 million households in the US own a dog, with a dog population close to 94.2 million (APPA, 2017). Most pet foods can be divided into three categories based on moisture content: dry, semi-wet or soft-moist, and canned (Kvamme & Philips, 2003). The vast majority (over 70%) of dog diets are dry products (< 10% moisture), which rely on a significant proportion of starch (30-60%) that must be processed (cooked) to form the final product. Starch can be an effective source of energy (glucose for metabolism), and it impacts the flavor and acceptability of the food product. Starch also provides structure, texture, and serves to agglomerate nutrients into a consistent product. As components of the food matrix, the nutritional utilization of starches depends in large part on their composition.

Starches are polysaccharides comprised of glucose units formed during photosynthesis, which are stored as granules in various parts of the plant (chloroplasts, tubers, roots, or grains). Starch granules are composed primarily of amylose (ca. 20-30%) and amylopectin (ca. 70-80%) (Zobel, 1988). Amylose is essentially a linear polymer consisting of hundreds to thousands of glucose molecules linked by α-1,4 glycosidic bonds. Despite common classification of amylose

as a linear molecule, there can be a small number of branches in the structure (Hizukuri, Takeda, Yasuda, & Suzuki, 1981). In contrast, amylopectin is a large branched structure that also contains α-1,4 bonds, and α-1,6 bonds every 20 to 25 glucose molecules that result in branch points (Zobel, 1988). Amylopectin can be comprised of 30,000 to 3 million glucose units and is responsible for the crystallinity in the raw starch (Zobel, 1988).

Different starch sources (cereals, tubers or legumes), as well as the arrangement of the crystalline structures, the amylose: amylopectin ratio, particle size, amylose-lipid complexes, degree of thermal processing (cooking), and interaction with other materials in the food matrix (sugar, protein, etc.) have been demonstrated to affect the rate and extent of starch digestion (Dhital, Warren, Butterworth, Ellis, & Gidleyet, 2017; Singh, Dartois, & Kaur, 2010; Bird, Lopez-Rubio, Shrestha, & Gidley, 2009). Important endpoints to determine the impact of dietary starch *in vivo* include nutrient utilization, stool quality, and glucogenic response.

When starch is cooked during pet food processing, gelatinization occurs (Cheftel, 1986). This is a process of hydrating the starch granule and unfolding the tightly packed crystals, which allows for hydrolysis by intestinal enzymes and improvement of starch digestion. Gelatinized starch also has an important function in croquette formation and texture development. In the animal, starch digestion releases glucose which is absorbed in the duodenum. This appearance of glucose in the circulation promotes insulin release by the pancreas. The degree of gelatinization has implications on utilization. Specifically, raw or less cooked starches are less digestible and contain a higher proportion of resistant starch. This has been shown to have some benefit for blood glucose control (Ribeiro et al., 2019) and colonic health (Jackson, Waldy, Cochrane, et al., 2020; Ribeiro et al., 2019; M. C. Peixoto et al., 2018). Understanding starch utilization and the

implications of processing on pet foods and nutrition is important to the health and wellbeing of dogs.

Dog digestive system and adaptation to starch-rich diets in comparison to obligate carnivores and other omnivores

Digestion and absorption of nutrients in dogs is similar to other monogastric mammals with a few minor differences. For more detail regarding the interplay between digestive physiology of the dog readers are referred to comprehensive reviews by Chang and Leung (2014) and the NRC (2006). As it pertains to starch, the mouth is the first step to digestion through mastication and lubrication. Unlike humans no starch hydrolysis occurs in the dogs' mouth as they do not produce salivary amylase (Simpson, Doxey, & Brown, 1984). The food bolus is transported by the esophagus' in peristaltic waves from the pharynx to the stomach, where it is bathed in hydrochloric acid and pepsin as the first step of enzymatic digestion principally affecting proteins. The acid-chyme leaves the stomach and enters the duodenum through the pyloric sphincter.

The preponderance of starch digestion begins as it reaches the duodenum where it is first cleaved into maltose and other oligosaccharides by pancreatic α-amylase. Then the maltose and oligosaccharides are hydrolyzed by maltase-glucoamylase, sucrase and isomaltase to glucose (Axelsson et al., 2013). The activity of alpha-amylase was reported to be 43-fold higher in the dog than in the cat (3,000 U/g vs 70 U/g wet weight) by Kienzle (1988 and 1993a). This enzyme activity increases in the pancreas and small intestine from weaning to adulthood, and alpha-amylase production adapts to changes in dietary starch in the dog (Kienzle, 1988). Noon et al. (1977) reported that maltase, sucrase, lactase, amyloglucosidase and cellobiase were active in

Beagle dogs. Additionally, dogs express proximal intestinal sucrase, maltase and lactase at 4.4, 4.6 and 3.1-fold higher than cats, respectively (Batchelor et al., 2010). Hore & Messer (1968) found that sucrase, maltase and lactase were present in the small intestine of both dogs and cats. However, lactase activity is lower in adult dogs and cats compared to young animals, and sucrase activity is low in both dogs and cats throughout their lives (Welsh & Walker, 1965; Prola, Dobenecker, & Kienzle, 2006; Kienzle,1993b). At the intestine, absorption of glucose after starch has been hydrolyzed takes place in the duodenum and jejunum. Both dogs and cats express intestinal brush boarder sodium-glucose transport proteins 1 (SGLT1; Axelsson et al., 2013; Wright, LOO, & Hirayama, 2011); and it functions two-fold higher in dogs than in cats (Batchelor et al., 2010). Relative to the cat, the dog is better adapted to starch-rich diets.

Digestion of cooked starch in the small intestine typically exceeds 95% (Murray, Fahey, Merchen, Sunvold, & Reinhart, 1999). Starch not digested in the small intestine is described as resistant starch (RS) and is fermented by colonic bacteria. The remaining product of digestion constitutes the feces which contains indigestible matter, microbial mass, and endogenous secretions.

Compiling data across several studies, average mean retention time of food in the dog was 25.6h ± 7.72 (Burrows et al., 1982; Fahey et al., 1990; Fahey et al., 1992; Lefebvre et al., 2001; Rolfe et al., 2002; Hernot et al., 2005; Childs-Sanford & Angel, 2006; Stein et al., 2008; De Cuyper et al., 2018; Koziolek et al., 2019). The total tract retention time (RT) of food is shorter in small dogs than large breeds (Hernot, Biourge, Martin, Dumon, & Nguyen, 2005), and varies according to the dogs' physiological condition (Rolfe et al., 2002; Lefebvre et al., 2001), food particle size (De Cuyper et al., 2018), and diet composition (fiber additions tend to shorten

RT; Burrows, Kronfeld, Banta, & Merritt, 1982; Fahey et al., 1990; Fahey et al., 1992) among other factors.

From a nutritional perspective, carbohydrates are not essential dietary requirements for dogs or cats, but both species have a metabolic requirement for glucose (Tanaka et al., 2005). Adult cats are obligate carnivores, in part because they require more amino acids, fatty acids, and pre-formed vitamins exclusive to animal rather than plant sources versus other omnivores and they are in a constant state of protein oxidation and gluconeogenesis (Verbrugghe & Bakovic, 2013). While dogs are also considered carnivores, they require less protein than cats (Verbrugghe & Bakovic, 2013). Dogs can synthetize glucose from amino acids or obtain it from the breakdown of carbohydrates, since their metabolic enzymes involved in both glycolysis and gluconeogenesis have high activity (Tanaka et al., 2005) and glucose from starches can effectively contribute to their daily energy level.

Pet owners are exposed to messages that their dog should be fed like their canid ancestor, the wolf (*Canis lupus*). Some similarities are bound to be present, but the modern canine has also evolved some subtle adaptations (Table 1.1). Childs-Sanford & Angel (2006) reported that Beagle dogs were similar to the maned wolf (*Chrysocyon brachyurus*) regarding transit time and diet digestibility when fed a low vs high animal protein diet. Although that study did not detect significant differences, genetic evaluation for key enzyme systems might.

While protein and overall diet digestive physiology may be similar between the dog and the wolf, utilization of starches and fats may provide a more definitive differentiation. Axelsson et al. (2013) reported genetic modifications in 10 genes in dogs related to key roles in starch digestion and fat metabolism in comparison to the wolf. They identified genes involved in starch digestion (α-amylase 2B; AMY2B and maltase-glucoamylase; MGAM), glucose uptake

(SGLT1) and a “candidate gene” for insulin resistance (Acyl-CoA synthetase medium chain family member 2A; ACSM2A) that initiates fatty acid metabolism that differed. Ollivier et al. (2016) confirmed that the gene AMY2B, which encodes for pancreatic α-amylase, an important enzyme involved in starch digestion, increased its expression in ancient dogs during the late Neolithic age. The AMY2B copy number was estimated to be 2 to 8-fold higher in the dog than in the wolf in regions of Europe and Asia (Ollivier et al., 2016). While starch utilization in canids appears to be adequate, the dog has adapted during domestication to better utilize dietary starch than the wolf.

The time in history at which certain breeds emerged in the evolution of the dog may also provide a clue to the shift in their ability to utilize starch. More ancient breeds such as the Samoyed and Greenland Sledge dogs had the lowest AMY2B activity (Arendt, Fall, Lindblad & Axelsson, 2014). Considering the Samoyed and Greenland Sledge dogs are breeds that did not have much genetic variation, and whose diets were primarily based on protein, there was less selection pressure on adaptation to starch based foods (Arendt et al., 2014) and thus minimized their dependence on AMY2B expression. The Dingo and Siberian Husky are breeds from regions that have only recently began agriculture production and they also express a low AMY2B copy number (Ollivier et al., 2016). Conversely, breeds such as the German Shepard dog and the Springer Spaniel that were developed in highly intensive agricultural regions had a larger expression of AMY2B (Arendt et al., 2014). In another study, Arendt et al. (2016) investigated dogs’ worldwide AMY2B copy number diversity and found that this gene is bimodally distributed in the dog population; those originating from agrarian regions had a higher gene copy number and those from non-agrarian regions had a low gene copy number. This would suggest that early ancestors of modern dogs transitioned from hunting prey to scavenging in waste dumps

near human settlements during the agricultural revolution, ultimately consuming a greater concentration of starches in their diets. This change in their ecological niche along with selection pressures associated with domestication may have led to more flexibility for calorie acquisition and enhanced their ability to utilize starches.

Factors affecting digestion of starch by dogs

Starch utilization by dogs has been studied for decades. Early in the last century Roseboom & Patton (1929) reported that after feeding dogs a high starch diet, their feces was void of undigested starch when evaluated by the iodine test. Further, Ivy, Schmidt, & Beazell (1936) discovered that dog pancreatic juice was comparable in amylolytic activity to duodenal drainage fluid from healthy humans. These authors also reported that starch disappearance was nearly complete when dogs were fed high starch diets. More recent studies have reported starch disappearance to be in excess of 95% in dogs (Twomey et al., 2003; Carciofi et al., 2008; Murray et al., 1999; Walker, Harmon, Gross, & Collings, 1994; Schünemann, Mühlum, Junker, Wilfarth, & Meyer, 1989; Moore, Fottler, Fahey, & Corbin, 1980). Rate and extent of starch digestion are affected by both intrinsic (i.e. structure of starch) and extrinsic factors (i.e. processing conditions).

Starches from any source (cereals, tubers or legumes) can also be classified into digestible or resistant to digestion. As noted above, the starch digested completely by the end of the small intestine (ileum) is considered digestible starch, whereas the fraction that escapes digestion in the small intestine is referred to as resistant starch (RS). This tends to be low in extruded pet foods (Corsato Alvarenga & Aldrich, 2020; Peixoto et al., 2018). Since resistant starches are fermented by saccharolytic bacteria in the colon and almost completely disappear in

the feces, starch enzymatic digestion is better characterized from ileal digestibility data. This type of data is scarce due to technical and ethical challenges of surgical cannulation of dogs.

Starch sources

The most common starch ingredients in pet foods are cereals like corn, sorghum, rice, and wheat, pulses like peas or lentils, and tubers like potato or tapioca, as well as their byproducts or fractionated products. Besides being affordable and efficient sources of energy, grains, pulses and tubers are functional ingredients for extrusion (Guy, 2001). Starch ingredients are typically included between 30 and 60% in an extruded kibble. However, if only one starch source was added to the recipe, that would list it as the first ingredient according to labeling regulations (AAFCO, 2019). For this reason, pet food companies often include three or more cereals or tubers with no more than 20% each to not exceed the animal protein ingredient level, which is preferred by the consumer. Legumes such as peas, lentils and chickpeas can be considered both a source of starch and vegetable protein. These are usually included at lower levels due to their low palatability and presence of oligosaccharides, which may cause excess fermentation in the large intestine and soften stool (Corsato Alvarenga, Aldrich and Holt, 2020). Other less common starch ingredients in pet foods include pseudo-cereals like buckwheat, quinoa, amaranth, which have improved nutritional quality (Alvarez-Jubete, Arendt, and Gallagher, 2010).

It is possible to assess the effect of starch ingredients on overall diet digestibility by comparing dry matter (DM) or organic matter (OM) apparent total tract digestibility (ATTD) of diets with similar ingredient or nutritional composition among starch sources. For example, Walker et al. (1994) produced extruded diets with similar ingredient composition and reported

that DM ATTD of the diet containing corn was lower than the diet containing rice, and the digestibility of the oat diet was lower than barley (Table 1.2). Since whole cereals were used at the same concentration, diets composed of corn and oats would have had higher amounts of fiber and less starch, which possibly led to decreased DM ATTD. Other studies have reported that diets with cereals like rice, sorghum and corn have similar nutrient disappearance (Murray et al., 1999; Carciofi, Sakomura, Kawauchi, & Vasconcellos 2010; Corsato Alvarenga & Aldrich, 2018), while barley (Murray et al., 1999) and legumes like lentils and peas were usually less digestible (Carciofi et al., 2008). Tuber digestibility varies by study (Table 1.2). There is a general trend towards some flours and cereals being more digestible, mostly due to their lower fiber contents and (or) fine milling prior to extrusion.

Chyme samples collected at the ileum can be used to determine the amount of starch which was digested by mammalian enzymes and absorbed in the small intestine. Walker et al. (1994) reported that starch ileal digestion of extruded kibbles based on corn, rice, barley and oats were over 98% with no difference among treatments due to carbohydrate source. Similarly, flours from cereal sources (barley, corn, rice, sorghum, wheat) and from potato were also reported to be over 99% digested at the dog ileum (Murray et al.; 1999). In both studies (Murray et al., 1999; Walker et al.,1994), the small starch portion that escaped ileal digestion and appeared in the colon represents the RS fraction, which was minimal.

Diets containing brewer's rice have been reported by several authors to be highly digestible by dogs when compared to other cereals (Moore et al., 1980; Kore, Pattanaik, Das, & Sharma, 2009; Twomey et al., 2014). The extent of starch digestion is also influenced by the rice variety. Belay, Shields, Wiernusz, Kigin, & Brayman (1997) reported that DM ATTD was greatest when dogs were fed a parboiled rice diet (pre-cooked rice), intermediate for white

medium grain rice, and lowest for brown rice (containing bran). In a second experiment, Belay et al. (1997) also reported that the more brewer's rice added to the diet in exchange for corn, the more digestible the diet. Rice included in pet foods has lower total dietary fiber (TDF) and higher starch content than other cereals (Kempe et al., 2004; Bednar et al., 2001), which leads to an overall increase in digested starch.

The differences in starch digestibility by dogs could be due to some intrinsic characteristics of starches. For example, the amylose: amylopectin ratio within the starch granule may affect starch digestion. Amylose is more susceptible to recrystallization after cooking than amylopectin (Wang et al., 2015), and its linear structure tends to form double helical structures (Wang et al., 2015) that are less available for enzymatic digestion (Tovar et al., 1990). Gajda et al. (2005) reported that an extruded high-amylose (HA) corn diet had lower ileal starch digestibility than extruded conventional corn diet, when fed to dogs. The starch granule size may also have an impact on digestibility. An *in vitro* study using a canine model found that the raw cereals ground using a 2-mm screen were better digested when the starch granules were small (oats and rice) vs large (corn, wheat; Bednar et al., 2001). Bednar et al. (2001) also reported that legume starches were less digestible than cereals, and all flours had a much higher starch digestibility than their whole grain counterparts. Plant cell walls provide a protective layer around starch granules that slow their digestion rate as compared to purified starches (Bhattarai et al., 2018).

Most starch sources studied to date are safe to be consumed by dogs, and most of them are highly digestible if properly processed (Moore et al., 1980; Schünemann et al., 1989; Walker et al., 1994; Wolter et al., 1998; Murray et al., 1999; Carciofi et al., 2008; Kore et al., 2009; Carciofi et al., 2010; Table 1.2). The digestibility ranking among studies may differ due to a host

of factors, such as the method of digestibility estimation, starch ingredient inclusion, processing conditions, and (or) individual variations between dogs or breeds. Within the cereal category, rice-based diets commonly have the highest overall nutrient digestibility (Twomey et al.,2014; Kore et al., 2009; Belay et al., 1997; Table 1.2), sorghum, corn and wheat containing diets tend to be intermediate (Alvarenga & Aldrich, 2018; Twomey et al., 2014; Kore et al., 2009; Table 1.2), while barley and oat diets have the lowest digestibility (Walker et al., 1994). Some unconventional starch ingredients like ancient grains millet or spelt have also been used in pet foods, and were also reported to be well utilized by dogs (Carciofi et al., 2010; Pezzali et al., 2020).

Processing conditions

Extrusion, thermal processing and milling degree

Starches in the presence of heat and water undergo a physical transformation called gelatinization. Extrusion is the most common pet food process (Spears & Fahey, 2004), and therefore it has received a greater research focus. Pet food extrusion is a medium to high moisture process that cooks the food matrix by heat generated from mechanical and thermal energies (Rokey, 2000). A process which results in nearly complete starch gelatinization along with a significant increase in digestibility(Svihus, Uhlen, & Harstad, 2005; Dust et al., 2004). The degree of gelatinization or starch cook is also affected by starch granule size (Chiotelli & Le Meste, 2002) and degree of milling (Bazolli et al., 2015).

The effect of cooking on starch digestibility is greater for some starch ingredients than others. Schünemann et al. (1989) reported that rice and corn were highly digested by dogs even in their raw form. Likewise, Moore et al. (1980) also observed that total starch digestibility by

adult dogs fed cooked (extruded) and uncooked rice and corn were similar (98.6% vs 98.0% for rice and 95.7% vs 94.3% for corn, respectively). In the same study, cooked oats had a higher digestibility compared to its uncooked form, but the difference was small (96% cooked vs. 94% uncooked). Wolter et al. (1998) also reported that both raw and gelatinized corn starches were well digested by dogs. Conversely, raw potato and tapioca were poorly digested by dogs (Schünemann et al., 1989; Wolter et al., 1998). Moreover, raw tapioca diet has been reported to cause an increase in intestinal transit time, a decrease in fecal pH, and diarrhea with mucous. This was possibly associated with anti-nutritional factors present in raw tapioca. However, thermally processed diets based on tubers have been reported to have high overall nutrient digestibility (Murray et al., 1999). Hence, data suggests that tubers should always be cooked to some extent, while cereals may be offered raw. To the best of our knowledge, there is no published data regarding feeding raw legumes to dogs. This is most likely due to the presence of protease inhibitors in legumes which are inactivated by heat. If this were not a factor, then one might extrapolate that legume starch would be poorly digested due to characteristics of the legumes cell walls and their starch granules (Bhattarai et al., 2017).

The extent of milling has a major role in starch and overall diet digestibility. As a general rule, coarser milled particles have less surface area to mass for starch enzymes adhesion and physical barriers limit the diffusion of the enzymes to starch. For example, Bazolli et al. (2015) reported that coarse ground corn and sorghum containing diets tended to be less digestible than the finely ground diets. In the same study, dogs fed finely ground rice had lower fecal lactate, suggesting that this cereal was almost entirely digested in the small intestine rather than escaping enzymatic digestion and being fermented in the colon. In contrast, dogs fed medium to coarse corn and sorghum diets produced feces with lower pH, lower concentrations of acetate and

propionate, and a higher concentration of butyrate. That study was one of the few that explored how different levels of grinding would affect starch conversion from extrusion processing and its impact on the animal. The uncooked starch resulted in increased RS and fecal SCFA proportions (Bazolli et al., 2015).

More recently, Peixoto et al. (2018) and Ribeiro et al. (2019) explored the combination of both grinding level and degree of extrusion cooking on Geriatric Beagle dogs. These authors produced two extruded corn-based dog diets: one coarsely ground with limited gelatinization (low SME; high RS), and the second finely ground with high gelatinization (high SME; low RS). The limited gelatinization may have been affected by increasing the extruder die opening, which would decrease resistance to flow and subsequently lower SME. In both studies there were positive effects on colonic health related to the less cooked food which indicates that a less processed (lower SME), coarser ground corn-based diet may yield more RS that could function as a prebiotic. It also suggests that the age-old practice of processing for the highest starch digestibility should be reconsidered.

Baking, pelleting and flaking

Pet food extrusion processing imparts two kinds of energy to the dough (thermal and mechanical), along with pressure, mixing, and addition of moisture (in-barrel moisture close to 30%; Baller, Pacheco, Peres, Monti, & Carciofi, 2018; Bazolli et al., 2015; Koppel, Gibson, Alavi, & Aldrich, 2014). Baking differs in that it only imparts thermal energy (Koppel et al., 2014). Thus, it is expected that baking leads to a lower conversion of starch. For this reason, it is anticipated that starch in baked foods may be less digestible than extruded diets due to the

absence of mechanical shear in this process. Though data in pet foods demonstrating this effect are unavailable.

Mild food processes like pelleting have a small effect on starch gelatinization when compared to extrusion or baking. Although pelleting is mostly used for livestock feed production and is uncommon in pet food, İnal et al. (2018) explored the utilization of iso-nutritional pet foods processed by pelleting and extrusion. They reported that the extruded diet contained 4 times more gelatinized starch than the pelleted form. As a result, extruded diets were better digested (84.2 vs. 81.2% DM digestibility, respectively) and were preferred (preference rate 66 vs. 34%, respectively) over pelleted diet.

Other less common processes that may be used to produce pieces of meal-type pet foods are toasting or flaking. One study found that starch gelatinization of green peas can be improved by soaking, toasting and infrared radiation, and was highly influenced by toasting prior to flaking (Yang, Zandstra, & Van Der Poel, 2008). With proper processing to reduce anti-nutritional factors and increase digestibility, dry peas can be efficiently used in dog diets (van Zuilichem & van der Poel, 1989).

Food matrix factors affecting starch utilization

A complete pet food contains all types of ingredients and their accompanying nutrients. There is the potential that non-starch ingredients and nutrients may interfere with starch digestion. If we simply consider the non-starch polysaccharide components such as soluble fibers, there is an increase in digesta viscosity and insoluble fibers may lead to mechanical irritation on the large bowel which could lower overall nutrient absorption (McRorie & McKeown, 2017). Cellulose is an insoluble (non-fermentable) fiber commonly used in pet foods

and has been reported to decrease starch and overall digestibility (Burrows et al., 1982; Kienzle, Dobenecker, & Eber, 2001). Similarly, Carciofi et al. (2010) reported that fiber rich fractions such as rice bran and wheat bran had the lowest nutrient ATTD by dogs compared to whole cereals. Alvarenga & Aldrich (2018) also found that an extruded dog diet formulated with close to 66% sorghum mill-feed fraction (high in insoluble fiber, lower starch content than other diets) dramatically reduced digestibility when fed to Beagle dogs.

Whole cereal and isolated flour digestibility differ due to the matrix surrounding the starch granules (Svihus et al., 2005). Flour ingredients tend to be more digestible because most of the bran and germ (mostly fiber, protein and lipids) are removed during milling; whereas, in whole cereals the lipids such as free fatty acids and phospholipids are present. These can limit granule swelling due to lipid hydrophobicity (Vasanthan & Bhatty, 1996), and can also form amylose-lipid complexes which resist α-amylase digestion (Holm et al., 1988). Additionally, phosphate monoesters present in potatoes (at a 0.09% level) were reported to increase paste viscosity and decrease gelatinization temperature of starch (Jane, 2009). This may have implications on other processing parameters in extrusion, such as screw speed and water or steam additions, which would require adjustments when tubers are included in large proportions.

Anti-nutritional factors present in some starch ingredients may decrease starch digestibility by dogs. Alpha-amylase inhibitors have been found in wheat, rye, triticale and sorghum, but not in rice, barley and corn (Schuppan, 2017; Saunders, 1975). Anti-nutritional factors are also present in tubers (Rekha & Padmaja, 2002) and legumes (Alonso, Aguirre & Marzo, 2000). Thermal processes are known to inactivate anti-nutritional factors to nearly 100% (Schuppan, 2017; Rekha and Padmaja, 2002; Alonso et al., 2000). In the work by Shünemann et al. (1989) the poor digestibility of raw potato starch may be explained in part by active anti-

nutritional factors. Besides alpha-amylase inhibitors, antinutritional compounds like trypsin inhibitors or more toxic compounds such as hydrocyanic acid (Prussic acid) results in cyanide release and dramatically affect performance. One study with dogs in Nigeria reported that a diet based on cassava had similar nutrient ATTD compared to the control, which demonstrated that the cyanide present in raw cassava was vaporized by wet heat treatment (Kamalu, 1991). Thus, there is a safety consideration to cooking tubers, legumes and some cereals independent of the starch utilization.

In vitro digestion methods

In vitro digestibility methods can be a valuable tool to evaluate starch digestion in ingredients and complete foods. *In vitro* methods provide a controlled glimpse into mechanisms that can be later evaluated *in vivo*. The *in vitro* methods are usually less expensive, less labor intensive, more rapid, and allow a greater number of samples to be evaluated. Though there are the limitations to the amount of information that can be obtained, and whether or not they truly reflect the animal's response. For example, there are several physiological parameters including gastric residence time, small intestinal passage rate, blood glucose clearance, hormones involved in digestion and individual variations in which the *in vitro* digestion does not consider (Dhital et al., 2017). Despite these potential shortcomings, there are many reports regarding *in vitro* starch digestion. When interpreting the results, it is crucial to understands that the outcome depends on the method chosen and varies due to incubation time, temperature, pH, buffers, enzyme activity and concentration, and other experimental factors such as mixing.

Starch *in vitro* digestion estimates the amounts of digestible starch and RS by difference. One can also classify digestible starch into rapidly (RDS) and slowly (SDS) digested starches.

The procedure described by Muir & O'Dea (1992, 1993) to determine digestible and RS were validated against *in vivo* data from humans with indwelling ileostomy and were correlated best after 15h of *in vitro* digestion. Using this as a starting point, research with dogs (Murray et al., 1999) considered the undigested starch after 2.5h, 15h and 24h for the RDS, SDS, RS which was then compared to *in vivo* results from ileally cannulated dogs. Their *in vitro* assay had slightly higher results (Table 1.3). Gajda et al. (2005) conducted a similar study; wherein, a conventional and high protein corn diet fed to ileal cannulated dogs were reported to have a starch ileal digestion comparable to that measured *in vitro*. However, the dogs which were fed amylomaize and high-amylose diets had a low starch *in vivo* digestibility (average 63.5%). Further, high-amylose corn (as a RS source) had poor *in vivo* digestibility and little fermentation, so the authors recommended against its use in dog foods. These studies are the only reports to date that compared starch digestion in complete dog diets with both *in vivo* and *in vitro* procedures. A major limitation to *in vivo* starch ileal digestion is the invasive nature of the surgical procedure to insert the cannula, the ethics of altering the dogs, and the long-term implications for the duration of the dogs' life. This explains why most studies opt for *in vitro* models alone.

The benefit of using *in vitro* starch digestion models is to understand how a given starch ingredient might behave relative to other ingredients. For example, Bednar et al. (2001) studied the *in vitro* starch digestion profile of various cereal flours and found that these had a much lower proportion of RS than their milled counterparts. In the same study, legumes had more RS than cereals and flours, and *in vitro* ileal starch and OM digestion were both lower in the legumes. The intact cell wall structure surrounding the starch granules of both legumes (Dhital, Bhattarai, Gorham, & Gidley, 2016) and cereals (Bhattarai, Dhital, Mense, Gidley, & Shi, 2018) was reported to reduce *in vitro* digestion by restricting amylase access to the starch itself.

There is need for an official and validated method to determine RS in pet foods. Englyst, Wiggins, & Cummings (1992) were some of the first to describe a method to quantify RDS, SDS and RS fractions in human foods. Since that time the method has been modified by several authors. The concept presented by Englyst, Wiggins, & Cummings (1992) was that starches digested by an enzyme cocktail in less than 20 and 120 minutes of incubation correspond to RDS and SDS fractions, respectively, and the starch portion not digested after 2h is considered RS. There is a recently described procedure that considers a more physiologically relevant time period of 4h for human small intestinal total starch digestion (McCleary et al., 2020). A commercial enzymatic kit has integrated this method (K-DSTRS; Megazyme International Ireland Limited, Ireland) and matched RS values of both a pure starch sample and starch containing foods with RS collected from ileal cannulated human subjects (McCleary & Monaghan, 2002). In the future it would be valuable to validate this methodology against *in vivo* intestinal digestibility data from dogs rather than extrapolation from humans.

Effects of starches on dog health

Obesity and glycemic control

In developed countries like the US, obesity has been considered the most problematic and insidious nutritional disease in both humans and pets (dogs and cats). Dogs also suffer from obesity and related diseases, including type 2 diabetes mellitus, cardiovascular diseases, and certain types of cancer (Chandler et al., 2017). The prevalence of overweight and obese dogs in the US was estimated to be between 19.7- 59.3% (Chandler et al., 2017). In its simplest description obesity develops from ingestion of calories that outpaces expenditure. These calories

can be influenced by the proportions of carbohydrates, lipids and proteins. Obesity in dogs and cats can be effectively managed by conscientious pet owners that practice portion control and plenty of exercise. Yet, the rate by which those calories enter the circulation can prime other metabolic responses which influence the storage of glucose as adipose tissue and also alter fat mobilization once deposited.

Diets based on cereals with lower digestibility and a lower glycemic index may be beneficial for diabetic or obese animals (Kimura, 2013; Carciofi et al., 2008). This has implications on metabolic utilization, and (or) the amount of starch that escapes digestion. By lowering the degree of starch gelatinization, less will be digested in the small intestine (Inal et al., 2017), thereby decreasing the rate and amount of glucose absorption into the circulation. Conversely, high starch diets with highly digestible starches rapidly increase plasma glucose and insulin release, which may lead to insulin resistance (André et al., 2017) and consequent development of diabetes. Alternatively, diets with rapidly digested starches might benefit malnourished or ill dogs that require quick nutrient support. Thus, starch level, source, composition, and level of gelatinization should be considered relative to the animal's physiological condition.

Legumes are reported to have lower starch digestibility due to their starch granule structure which express a C-type X-ray diffraction pattern (Sandhu and Lim, 2008), as well as their cell wall structure (Bhattarai et al., 2017) and amylose characteristics (Shin et al., 2004). This may affect the rate at which glucose is absorbed in the SI and insulin is secreted by the pancreas. A study investigating the effect of adding legumes to cereal extrudates confirmed that legumes had a decreased glucose absorption rate during *in vitro* digestion (Patil et al., 2017). *In vivo* studies with dogs have demonstrated similar effects. Adolphe et al. (2012) reported that

dogs fed diets rich in peas had a lower glycemic index than diets with rice or barley. Carciofi et al. (2008) reported that sorghum and lentil-based diets had the lowest glucose time to peak and both glucose and insulin peaks were delayed relative to dogs fed corn, rice and cassava diets. Clearly, the type of starch influenced the glycemic response. Feitosa et al. (2016) also compared sorghum to corn in diets fed to obese dogs and observed that the dogs fed the sorghum-based diet had lower levels of fructosamine, which is a stable form of glycosylated protein that correlates with blood glucose level. In their work a breed effect was also observed; wherein, Dachshunds had both lower cholesterol and fructosamine than Beagle dogs. This suggests that glucose metabolism may not be uniform across canine breeds and should be evaluated in a wider cross section of the dog population to better understand the breadth of responses.

Beyond starch ingredient selection for glycemic response, other nutrients may interact or influence blood glucose and insulin. André et al. (2017) found that a high carbohydrate-medium protein diet fed to obese dogs had a greater post-prandial insulinemia and area under the curve for both insulin and glucose than a high protein-medium carbohydrate food. Some amino acids can be converted to glucose through gluconeogenesis, while starches are digested to glucose which is a direct energy substrate. In the same study, André et al. (2017) induced weight loss in dogs using the high protein-medium carbohydrate diet, and there was an improvement in insulin sensitivity. It would have been interesting to determine insulin sensitivity in dogs after losing weight with the high carbohydrate-medium protein diet as well. In addition, the study would have been more powerful if diets were formulated with the same ingredients. This study confirmed that there may be a benefit to shifting diet protein and starch composition.

In a study with 20 different cereal-based dog foods, Nguyen, Dumon, Biourge, & Pouteau (1998) found that both crude protein and starch contributed to an increase in the area

under the insulin curve, while crude fat tended to decrease it. Conversely, Hewson-Hughes (2011) found that a diet with 41.4 % NFE had the same effect as a diet with 12.3 or 31.0% NFE (starch replaced with protein and fat) on plasma glucose and insulin of dogs, suggesting that diet formulation did not affect glucose metabolism. However, when cats were fed the same diets, they had a higher plasma glucose concentration after 10h of feeding. Fat was expected to contribute to a lower glycemic response because glycerol (from the triacyclglycerol backbone) requires ATP to be converted into glucose, while starches are directly broken down into glucose in the intestinal lumen (Gropper & Smith, 2012).

Besides controlling glycemic response, fats may also induce satiety (Shauf et al., 2018). Schauf et al., (2018) determined the effects of feeding a high carbohydrate and a high fat diet at the same energy levels to Beagle dogs on levels of satiety hormones. Satiety hormone glucagon-like peptide 1 (GLP-1) was greater in plasma of dogs fed a high-fat diet at the fasting state and after 180 min of feeding, compared to the high carbohydrate diet. Thus, indicating that fat had a greater impact on satiety in the long term.

This theory for optimizing nutrition to control glycemia and ultimately weight could result from a low to moderate starch formulation with starch ingredients high in SDS and RS, and low in RDS, like barley (Murray et al., 2001), legumes (Bednar et al., 2001), or low glycemic index ingredients such as sorghum, peas and lentils (Carciofi et al., 2008). Once essential amino acid and fatty acid requirements are met, protein and fat might also be used as a means to control glycemia and insulinemia through gluconeogenic mechanisms. Fat also plays a role in long term satiety (Schauf et al., 2018), which is important to controlling calorie intake. In addition, fiber inclusion can dilute caloric density and lower nutrient absorption. These adaptations would complement efforts to control food intake.

Resistant starch and colonic health

The concept of RS has been a popular topic in nutrition for more than two decades, although only a few published studies in pets can be found in the scientific literature. Resistant starches include all starch or products of starch degradation that escape small intestinal digestion (Englyst et al., 1989; Muir and O'Dea, 1993). At present there are five types of RS described (Dupuis, Liu, & Yada, 2014; Fuentes-Zaragoza, Riquelme-Navarrete, Sánchez-Zapata, & Pérez-Álvarez, 2010). Resistant starch I (RS I) describes the starch that is physically inaccessible to enzymes due to the presence of cell walls (Hernández, Emaldi, & Tovar, 2008). This includes partially milled cereals which would contain a higher amount of RS I (Dupuis et al., 2014) than more finely milled cereals. Resistant starch II (RS II) are native starch granules that are protected from digestion by the conformation or structure of the starch granule (greater in ungelatinized or uncooked starch). Food processing with heat and moisture reduces RS due to starch gelatinization, but upon cooling a portion of starch may retrograde and yield RS III (Faraj, Vasanthan, & Hoover, 2004). Limited resistant starch III may be formed during cooling and drying after extrusion, since starch needs some moisture and storage time to recrystallize (Kim, Tanhehco, & Ng; 2006). Resistant starch IV comprises starches chemically modified and are uncommon or nonexistent in pet foods. Finally, RS V is an amylose-lipid complex which forms when the fatty acid hydrophobic tails from lipids associate with the internal hydrophobic helix of amylose which has leached from gelatinized starch (Ai et al., 2013). These complexes restrict the ability for enzymatic hydrolysis.

Dhital et al. (2017) proposed a new classification regarding the digestion mechanism of resistant starches. There are two stages of starch digestion that determine its resistance; the first

is the rate at which the enzyme binds to substrate, or its access; and the second is the rate at which substrate is converted to product. Starch can also be resistant due to a combination of these two stages. It is important to understand that every starch, with exception of chemically modified starches, can be entirely digested given the right conditions of time, environment and combination of exo- and endoenzymes (Dhital et al., 2017). For this reason, the physiological or *in vitro* conditions are essential to their classification as resistant starch.

Functionally, RS were reported to promote satiety and decrease post-prandial plasma glucose and insulin peaks in pigs (Da Silva et al., 2014), and to stimulate production of SCFA with emphasis on butyrate in dogs (Beloshapka, Alexander, Buff, & Swanson, 2014; Peixoto et al., 2018; Ribeiro et al., 2019; Jackson et al., 2020), cats (Jackson, Waldy, & Jewell, 2020), and pigs (Umu et al., 2015; Haenen, Zhang, Souza da Silva, et al., 2013). Most studies have measured endpoints such as pH and SCFA as indicators of fermentation (Murray et al., 1998; Bednar et al. 2001; Murray et al. 2001 ; Spears and Fahey 2004 ; Gajda et al. 2005; Goudez, Weber, Biourge, & Nguyen, 2011; Beloshapka, Alexander, Buff, & Swanson, 2014; Jackson, Waldy, & Jewell, 2020; Jackson et al., 2020), few have explored the effect of RS on glycemic response (Kimura et al., 2013; Ribeiro et al., 2019), and only a few on the microbiome (Jackson, Waldy, Cochrane, et al., 2020; Beloshapka et al., 2014) and metabolomics (Jackson, Waldy, Cochrane, et al., 2020). Glycemic response is an important metabolic consideration. Kimura (2013) found that a canine glycemic response of β-cyclic dextrin was much lower than that of a soluble starch, suggesting that ingestion of resistant starches has the potential to benefit glycemia and possibly reduce the incidence of type 2 diabetes mellitus. Though this is speculation and would need to be confirmed.

Resistant starches can be fermented by GI microbes which produce SCFA including acetate, propionate, and butyrate, among others. It is desirable that butyrate concentration increases because it is almost entirely used by colonocytes as an energy source, whereas acetate and propionate are transported to the liver through the portal vein (Bergman, 1990; Haenen et al., 2013) to be converted to energy substrates. Further, butyrate is thought to inhibit division of cancer cells and to stimulate the proliferation of colonic mucosal cells (Barnard & Warwick, 1993). These SCFA also decrease colonic pH, which directly affect the microbiome composition selecting more beneficial bacteria at the expense of the pathogenic strains (Hooda, Vester Boler, Kerr, Dowd, & Swanson, 2013; Murray et al., 2001; Vernia et al., 2003). Hence, butyrate benefits colonic health.

Understanding the properties of starches that make them resistant to digestion, how they are utilized, as well as ways to retain RS during food manufacturing is of substantial interest. The first study to determine the effects of different extrusion cooking levels on starch availability of common pet food cereal ingredients *in vitro* reported that low processed ingredients did not favor production of butyrate (S. M. Murray et al., 2001). But, there were increases in total SCFA from fiber and starch fermentation using dog ileal chyme. From all the ingredients tested, whole barley, oats and potato flour led to the most SCFA *in vitro* production using dog ileal chime for fermentation (Bednar et al., 2001, Murray et al. 2001).

Studies *in vivo* with both dogs and cats have reported increased fecal butyrate when these animals were fed RS II from corn and rice (Jackson, Waldy, & Jewell, 2020; Jackson, Waldy, Cochrane, et al., 2020; Ribeiro et al., 2019; M. C. Peixoto et al., 2018). Hence, there is indication that mildly processed foods with cereals retain RS and benefit health by increasing butyrate production. Peixoto et al. (2018) attempted to retain RS in extruded products by controlling the

extruder die opening and grind size. Their low mechanical energy processing combined with coarse particles size did not fully cook the starch; thus, retaining some as raw which is high in RS. Although only a small portion of RS was retained (1.46%), they observed improved local immunity and increased fecal SCFA with higher butyrate concentration when dogs were fed the high RS treatment. Similarly, Ribeiro et al. (2019) produced two corn-based diets with a low and high RS (0.22 vs 1.53%, respectively). In their study, dogs fed the high RS diet produced feces with lower pH, indicating more fermentation, and there was an increase in acetic (16.6 %), propionic (45.1%), and butyric (54%) acid concentrations. Further, lowered mean blood glucose was only noted in older dogs.

In a recent publication, Jackson et al. (2020) found that a diet produced with low extruder shear (high RS) by decreasing extruder shaft speed led to greater fecal butyrate concentration after 6 weeks of feeding relative to an identical recipe produced with high shear (low RS). Metabolomics results indicated that feces from dogs fed the low shear food with more starch derived glucose, maltotetraose and maltotriose. This led to a greater bacterial species richness (alpha-diversity) and a difference in beta-diversity. Twelve bacterial classes were altered in dogs and six microbe genera were correlated with at least three of the RS derived sugars. Most changes were related to an increase in bacteria from the phyla Firmicutes for those dogs fed the high RS diet. In their study there was no direct measure of RS in the foods; rather, it was estimated from viscosity measured by a rapid visco analyzer (RVA) procedure.

Resistant starches may also be provided as supplements rather than being an intrinsic part of the food. Beloshapka et al. (2014) studied the effects of supplementing biscuits containing 0 g, 2.5 g, and 5 g RS on the gut health of Miniature Schnauzer dogs. When dogs were fed up to 2.7% (5 g) RS per day, no treatment effects on fecal output, fecal scores, fecal SCFA, ammonia

and (or) microbiota composition were observed. As mentioned above, other researches have reported positive gut effects from cereal RS consumption (retained by mild extrusion processing) by dogs at a much lower concentration (Jackson, Waldy, Cochrane, et al., 2020; Ribeiro et al., 2019; M. C. Peixoto et al., 2018). This suggests that the source and type of RS are relevant and present different fermentation characteristics. None of this information was provided by Beloshapka et al. (2014), so it is difficult to draw conclusions as to why there were no dietary effects when dogs consumed up to 2.7% RS per day.

There is evidence that starch fermentation in the colon depends on both dog breed and source. Cutrignelli et al. (2009) studied the fermentation pattern of starch substrates using fecal inoculum in two dog breeds: German Shepherd dogs and Neapolitan Mastifs. Substrates rich in rice and potato were more readily fermented than corn and spelt, and feces from the Neapolitan Mastifs had the greatest organic matter disappearance, as well as faster fermentation rates.

The ability to efficiently ferment RS in the colon may be related to dog size. Goudez et al. (2011) found that breed was a significant factor in stool quality by supplementing the basal diet with RS from high-amylose corn to small and large breed dogs. Regardless of the RS supplementation (2.5-7.4%), fecal scores of the Miniature Schnauzers were always optimal, whereas the Giant Schnauzer and German Shepherds (larger dogs) had soft stools in direct proportion to RS intake. Large dogs seemed to be more sensitive and quite possibly should not be fed diets with more than 2.5% RS based on this research.

To our knowledge, there is only one study with dogs that compared treatments with more than one type of RS (Murray et al., 1998). In this work, dogs were fed three enteral formulas with the same composition except for the carbohydrate source; a control with maltodextrin, a de-branched amylopectin-lipid complex (V-complex) made of corn oil and monoglyceride, and a

retrograded high-amylose corn starch (RS III). The RS III treatment had lower starch ileal digestibility as expected, and crude fat digestibility was lowest when dogs were fed the V-complex diet, possibly because fat was partially trapped within debranched amylopectin. Stool production increased and were softer when dogs were fed the RS III diet. This suggests more extensive fermentation, although fermentation end-products were not measured. It would be interesting to determine how these different sources of RS (III and V) affect the microbiota.

Resistant starches in pet diets have not yet been fully explored. Studies focused on the effects of different types and sources of RS on the colonic microbiome and fermentation products are missing. Also, studies reporting the effects of RS on satiety hormones in dogs are lacking. Further, the recommended amount of RS for each dog size (small, medium and large) has not yet been determined, but it is known that large breeds are more sensitive to colonic fermentation. Lastly, pet food processes such as baking could be utilized to improve RS yield, since the biscuits are cooked with a single energy source (thermal). Although extrusion is a highly efficient cooking process, it effectively eliminates RS in commercial foods (Corsato Alvarenga & Aldrich, 2020). This low yield (~1.5%) of RS in the dry kibble after extrusion cooking was enough to promote some gut health in medium sized dogs (Peixoto et al., 2018; Ribeiro et al., 2019; Jackson et al., 2020).

Conclusions

Cereals, tubers and legumes are important starch containing ingredients in dry dog foods. Their starch aids texture development and expansion of extruded kibbles and provides viscosity and binding to other food forms. The starch from cereals can be a readily available and economical source of energy. While not nutritionally essential per se, dogs do have a metabolic

requirement for glucose which can be met either by dietary starch directly from absorption or through gluconeogenesis. Genomic research reveals that the dog has evolved to be more amenable to starch-rich diets than wolves. All natural starch sources can be effectively utilized by dogs with some cooking. The digestibility of most cereal starches is modestly improved by thermal processing, while tuber and legume seed starch digestibility is greatly improved. Legumes tend to be more resistant to digestion due to their starch granule structure and surrounding cell walls. Factors including amylose: amylopectin ratio and interaction with other nutrients may also affect the extent of digestion. Moreover, ingestion of slowly or resistant to digestion starches may help control glycemia and insulinemia. Resistant starches also modulate the growth of beneficial bacteria in the colon, which generate SCFA and improve colonic health. In the future, resistant starches should be better characterized and incorporated in commercial pet foods in order to positively affect canine health.

There is still much to explore about resistant starches and its health benefits in dogs, as well as process inputs required to retain RS in foods in a predictable manner. Obese and diabetic dogs may benefit from a diet with low to moderate starches, with the selection of low glycemic index starch ingredients like legumes, some tubers and some cereals. Conversely, dogs needing a rapid energy intake may benefit from a high starch diet with rapidly digestible starches. Studies regarding utilization of beans, pulses, and ancient grains by dogs are lacking, as well as studies with more food processes beyond extrusion. Knowledge about nutrition availability, food processing, and ingredient physical characteristics are essential to match ingredient, nutrient, and product to produce high quality nutrition that dogs and their owners appreciated.

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Table 1.1.

Differences in Nutrient Digestion and Genes related to Glucose Absorption or Digestion between the Dog and the Wolf.

Digestion and Genes	Dog vs Wolf	Reference
DM[1] digestibility	69.9% dog, 64.7% maned wolf	Childs-Sanford & Angel (2006)
Energy digestibility	3,641 kcal/kg dog, 3,331 kcal/kg maned wolf	
Transit time	14.2 h dog vs 13.5 h maned wolf to recover 50% titanium dioxide	
α-amylase gene (AMY2B)	Expression 2 to 8-fold higher in the dog	Ollivier et al., 2016
	Higher activity in the dog	Axelsson et al., 2013
Glucose uptake gene (SGLT1)	Higher activity in the dog	
Maltase-glucoamylase gene (MAGAM)	Higher activity in the dog	

[1]Dry matter.

Table 1.2.

Dry Matter (DM) Apparent Total Tract Digestibility (ATTD) and Starch Ileal Digestion of Different Starch Ingredients in Extruded Diets by Dogs.

Starch Ingredient	Starch Ingredient Inclusion in the Diet (%)	Dog Breed	DM ATTD, %	Starch Ileal Digestion, %
Rice (brewers)	67.0[a], 47.1[b], 59.6[c], 30.0[d], 45.7[e], 77.9[g], 70.5[h], 44.1[i], 52.1[k]	Mongrel dogs[a], Beagle dogs[b,c], Mixed-breed[d,e,g**,k], Spitz dogs[h], Hound bloodline[i]	91.5[a], 79.0[b*], 89.2[c], 86.6[d], 82.4[e], 90.9[g], 89.4[h], 83.9[i], 94.0[k]	99.5[a], 99.8[i]
Corn (maize)	67.0[a], 53.5[b], 66.7[d], 53.5[e], 31.9[f], 70.5[h], 43.6[i], 34.0[j], 53.5[k], 50.0[l]	Mongrel dogs[a], Beagle dogs[b,j,l], Mixed-breed[d,e,k], Spitz dogs[h], Hound bloodline[f,i]	87.2[a], 82.1[b*], 84.9[d], 78.6[e], 77.1[f], 83.8[h], 85.4[i], 82.2[j], 92.0[k], 91.9[l]	99.4,[a] 89.9[f], 99.5[i]
Sorghum	53.4[b], 30.0[d], 59.3[e], 70.5[h], 44.2[i], 55.2[k], 64.7[m]	Beagle dogs[b,m], Mixed-breed[d,e,k], Spitz dogs[h], Hound bloodline[i]	79.9[b*], 86.6[d], 79.0[e], 83.1[h], 79.7[i], 94.0[k], 81.1[m]	99.7[i]
Millet	30.0[d], 70.5[h]	Mixed-breed[d], Spitz dogs[h]	85.2[d], 81.6[h]	n.a.
Wheat	49.1[i***]	Hound bloodline[i]	83.5[i]	99.8[i]
Barley	67.0[a], 51.9[i]	Mongrel dogs[a], Hound bloodline[i]	84.6[a], 82.5[i]	98.8[a], 99.4[i]
Oats	67.0[a]	Mongrel dogs[a]	70.4[a]	98.5[a]
Cassava	42.5[e], 70.0[g], 50.0[l***]	Mixed-breed[e,g**], Beagle dogs[l]	83.1[e], 91.0[g], 91.2[l]	n.a.
Potato	50.4[i***]	Hound bloodline[i]	83.6[i]	99.6[i]
Lentil	69.5[e]	Mixed-breed[e]	74.5[e]	n.a.
Pea	66.3[e]	Mixed-breed[e]	76.1[e]	n.a.

[a] Walker et al., 1994
[b]Bazolli et al., 2015; *values reported from grain medium grinding (451 um) in this study.
[c]Belay et al., 1997
[d]Carciofi et al., 2010
[e]Carciofi et al., 2008
[f]Gajda et al., 2001
[g]Kamalu et al., 1991; [§]6-week old dogs
[h]Kore et al., 2009 (don't specify if they use brewers rice, or other type of rice)
[i]Murray et al., 1999; ***Starch sources used as flours.
[j]Schauf et al., 2018
[k]Twomey et al., 2003
[l]Wolter et al., 1998; ***wet diets on DM basis (%)
[m]Alvarenga and Aldrich, 2018

Table 1.3.

In Vitro vs in Vivo Starch Digestion Methods of Extruded Kibbles (reported on a Dry Matter basis).

Authors	**Treatments**	***In Vitro* Method**	***In Vitro* RS Results**	***In Vivo* Method**	***In Vivo* RS Results[1]**
Murray et al., 1999	Extruded diets with different starch ingredients	Muir and O'Dea (1992, 1993)	Barley: 1.2% Corn: 1.1% Potato: 1.2% Rice: 1.0% Sorghum: 1.3% Wheat: 0.9%	Six purpose-bred Ieally cannulated mature female dogs with hound bloodlines	Barley: 0.62% Corn: 0.47% Potato: 0.43% Rice: 0.18% Sorghum: 0.33% Wheat: 0.22%
Gajda et al., 2001	Extruded diets with different corn hybrids	Muir and O'Dea (1992, 1993)	[2]CONV: 9.9% [2]AM: 15.3% [2]HA: 13.5 % [2]HP: 9.9 % [2]HPLP: 10.0 %	Five purpose-bred Ieally cannulated female adult dogs with hound bloodlines	[2]CONV: 10.1% [2]AM: 37% [2]HA: 36% [2]HP: 10.5 % [2]HPLP: 17.7%

[1]Calculated by subtracting 100% - starch ileal digestion.
[2]CONV: conventional yellow dent corn; AM: amylomaize; HA: high-amylose; HP: high-protein corn; HPLP: high protein, low phytate corn.

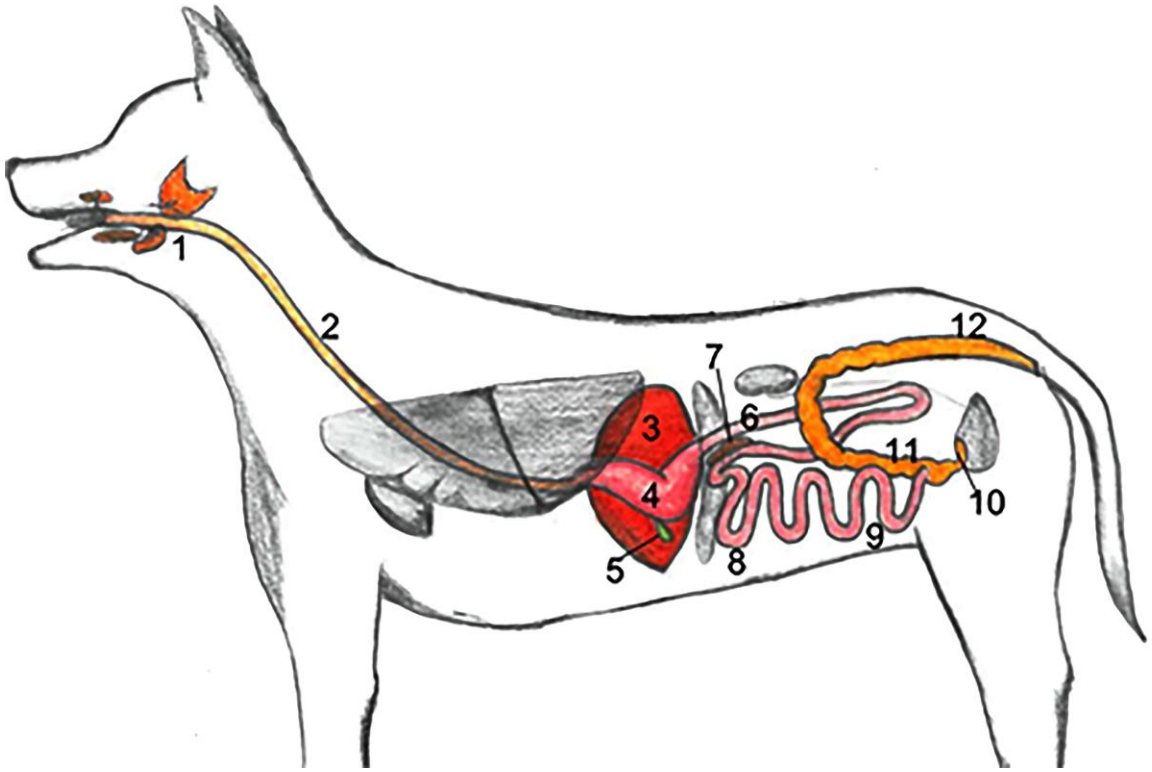

Figure 1.1

Schematic drawing of the dog's digestive system. 1: salivary glands; 2: esophagus; 3: liver; 4: stomach; 5: gallbladder; 6: duodenum; 7: pancreas; 8: jejunum; 9: ileum; 10: cecum; 11: large intestine; 12: rectum.

Chapter 2 - **Starch characterization of commercial extruded dry pet foods**

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Abstract

Starches provide an effective energy source for dogs and cats and can affect health according to its inclusion and extent of digestion. The starch fraction that escapes small intestine (SI) digestion is called resistant starch (RS) and is desirable due to its prebiotic function. Starch is not an essential nutrient for dogs and cats and thus is not reported on commercial pet food labels. Hence, the objective of this work was to characterize starches in commercial pet foods. The top five pet food companies by sales were selected to represent US pet foods, which were divided into four strata with a sampling frame of 654 foods: dog grain based (372 foods), dog grain free (71 foods), cat grain based (175 foods), and cat grain free (38 foods). Five random foods within each stratum were purchased (20 total). Starch analyses (total starch, resistant starch and starch cook), as well as nutrient analyses were conducted on all foods. Total starch, RS and starch cook means were compared using a 2-group Z-test on dog vs cat and grain-based vs grain-free diets, and differences were considered significant at a $P < 0.05$. Total starch was higher ($P < 0.05$) in dog than cat food, and starch cook was greater ($P < 0.05$) in grain-free diets. A regression analysis showed that NFE was a good predictor of total starch. Resistant starch was low and not different among groups. A post-hoc test showed that a total sample size of at least 28

diets per group would be required to detect differences in RS between GF and GB diets, if one exists.

Keywords Pet food, extrusion, grain-free, starch, gelatinized, resistant starch.

Introduction

The US pet food industry is a growing market expected to exceed USD 30.01 billion by 2022 (Zion Market Research, 2017). Most dogs and cats are fed dry food (9.2 billion US dollars in sales in 2014; Statista, 2016), and the greatest part of it is produced through extrusion. This type of processing involves cooking with steam, water and shear. It also requires some amount of structure forming ingredients like starches (Guy, 2001) to promote food particle binding, texturization, improvement in palatability, and to aid in expansion of the kibble.

Starch is not an essential nutrient for dogs and cats, but it can impact health in different ways according to its inclusion, type, and processing. The more cooked or gelatinized, the more rapidly the starch is digested (Murray et al., 2001). This has implications on metabolic utilization, and (or) the amount of starch that escapes digestion. Rapidly digested starches can promote high blood glucose/insulin peaks with subsequent fat deposition (Coulston et al., 1983). Conversely, the indigestible starch, or resistant starch (RS) can serve as substrate for colonic fermentation yielding short-chain fatty acids of which butyrate can be used as a direct energy source for colonocytes (Bergman, 1990; Haenen et al., 2013). Starches may be inaccessible to digestive enzymes due to their tightly packed physical conformation, or physical barriers associated with the granule like cell walls and protein bodies (Dhital et al., 2017).

There are differences in the digestion profile among starch sources for a number of reasons. For example, common cereals like corn, rice or wheat can have polyhedral and (or) oval

starch granules which contain pores and channels that create adhesion sites for hydrolytic enzymes (Dhital et al., 2017). Some cereals like sorghum may be more difficult to digest than corn or rice due to tight bonding of protein bodies to the starch granule. Similarly, legume seeds are known to be high in naturally occurring RS, partly because their starches are trapped inside the cotyledon cell parenchyma (Berg et al., 2012; Würsch, 1986). Tuber starches like potato may also have some resistance to enzymatic digestion because its granules are large and smooth (Martens et al., 2018; Dhital et al., 2017). However, all these reports have been conducted with starch ingredients alone, but pet foods are composed of other ingredients which are ground, mixed and then cooked or processed in some manner. Due to morphological differences in starch ingredients used in pet foods and the interference of other ingredients and processing, it would be valuable to characterize these food starches in a complete food.

There is no information required on pet food labels regarding starch percentage, extent of digestion, and (or) resistant starch concentration. Starch is not required nor allowed on the guaranteed analysis by current labeling regulation (AAFCO, 2019). Typical carbohydrate levels (starches and fibers) in dry extruded dog foods range from 30-60%, while starches in commercial cat foods are included up to 35% on a DM basis (Gross et al., 2010). Nutritionists and pet food scientists commonly estimate starch content using the NFE (nitrogen-free extract) calculation (NFE= 100 – moisture – crude fiber – crude protein – crude fat – ash; Gross et al., 2010). This equation may overestimate starch content, as most of the nutrient analysis in pet foods are crude estimates of their true value and may not account for their total contribution. Knowing the true starch content of pet foods, and how much of it is digested, would be valuable information for diet development and future research. No research has been published previously characterizing and comparing the various starch components and methodologies of analysis in commercial

complete pet foods. Further, there are no studies comparing the digestible starch and RS of grain-based foods and those containing elevated levels of tubers and legumes as their sole starch sources. Thus, the objective of this study was to determine the total starch content and its fractions (digestible and resistant starches) in dog and cat foods, and those that are grain-free (GF) and grain-based (GB) diets sold in the USA. The hypotheses were: 1. Dog foods would contain more starch than cat diets; 2. Extruded foods would be extensively cooked to a point that resistant starch would be almost nonexistent and thus insufficient to promote colonic health; and 3. GF diets would have more resistant starch in comparison to GB diets.

Materials and methods

Sample selection

The top five pet food companies by sales (Pet Food Industry, 2017) were selected to represent the majority of US pet foods in this study. These companies, in decreasing order of sales, were: Mars Petcare, Nestlé Purina Petcare, Big Heart Pet brands, Hill's Pet Nutrition and Blue Buffalo (Pet food Industry, 2019). A list of all dry complete extruded pet foods, excluding prescription diets, of the top five companies was created. Pet foods were divided into four strata with 654 foods composing the sampling frame: dog GB (372 foods), dog GF (71 foods), cat GB (175 foods), and cat GF (38 foods). Four lists with 10 random samples within each stratum were created using a randomization program. These lists were taken to pet stores in Manhattan, KS, and 5 foods present in the list within each stratum were purchased (20 total), according to store availability (Table 2.1). Grain based diets contained combinations of brewers rice (8 foods), brown rice (2 foods), corn (5 foods), wheat (3 foods), barley (4 foods), oats (4 foods), and some also included peas and potato starch. The GF foods had one ingredient or a combination of some

of the following: peas (5 foods), pea starch (4 foods), sweet potatoes (4 foods), potatoes (4), potato starch (1 food), tapioca starch (3 foods), chickpeas (2 foods), and lentils (1 food).

Nutrient analysis

All food samples were ground to 0.5 mm in a laboratory fixed blade impact mill (Retsch, type ZM200, Haan, Germany) prior to nutrient analyses. Ash (AOAC 942.05), nitrogen (AOAC 990.03; multiplied by 6.25 factor to estimate crude protein), fat by acid hydrolysis (AOAC 954.02), and total dietary fiber (TDF; TDF-100A kit; Megazyme International Ireland Limited, Ireland) were measured on each sample in order to determine NFE, using the following calculation: NFE = 100(%)- ash(%) – moisture(%) – protein(%) – fat(%) – TDF(%). Total starch and starch fractions (resistant and digestible starches) were analyzed with enzymatic digestion followed by colorimetric assays using kits (Total Starch Assay kit & Resistant Starch Assay kit, respectively; Megazyme International Ireland Limited, Ireland). Total starch was reported as both measured (using the total starch assay kit) and calculated (sum of digestible and resistant starches quantified by the resistant starch assay kit). Starch cook was analyzed by an enzymatic procedure as described by Mason et al. (1982). Briefly, two samples were prepared. One was boiled for 20 min with DI water. The second was equilibrated with DI water at 25ºC for 20 min. Then, buffer was added along with glucoamylase enzyme solution to both samples and they were incubated for 70 min at 40ºC. Free glucose in each sample was measured using a biochemistry analyzer (YSI 2900D, Xylem Analytics, Ohia, U.S.A.), and the level of gelatinization (%) calculated as a proportion of free glucose in the tested sample (gelatinized) to the free glucose in the boiled sample (total starch).

Statistical analysis

The study was conducted using stratified random sampling. The averages of each analysis within each stratum were calculated according to Lohr (2009). Treatment means were compared using a 2 group Z-test with a significance level of $\alpha = 0.05$. A regression analysis was conducted between dietary starch content measured by the total starch procedure (total starch measured) vs resistant starch procedure (total starch calculated = digestible + resistant starch), and NFE vs total starch measured, using the proc reg procedure of Statistical Analysis Software (SAS, v. 9.4; Cary, NC).

Results & discussion

The premise of this work was to characterize starch in commercial pet foods to gain some understanding of what is typical regarding starch components to aid further diet development, and to conduct future research in this area. Nutrients were measured in all diets in order to determine NFE, which is a common and rapid method to estimate starch content in diets. As expected, all nutrient levels met the specified guarantees identified on the label (information not shown). Nitrogen-free extract was calculated using TDF instead of crude fiber, which consists of a more accurate measurement of fibrous components in the food. The regression analysis between total starch measured by the total starch assay kit (Megazyme International Ireland Limited, Ireland) and NFE ($P < .0001$) was:

$$NFE = 1.04 \times TS + 3.50$$

The adjusted R^2 and standard error of this regression analysis were 0.94 and 0.0601, respectively. This indicates that NFE correlates well with total starch and thus it is a good

estimation of starch content. Likewise, total starch calculated (TS_{calc}) was also highly correlated to total starch measured (TS):

$TS_{calc} = 0.944 \times TS + 2.08$; adjusted R^2=0.91, standard error= 0.0675.

The first hypothesis stated that dog foods would contain more total starch than their feline counterparts, due to cats' obligate carnivore nature and higher requirement for protein (NRC, 2006) which would result in a lower starch concentration in their diet. This was confirmed; wherein, total starch measured and calculated in cat diets were lower ($P< 0.05$) than dog diets (Table 2.2). The total starch (measured) difference between dog and cat foods, with 95% confidence, was estimated to be between 2.26% and 9.87% within the studied sampling frame. When grouping treatments as GB vs GF, total starch levels were not different.

In the present study we found that commercial diets averaged above 87.5 % starch cook, and there was a difference ($P < 0.05$) in starch cook between GB and GF diets (87.5 vs 94.1%, respectively). Tubers compose a large fraction of GF diets, and it was expected that tuber starches would have a greater degree of cook, since they have a higher water solubility index (Nuwamanya et al., 2011; Mishra and Rai, 2006) and gelatinize at a lower temperature than cereals (Mishra and Rai, 2006). Pezzali and Aldrich (2019) found that a GF dog food with a blend of tapioca starch, potato and peas required lower extruder thermal energy to produce kibbles with similar bulk density when compared to an ancient grain diet (composed of spelt, millet and sorghum), and the degree of starch cook of the GF treatment was also high and comparable to the present study (96.8% vs 94.1%, respectively).

An important premise of this study was to determine the average level of RS in commercial diets. In order to compare starch fractions of different formula diets, digestible and resistant starches were calculated as a percentage of the total starch, so they would be on the

same basis. The second hypothesis stated that commercial extruded diets would be low in RS, and indeed the RS levels of all commercial diets were observed to be less than 1% of the starch content (0.945 vs 0.703% resistant starch in dog vs cat diets, respectively; Table 2.1). This may not be sufficient RS to promote colonic health. Peixoto et al. (2018) were able to detect positive differences in colonic fermentation with 1.46% RS as a percent of total kibble weight, which increased butyrate production and improved nutrient absorption. Another beneficial effect from resistant starch is the reduction of the glycemic index of the food (Kimura; 2013), which decreases the rate of insulin release and positively impacts health. This can help reduce the incidence of obesity and type 2 diabetes.

The third hypothesis was that GF would have more RS than GB diets. Tubers and legumes are common ingredients in GF diets, and they are known to have some resistance to α-amylase digestion due in part to low or absent starch granule pores, while most cereal starches have pores and channels that increase surface area for enzyme adsorption (Martens et al., 2018; Dhital et al., 2017). Also, most legumes have a protein matrix tightly bonded with starch granules, which create a physical barrier to enzymatic digestion (Dhital et al., 2017; Berg et al., 2012). Moreover, type C starch present in legumes has a lower swelling capacity than cereals or tubers (Wani et al., 2016), and a higher amylose content (Martens et al., 2018), which contribute to enzymatic resistance. Hence, one would expect GF diets to be higher in RS than a GB recipe. However, in the present study there was no difference ($P > 0.05$) in RS between GB and GF diets (0.83 vs 1.06%, respectively). Although RS content was numerically greater in grain-free diets, the analytical technique employed has a high degree of variation when RS levels are below 2%. This high variability could influence the ability to detect differences. A retrospective power analysis (post-hoc) using RS as the endpoint of GF vs GB diets, showed that statistical analysis

of the present study (using 10 diets as sample size) resulted in a power of only 0.52. This means that the study had a 52% probability to correctly lead to rejection of a false null hypothesis (RS in GB = RS in GF diets). In order to obtain a power of 0.80, with a significance level of 0.05, it would require 28 observations per treatment to detect some difference, if one exists. It is important to note that a statistical difference in dietary RS does not necessarily mean it would have biological significance in the animal.

Conclusion

In this study all the commercial diets tested had a very low RS level (close to or less than 1% of the total starch), which is less than what would be considered sufficient to promote colonic health. The level of starch cook did not reflect the amount of RS in the foods, possibly due to the analytical procedures themselves. When grouping treatments as GF vs GB diets, there was no difference in total starch and RS levels, whereas a difference in total starch content between dog and cat foods was observed. Another important conclusion from this work was that regression analysis of NFE and total starch (calculated) showed that these were good predictors of total starch measured. Although an expanded and uniform kibble is aesthetically pleasing, a less expanded, denser kibble with less gelatinized starch might yield more RS. This work would suggest that different processing considerations than currently used in commercial products would be necessary to shift starch toward greater RS and thereby benefit colonic health.

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Table 2.1.

Nutritional Composition of the Commercial Diets Used in the Study.[1]

Food	Species	Category[2]	Crude Protein	Fat, Acid hydrolysis	Ash	TDF	Starch	Moisture	NFE[3]
1	Dog	GB	28.8	16.6	7.75	10.34	24.4	6.15	30.4
2	Dog	GB	27.1	10.5	9.20	9.02	32.5	6.82	37.3
3	Dog	GB	29.9	12.9	7.24	6.10	27.9	6.31	37.6
4	Dog	GB	29.2	16.7	7.42	7.43	26.9	5.52	33.7
5	Dog	GB	26.2	15.7	6.66	8.99	30.1	6.22	36.3
6	Dog	GF	23.7	14.3	8.46	9.88	28.3	6.20	37.5
7	Dog	GF	22.5	13.3	6.02	11.46	32.9	6.35	40.3
8	Dog	GF	31.0	16.4	7.83	10.11	22.7	6.30	28.3
9	Dog	GF	22.7	10.7	8.81	8.05	37.5	6.28	43.5
10	Dog	GF	33.2	20.0	7.21	11.83	16.2	5.99	21.7
11	Cat	GB	36.7	17.9	7.03	6.54	23.9	4.04	27.8
12	Cat	GB	31.9	13.0	7.05	10.59	25.8	6.06	31.4
13	Cat	GB	33.3	10.8	7.08	10.20	27.3	4.89	33.7
14	Cat	GB	36.2	11.2	5.86	13.56	19.9	6.07	27.1
15	Cat	GB	36.7	15.2	8.24	10.90	19.4	5.59	23.4
16	Cat	GF	43.8	16.7	7.93	7.92	15.8	4.25	19.4
17	Cat	GF	33.8	11.5	7.04	11.73	22.0	5.81	30.1
18	Cat	GF	37.3	15.5	7.88	7.88	21.1	5.74	25.7
19	Cat	GF	41.4	14.4	7.03	8.18	20.9	4.62	24.4
20	Cat	GF	33.5	14.1	8.23	9.90	22.7	5.63	28.7

[1]All nutrients reported on a percentage as-is basis.

[2]GB = grain based; GF= grain-free.

[3]NFE was the only calculated component.

Table 2.2.

Total, Digestible and Resistant Starches of Dog vs Cat Diets, and Grain-Based (GB) vs Grain-Free (GF) Diets.

Item, %	**Dog**	**Cat**	**SEM**	**T**	***P*-value**
	n= 10	**n= 10**			
Total starch, measured	30.1	24.0	1.94	3.1218	0.0018
Total starch, calculated	31.4	25.1	2.16	2.9122	0.0036
Resistant starch[1]	0.945	0.703	0.2212	1.0950	0.2735
Digestible starch[1]	99.0	99.3	0.22	1.1418	0.2535
Starch cook[2]	88.3	89.2	2.51	0.3498	0.7265
Item, %	**GB**	**GF**	**SEM**	**T**	***P*-value**
	n= 10	**n= 10**			
Total starch, measured	28.4	26.7	1.14	0.6361	0.5247
Total starch, calculated	29.9	26.4	1.52	1.6016	0.1092
Resistant starch[1]	0.828	1.062	0.1602	0.6360	0.5248
Digestible starch[1]	99.2	98.9	0.16	0.9158	0.3598
Starch cook[2]	87.5	94.1	1.46	3.9030	<.0001

[1]Resistant and digestible starches were calculated as percentages of the total starch.

[2]Starch cook calculations were based on total starch and starch gelatinized measured at a commercial laboratory (Wenger Technical Center; Wenger Mfg., Sabetha KS, US

Chapter 3 - **A Model that Predicts Resistant Starch in a Dog Kibble using a Small-Scale Twin-Screw Extruder**

Abstract

Resistant starches (RS) escape enzymatic digestion in the small intestine and are fermented by colonic saccharolytic bacteria, promoting gut health. These RS are usually included in the diet as either retrograded or chemically modified starches (types III and IV, respectively). The objective of the present study was to modify extrusion processing parameters to yield greater RS in the kibble and create a model to predict its concentration. A single nutritionally complete diet (AAFCO, 2019) for adult dogs at maintenance was formulated. The recipe was extruded through a small-scale intermeshing co-rotating twin-screw extruder (Evolum 25; Clextral, Firminy, France) as a central composite design with 6 central points (replicates) and 20 total samples collected. There were three factors at three levels (-1, 0 and 1): corn particle size (PS), extruder shaft speed (SS) and in-barrel moisture (IBM). The remaining fixed inputs were kept constant across treatments. Food samples were collected off the extruder once steady state of moisture and extrudate temperature at the die were achieved. Resistant starch and starch cook were measured with enzymatic procedures and RVA was measured with a RVA 4800 (Perten and PerkinElmer Instruments, Springfield, IL) and reported as AUC of each starch transformation peak. A model to predict RS was created by backwards elimination using the REG procedure, and Pearson correlations among extrusion cooking parameters and starch analyses were obtained by the CORR procedure from SAS (v.9.4). The RVA of two samples of extreme temperatures and specific

mechanical energies (SME) were plotted. Resistant starch could be predicted with an R^2_{adj} of 0.834 ($P < .0001$; MSE= 0.013; CV= 11.04%):

Predicted RS= 0.018*PS + 0.00161*SS + 0.0601*IBM – 0.000013*SS*PS – 3.78

The RVA data confirmed starch transformations. Both SME and extrudate temperature had a high negative correlation with RS and RVA AUCs. All data results corroborated that the decrease in thermomechanical energies during the extrusion process favors RS retention in the kibble. Future work should focus on improving this model by using a wider range of corn particle sizes and applying it to large-scale extruders.

Keywords: dog, extrusion, kibble, corn, resistant starch, viscosity.

Introduction

Extrusion is the most common process through which pet foods are produced. It can be classified as a medium shear (screw speed above 100 rpm), medium temperature (55 to 145 ºC), and medium moisture (15 to 30%) process (Riaz, 2000). Before cooking, cereals and other dietary ingredients are ground and mixed, then fed to a preconditioner (PC) where water and steam are added and mixed with the dry recipe. This step hydrates the mix and starch granules begin to swell. From the PC the mix is fed into the extruder barrel, the primary cooking apparatus. At this stage, pressure increases, screw rotation transfers mechanical energy to the dough, and additional steam may be added. The moisture content and energy transfer enable the dough to transition from a glassy to rubbery state which traps water droplets within the melt. Water droplets expand as these vaporize when the material exits the extruder and product goes from high pressure to atmospheric pressure (Moraru and Kokini, 2003). Post extrusion, kibbles are dried to less than 10% moisture and coated with fat and flavors.

The extrusion process conditions such as water and steam additions combined with mechanical energy promote starch gelatinization (Baller et al., 2018). Past studies have demonstrated decreasing the amount of mechanical energy (Jackson, Waldy, Cochrane, et al., 2020; Ribeiro et al., 2019; M. C. Peixoto et al., 2018), increasing process water content addition (Baller et al., 2018), and increasing the starch ingredient particle size (Ribeiro et al., 2019; M. C. Peixoto et al., 2018) minimize starch gelatinization, which retains some resistant starches (RS). Resistant starches represent the starch fraction that escapes small intestinal digestion and undergoes fermentation by saccharolytic bacteria within the colon to produce short chain fatty acids (SCFA). Previous data have shown an increased butyrate production from consumption of RS from low shear extrusion at a low concentration (Jackson, Waldy, Cochrane, et al., 2020; Ribeiro et al., 2019; M. C. Peixoto et al., 2018). It is common in the human food industry to produce RS through chemical or enzymatic modification of raw starches and their effects on health have been extensively studied (Shen et al., 2017). In pet foods, some studies have explored the health benefits of supplementing RS in dog diets across various breeds (Beloshapka et al., 2016; Goudez et al., 2011) and a few have attempted to retain RS in extruded foods (Jackson, Waldy, Cochrane, et al., 2020; Ribeiro et al., 2019; M. C. Peixoto et al., 2018). The latter represents an opportunity in the pet food industry. Modifying the process to retain some of the native crystalline resistant starch (type II) and(or) develop retrograded starch (type III) may be more cost effective, as well as provide a health attribute to the food. Therefore, the objectives of the present study were: 1. maximize RS of corn in the final kibble by controlling three processing factors including particle size, water input at the extruder barrel, and extruder shaft speed, 2. create a response surface model that predicts RS concentration based on process inputs, and 3. correlate physical analyses of kibble factors with chemical methods for starch conversion.

Materials and methods

Diet

A single diet was formulated (Concept5©; CFC Tech Services Inc., Pierz, MN, U.S.A.) to meet the nutrient requirements for adult dogs at maintenance (AAFCO, 2019). For the purpose of this study, only the dry ingredients were used in the grain mix, and any flavors, fats or oils were excluded. This was chosen because the focus of the present work was not to feed diets to dogs, but to assess the effects of extrusion processing on starch transformation. Whole yellow corn (Cargill, Wayzata, MN, U.S.A.) was the sole starch ingredient and no fiber ingredients were added to the formula (Table 3.1). The remainder of the dry mix included chicken meal (Tyson, Springdale, AR, U.S.A.), amino acids, minerals and vitamins (DSM Additive Mfg., Parsippany, NJ, U.S.A.).

Extrusion processing

Extrusion was conducted in a small-scale intermeshing co-rotating twin-screw extruder (Evolum 25; Clextral, Firminy, France) set up as a 24 length to diameter screw design with twin 25 mm-diameter screws (Appendix A.1). This system utilized a 25L dual cylinder preconditioner with steam and water injection. The rationale for using this small-scale extruder was to optimize data collection with a small amount of raw material across a wide range of settings that would correspond to a larger scale extruder.

The experiment was conducted as a non-rotatable central composite design with 6 replicated center points and a total of 20 runs (Johnson and Milliken, 1989; Table A.1.). The factors were tested at 3 levels (low, medium, high) on the same recipe at three mill screen sizes (0.793 mm, 1.19 mm and 1.586 mm), three pre-conditioner moisture contents (target 20%, 25%

and 30% water added to the grain mix) at constant PC feed rate and three screw speeds (400, 800 and 1200 rpm).

The 6 central point replicates were run sequentially (sample 1 to 6; Table A.1.) to facilitate processing. Each time replicates were switched, extruder shaft speed was either increased to 1200 rpm, or decreased to 400 rpm for 5 minutes, then changed back to 800 rpm. This led to some variation in the process as would be expected for replicates not run sequentially. Samples were collected after 5 minutes of setting changes to allow for leveling to steady state condition for moisture and extrudate temperature inside the die. The other single replicates were run in a random order (Table A.1.).

Processing details and data collection

Corn was ground through a hammermill at three screen sizes: 0.793 mm, 1.19 mm and 1.586 mm in order to produce a fine, medium and coarse grind size. In sequence, ground corn was mixed with the other ingredients of the "grain mix" (Table 3.1.) using a 68 kg capacity ribbon mixer (Model 9, Wenger Mfg., Sabetha, KS), and then the complete grain mix was ground again in a hammermill (Jacobson 120-D portable hammermill; Carter Day International Inc., Minneapolis, MN) with a 1.586 mm screen. The grinding of the grain mix was done to assure no large pieces would interfere with the dough flowing through the extruder die.

Extruder data for input and output variables were recorded every 5 minutes (twice) during each run then averaged (Table 3.2.). The dry mix was delivered to the PC at a constant feed rate of 30 kg/h with a PC shaft speed set to 100 rpm for every run. The PC steam flow rate was set to 4.5 kg/h, while PC water was varied from 1.5 to 4.5 kg/h in order to achieve the target moisture by the operator. Extruder water was also kept constant at 2 kg/h in all treatments and no

steam was added to the extruder. At the end of the extruder barrel knife speed was fixed at 900 rpm with an 8.38 mm die opening. Kibbles were dried in a convection oven (Model FP 240; Binder Inc.; NY, U.S.A.) at 100 ºC until moisture content was below 10%. There was no coating step because these treatments as diets were not intended to be fed to dogs.

During extrusion, a total of 20 samples were collected at the exit of the extruder barrel (off the extruder; OE) once production reached steady state (after 5 minutes of changing the settings), immediately flash frozen in liquid nitrogen, and kept at -70°C until RVA analysis was performed. Samples were placed into 30 g whirl-paks and collected at two time points during each run to assure representative sampling, which were then used for RVA and starch cook analyses. Samples collected off the drier (OD) were used for kibble measurements, texture analysis, and resistant starch determination.

Output parameters collected at the extruder panel were motor load, PC temperature, pressure and extrudate temperature measured by a penetrating probe between the die insert and final head of the barrel, while in-barrel moisture (IBM) and specific mechanical energy (SME) were considered as intermediate variables (Table 3.2.). The SME was determined according to Equation 3.1:

Equation 3.1

$$\text{SME } (\frac{W*h}{kg}) = \frac{\text{Power (Watts)}}{Mass\ Throughput\ (kg/h)}$$

Where motor power was measured by the solid-state extruder motor drive, and mass throughput was calculated as the total wet mass flow rate (dry feed rate + water and steam inputs

at PC and extruder). The steam input was corrected for calculated steam loss before the extruder. In-barrel moisture was determined according to Equation 3.2:

Equation 3.2

$$IBM\ (\%)\ =\ \frac{[(Mf\times Xf)+Mps+Mpw+Mes+Mew]}{(Mf+ps+Mpw+Mes+Mew)}$$

Where: Mf = dry feed rate (kg/h); Xf = wet basis moisture content of the feed material (%); Mps = steam injection rate in the pre-conditioner (kg/hr); Mpw= water injection rate in the pre-conditioner (kg/hr); Mes = steam injection rate in the extruder (kg/hr), and Mew= water injection rate in the extruder (kg/hr).

Particle size analysis

Particle size of the corn and raw mix were determined by a Morphologi G3 instrument (Malvern Panalytical; Malvern, United Kingdom) using 5 x magnification, 5 mm^3 sample size and one bar of air pressure for dispersion of the sample. Information collected and respective descriptions are below:

Circle equivalent (CE) diameter volume distribution: the average 3D image of the particles converted to a 2D circle of equivalent area. The median diameter based on a volume distribution (v, 0.5) of each corn grind size was reported (Table A.2.).

High sensitivity (HS) circularity: $4\pi A/P^2$; where A is the particle area, and P its perimeter. Measurements ranged from 0 to 1 and describe the deviation from a perfect circle (the closer to 1, the more it approximates to a perfect circle). The median HS circularity based on a number distribution (n, 0.5) of each grind size was reported (Table A.2.).

Aspect-Ratio-Number Distribution: average width/length of the particles. It ranges from 0 to1. A rod would have a low aspect-ratio. Data were reported as the median of aspect-ratio number distribution (n, 0.5; Table A.2.).

Elongation: calculated as 1 – aspect-ratio. This ranges from 0 to 1 and measured the average particle elongation (the closer to 1, the longer it is). Results represent the median elongation of all the particles (n, 0.5) measured of each grind size (Table A.2.).

Particle size distribution of the raw mix and corn samples were determined by rotating tapping sieve analyses (Ro-tap; Baker and Herrmann, 1995). This procedure involved 100 g sample and rotating-tapping time of 10 minutes. There were 14 sieves in the stack with screen sizes of 3,360, 2,380, 1,680, 1,191, 841, 594, 420, 297, 212, 150, 103, 73, 53 and 37 μm.

Kibble measurements and texture analysis

Kibble dimension measurements were performed on 20 kibbles per run. Each kibble was randomly selected, then diameter and length were each measured twice with a digital caliper, averaged, and weighed on an analytical balance (Ohaus, Explorer: E1RW60, OHAUS, Parsippany, NJ). With this information, piece density (g/cm^3) and sectional expansion index (SEI; cm^2_e/cm^2_d) were calculated. Volumetric expansion index (VEI) was calculated according to Alvarez-Martinez et al. (1988):

Equation 3.3

$$VEI = \frac{\rho d \times (1 - Md)}{\rho e \times (1 - Me)}$$

Where: ρd = extrudate density inside the die; Md= moisture content of the extrudate in the die; ρe= apparent density of the wet kibble; and Me= moisture content of the wet kibble. Moisture content inside the die (Md) equaled IBM, while moisture content of the extrudate after exiting the die (Me) was calculated as IBM minus steam loss. Steam loss was estimated according to Levine (1997). Density of the kibble inside the die (ρd) was calculated using a model of Singh (2013; http://rpaulsingh.com/problems/what_if/exdensity.html). Lastly, longitudinal expansion index (LEI) was calculated as a function of VEI divided by SEI.

Kibble hardness was considered the peak force required to break the kibble at the primary significant fracture. This was determined on 30 kibbles per run using a texture analyzer (TA-XT2; Texture Technology Corp., Scarsdale, NJ, U.S.A.) equipped with a 50 kg load cell. A 25 mm cylindrical probe was used to compress kibbles at a 50% strain level.

Starch analyses

Kibbles were subjected to RVA analysis as an indicator of process effects on starch biopolymers. Briefly, uncoated wet kibbles (OE) were frozen at -70^{o}C until analysis, and then ground through a meat mincer and sieved using a 400 mm sieve screen size. Prior to the RVA analysis, sample moisture was determined, then 2 g was diluted with 25g deionized distilled water in an aluminum cup containing a plastic paddle. The RVA (RVA 4800; Perten and PerkinElmer Instruments, Springfield, IL) was performed for 23 min. Samples were equilibrated at 25 ^{o}C for 2 min at 960 rpm, then the speed decreased to 160 rpm and temperature increased to 95^{o}C between 2 and 10 min, then held at 95 ^{o}C for 3 minutes then decreased to 25^{o}C from 13 to 18 min. The RVA data are reported as area under the curve (AUC) for each peak (cold peak, raw

peak and setback). The ratio of raw:cooked starch was also calculated by dividing the AUC of the raw peak by the AUC of the cold peak.

Resistant starch determinations were performed according to an enzymatic assay with glucose measured by colorimetry (K-RSTAR; Megazyme International Ireland Limited, Ireland). Degree of gelatinized starch was determined by a modified glucoamylase test based on a 70-minute enzymatic hydrolysis (Mason, Gleason and Rokey, 1982) with a blend of amylase and amyloglucosidase from *Aspergillus niger* (Hazyme DCL; DSM corporation, Heerlen, Netherlands), and glucose quantified by a YSI Model 2700 glucose analyzer (YSI; Yellow Springs, OH, U.S.A.).

Statistical analysis

The surface response model of central composite data from small-scale extrusion was first analyzed for lack of fit with the RSREG procedure from Statistical Analysis Software (SAS; v. 9.4, SAS Institute Inc., Cary, NC, USA). When lack of fit was not significant ($P > 0.05$) data were analyzed by the REG procedure (SAS; v. 9.4, SAS Institute Inc., Cary, NC, USA). The main effects of particle size (PS), extruder shaft speed (SS), and in-barrel moisture (IBM) were added to the model, as well as cross-products and quadratic terms. The model with the best fit (lowest P-value) was obtained by backwards elimination. When a main effect was eliminated from the model, but either cross-product or quadratic term were significant, the main effect was added back. The intercept was kept in the model even when it was not significant. This procedure was applied to RS, starch cook and RVA as dependent variables. When a model was significant ($P < 0.05$) with an adjusted $R^2 > 0.50$, a surface response plot was created using the proc G3D procedure from SAS (SAS 9.4, Cary NC). Pearson correlations were performed

between starch measurements, kibble data and extrusion parameters using the CORR procedure from SAS (SAS v 9.4, Cary, NC). Additionally, two regression equations were constructed using the REG procedure from SAS between RS and SME, and RS and dough T at the end of the extruder barrel.

Results

Particle size

Geometric mean particle size measured by the Morphologi G3 for the three ground corn samples and grain mixes were lower than expected (Table A.2.), and distributions were bimodal for corn ground at the three levels (Figure 3.1). Corn ground using a 0.793, 1.19, and 1.586 mm sieve size had a mean geometric diameter ± standard deviation of 158.6 ± 2.05, 174.8 ± 2.24 and 221 ± 2.6 µm, respectively, and the corresponding particle sizes of the grain mix (corn mixed and ground with other ingredients) were 161.6 ± 1.99, 172.7 ± 2.08 and 195.3 ± 2.24 µm.

Extrusion processing

The intermediate extrusion parameters IBM and SME were targeted to have the greatest variation according to each treatment combination. The 6 replicates had an IBM of 32.4%, while SME varied from 30.2 to 32.5 Wh/kg (Table 3.2.). In-barrel moisture was the lowest on treatments 12, 11, 19, 16 and 17 (average 29.8% ± 0.87), and the highest in treatments 14, 15, 9, 19 and 20 (average 35.3% ± 0.70). Specific mechanical energy was intentionally targeted to change according to water additions and shaft speed modifications, ranging from 11.6 to 52 Wh/kg (Table 3.2.). Treatments with the highest SME (> 50 Wh/kg) were those with the high

extruder shaft speed setting, and either low or intermediate IBM. Conversely, the lowest SME values (11.6, 15.7 and 12.8 Wh/kg) were obtained with the low extruder shaft speed setting, and either high or intermediate IBM.

Kibble measurements and starch analyses

Kibble volume and density for the 20 runs ranged from 0.995 to 1.429 cm^3 (average 1.21 cm^3 ± 0.125) and 0.377 to 0.732 g/cm^3 (average 0.463 g/cm^3 ± 0.076), respectively (Table A.3.). Kibble expansion (SEI) ranged from 1.328 to 2.082 times the die size (average 1.738 ± 0.2109), and VEI and LEI ranged from 0.719 to 1.68 cm^3_e/cm^3_d (average 1.27 ± 0.228), and 0.385 to 0.961 cm^2_e/cm^2_d (average 0.732 ± 0.119), respectively. Volumetric expansion index (VEI) represents the overall kibble expansion, while SEI and LEI are two components of expansion. Finally, hardness ranged from 6.37 to 16.41 kg (average 10.15 kg ± 2.861; Table A.3.).

Although the 20 treatments were produced with the same basal recipe, their total starch content varied from 48.8 to 65.9% (mean 57.7% ± 4.25; Table 3.3.). This was likely due to the high variability intrinsic to the total starch assay. Starch cook of the raw grain mixes with fine, medium and coarsely ground corn were, respectively, 11.0, 10.7 and 9.5%, and after processing these ranged from 83.3 to 99.7%; (mean 91.5% ± 5.13; Table 3.3.). The RS content of the raw grain mixes with fine, medium and coarsely ground corn were 1.20%, 1.05% and 2.28%, respectively, and RS of the diets after extrusion processing varied from 0.24 to 1.48% (mean 0.79 ± 0.278; Table 3.3.).

Each stage of change in viscosity measured by RVA was calculated as follows: The area under the curve (AUC) between minute 0.4 and 6 was the cooked starch AUC; the AUC between 6.1 and 14 minutes represented the raw starch AUC, while setback viscosity (high molecular

weight starch) was measured as the AUC between minutes 14.1 and 23. Although starch is responsible for the majority of viscosity changes, other nutrients such as protein or fiber may interfere with the analysis, thus in the present study peak viscosities were not relevant and not reported. To illustrate the RVA curves, two samples with extreme extruder barrel temperatures at the end of the barrel were plotted (Figure 3.2). The lower energy sample (number 15) showed little to no initial viscosity, a pronounced native starch peak where the RVA temperature increased above 60ºC, and a high setback viscosity. In contrast, the sample produced at higher energy (number 16) exhibited a large initial peak and no indication of a native starch (raw starch) peak where the RVA temperature increased. The setback viscosity of sample 16 was lower than sample 15. The ratios between the RVA raw:cooked AUC were calculated as another means to estimate the extent of starch cooked relative to raw starch, and these were 2.80 and 0.37 in extreme samples 15 and 16, respectively (mean 1.76 ± 0.681; Table 3.3.).

Correlations between starch analyses and processing parameters

Resistant starch was less than 50% negatively correlated ($P < 0.05$) with starch cook and RVA cooked AUC, and also less than 50% positively correlated ($P < 0.05$) with RVA raw:cooked AUC and setback viscosity (Table 3.4.). Conversely, the starch percent cook results had an inverse relationship with RVA raw AUC, raw:cooked AUC, and setback viscosity. Setback viscosity had the greatest correlations with RS and starch cook among other RVA parameters, indicating that the greater the content of native starch, the greater the final viscosity due to the presence of longer amylose and amylopectin molecules.

Volumetric expansion index (VEI) had a strong negative correlation with IBM (r=-0.89), which meant that a lower moisture content led to greater overall kibble expansion within the

experimental parameters (Table 3.5.). Longitudinal expansion index (LEI) was mostly affected by dough temperature (r=0.61) and IBM (r=-0.733), while SEI did not have a significant correlation with any of the parameters tested.

Resistant starch had a strong negative correlation with dough temperature at the end of the barrel, shaft speed and SME (r= -0.829, -0.724 and -0.862, respectively), and a less strong positive correlation with IBM (r= 0.48; Table 3.5.). The regression equations of interest follow below:

RS= 1.425 – 0.02083 x SME (R^2_{adj}= 0.73)

RS= 5.701 – 0.04179 x Dough T at the end of barrel (R^2_{adj}= 0.674)

Starch cook had a low correlation with all parameters tested but was significant ($P <$ 0.05) and positively correlated with dough temperature and SME and negatively correlated with IBM. The RVA AUC of cooked and raw starches were mostly affected by dough temperature and SME, but not IBM. Lastly, setback viscosity had a strong negative correlation with dough temperature and SME.

Surface response plots and model building

Dry kibble RS was the primary endpoint. For model building, the actual measured rather than target parameters were used. The independent variables were PS at 161.5 um, 172.7 um and 195.3 um mean geometric diameter, IBM at 29.8, 32.5 and 35.3%, and SS at 400, 800 and 1200 rpm, representing the low, medium and high settings. The IBM obtained in each level had to be averaged in order to be used in the model building. The model using these variables as predictors for RS content in the dry kibble was linear ($P < .0001$) with non-significant lack of fit ($P =$ 0.1136). After backward elimination and regression analysis testing the main effects, their cross-

products and quadratic terms the resultant significant terms were SS (P = 0.1439), PS (P = 0.0033), IBM (P = 0.0003), and SS*PS (P = 0.0427). The final model (P < .0001) with an adjusted R^2 of 0.834, MSE 0.0129, 11.04% coefficient of variation (CV), was:

Equation 3.4

RS= 0.018*PS + 0.00161*SS + 0.0601*IBM – 0.000013*SS*PS – 3.78

Due to difficulties visualizing a 3D surface response plot using 3 independent variables (PS, SS, and IBM), two variables were plotted against RS for each (Fig 3.3-3.5).

The final model to predict viscosity (RVA) with endpoints cooked peak and raw:cooked ratio was not significant (P > 0.05). The model to predict RVA raw peak AUC was significant (P = 0.0084) after backwards elimination with IBM, SS, PS and IBM*SS as part of the final model; but the graphs were not created because the adjusted R^2 was low (0.464) with CV=12.5% and mean AUC 24,458:

RVA Raw AUC = -858.6*IBM – 73.6*SS – 108.6*PS + 2.1*SS*IBM + 76,610.

Gelatinized starch (% cook) had a non-significant lack of fit (P = 0.2509), and the model was significant (P = 0.0401) after backwards elimination. However, the predictor IBM was not significant (P = 0.4779) and both SS and their cross-product (SS*IBM) had only a tendency (0.10 > P > 0.05) to be significant. The final model also had a low R^2adj (0.283):

Starch cook= 0.0855*SS + 0.889*IBM – 0.00245*SS*IBM + 57.94

Discussion

To date, the present study is the first published work to determine the effect of pet food processing parameters on RS yield and to create a model that would predict RS content in a dry

extruded kibble. A secondary goal was to explore methods of starch gelatinization, correlate these among themselves, as well as correlate extrusion processing parameters with starch transformation measures and kibble parameters.

The RS targeted in the present study was likely a combination of types II and III (Dupuis et al., 2014; Fuentes-Zaragoza et al., 2010). Evaluation of the starch granule physical structure would be necessary to define whether most RS was naturally occurring due to its tightly packed structure (type II) or retrograded (type III). Recently, a broader classification was suggested which groups RS according to its kinetic properties of digestion. Both types II and III would be categorized as starches with digestion rate limited by the granules tightly packed structure (Dhital et al., 2017).

The ideal concentration of RS that benefits dog colonic health without decreasing stool quality still hasn't been established for all breeds. Although it is known that large dogs may produce loose stools when fed a diet with RS as low as 2.5% (Goudez et al., 2011). In the present study the raw grain mix with the most RS (Grain mix 3) had only 2.2% RS. Thus, the maximum RS in a treatment could not have been over 2.2%. Ideally, we would extrude a dog food with the minimal energy necessary to destroy antinutritional factors and pathogens, while minimizing starch cook. This is challenging since extruded pet foods need hydration as well as thermal and mechanical energies in order to form an expanded kibble.

Starch granules of cereals like corn, wheat or rice have an X-ray diffraction type A and also contain pores and channels that facilitate alpha-amylase adhesion and digestion of the substrate (Dhital et al., 2017; Fannon et al., 1992; Fannon et al., 1993). Conversely, tubers have large smooth starch granules with less enzyme adhesion sites, and legume starches are trapped within cotyledon cells, which are little disrupted after thermal processing (Berg et al., 2012).

Although tubers and legumes tend to be higher in naturally occurring RS than cereals (Liu et al., 2006), corn was chosen as the sole starch ingredient for two reasons: 1.) it is one of the most common cereal grains used in pet food due to its high apparent total tract digestibility coefficients, palatability and low cost. 2.) by choosing one starch source and not adding any fiber ingredients the effect of recipe would be minimized which would focus the effects of processing on RS retention. Moreover, whole ground corn was selected instead of corn flour because it has lower *in vitro* starch digestibility when compared to fine milled flours (Heaton et al. 1988).

It is well known that raw cereals, legumes or tubers possess a greater amount of RS than their cooked forms (Schuenemman et al., 1998; Murray et al., 2001), but thermal processing is usually required to destroy antinutritional factors (Nikmaram et al., 2017), increase acceptability (Schuenemman et al., 1998), and microbiological safety of products. Thermal and mechanical processing with some moisture gelatinizes the starch making the α-glucan chains less ordered and more available for enzymatic adsorption and digestion (Warren et al., 2011; Oates, 1977). The RS of kibbles in the present study had a high and inverse correlation with dough temperature at the end of the extruder barrel confirming that the lowest thermomechanical energy from the process resulted in the most RS. There are many processing inputs that contribute to changes in extrudate temperature such as feed rate, steam and water additions, extruder shaft speed, and die open area. According to our model that was built to predict RS all the treatment parameters were significant.

When corn is extensively ground, starch gelatinization and digestion are improved due to a large surface area to mass of the small particles. This happens because the starch ground to smaller particles becomes more exposed to hydration, which increases gelatinization. Conversely, coarsely ground corn has a lower surface area to mass which slows water

penetration and starch gelatinization, thereby decreasing its digestion (Ribeiro et al., 2019; Peixoto et al., 2017). Grain milling also destroys cell walls and disrupts the protein matrix making starch more available (Al-Rabadi et al., 2009). For these reasons, an important factor selected to construct the surface response model for RS in dry extruded kibbles was the corn grind size. In the present study, the largest mean geometric diameter was less than initially targeted. This likely happened because the hammermill used was more effective than expected, and the corn (and the ration it was contained within) was ground through the hammermill with a 1.59 mm screen size twice. A better model may have been created if wider differences among mean geometric diameter had resulted. In future work using a larger screen size would be recommended. Ribeiro et al. (2019) and Peixoto et al. (2017) reported that extruded dog food produced with raw corn ground to geometric mean diameter of 224 (low) and 312 μm (high) and at high and low SME inputs yielded 0.21-0.22 and 1.46-1.54% RS, respectively. In their work the lowest geometric mean was greater than the grain-mix coarsely ground in the present study. Moreover, in their studies the SME was controlled by differing the die open area rather than altering screw speed in the present work, and both methods were effective in controlling SME. Bazolli et al. (2015) reported that fine, medium and coarse maize (360, 452 and 619 μm mean geometric diameter, respectively) produced foods with a starch gelatinization of 79.9, 73.8 and 63.2%, respectively. The starch cook values they obtained were wider than ours due to the greater differences in particle size. While RS was not measured in Bazolli's work, it would likely be above what was determined in the present study. Nevertheless, particle size still had a significant effect on RS concentration when plotted against shaft speed and in-barrel moisture; wherein, the RS prediction was the greatest at the largest mean geometric diameter.

Water, which is required for starch gelatinization, has a quadratic effect on starch cook: too little water does not provide enough hydration of the starch granule and subsequently the kibble will have small cell structure and little expansion (Baller et al., 2018). On the other extreme, too much water decreases temperature in the extruder and therefore starch cook, acting as a plasticizer (Lin et al., 1997). When water is below what is required to hydrate the dry mix, the mechanical shear inside the extruder barrel can cause starch damage which will create a premature RVA peak in cold water, meaning that starch has been mechanically damaged. This phenomenon was present in the RVA profile of sample 16 (Figure 3.2.). Baller et al. (2018) demonstrated that 22% and 37% IBM were the extremes because neither resulted in kibble expansion or to have a good cell structure according to scanning electron micrographs. Kim et al. (2006) reported that extruded wheat flour with the most RS was produced with excess water. At the other end of the spectrum, Robin et al. (2016) reported that corn starch extruded at both high and low SS (600 and 300 rpm, respectively) at lower moisture content (17% water) yielded the most RS. In their study, the low water content was likely not enough to cook the starch, and the treatment with more water (at 22%) caused greater starch gelatinization. The treatment in the present study with high IBM, low SS and high PS yielded the greatest predicted RS content. The IBM at 36% likely helped to dissipate energy.

Extruder shaft speed (SS) is a controllable input that affects specific mechanical energy directly. Simultaneous to a higher SS and increased mechanical energy in the process the residence time decreases. This exposes the starch to a shorter cook time in the extruder barrel. Therefore, altering shaft speed can have mixed results. Gonzales-Soto et al. (2006) reported that RS content of corn and mango were higher when extruded at a SS of 30 rpm as compared to 65 rpm. Kim et al. (2006) did not find a difference in RS of wheat flour extruded at the same

moisture contents and different shaft speeds. This is likely because they increased SS at 50 rpm increments, which was not a significant change to affect RS yield. In the present study, increasing the shaft speed in 400 rpm increments led to significant cross-product effects with particle size on RS yield.

Among methods used to determine the degree of starch gelatinization starch cook was the least consistent. This happened because even raw corn possesses pores and channels that facilitate enzyme adhesion and digestion (Dhital et al., 2010). Thus, the starch cook method in corn does not account for only the portion that was gelatinized. A better method to estimate starch gelatinization in this study was RVA as it correlated highly with dough temperature and SME. The RVA provides a broader characterization of starch transformations during extrusion. With cold water raw starch does not swell and presents a low viscosity, while high molecular weight starch derivatives produced from chain scission during extrusion easily swell and increase viscosity (Whalen, 2007). This was well illustrated in the RVA plots (Figure 3.2.) where the treatment with the highest thermomechanical energy had a significant initial cold swelling and the less cooked sample had no cold swelling. As the RVA temperature increased to the gelatinization range of corn starch (mid 60ºC; Biliaderis et al., 1980) the sample that preserved some raw starch had an increase in relative viscosity, while the treatment that suffered more thermomechanical energy had little to no raw starch left to promote a hot viscosity peak. Samples 15 and 16 that were used as examples of RVA plots also had the highest and lowest IBM, respectively. The first sample had a much higher setback viscosity than the latter. According to Whalen (1999), extruded puffs produced with high and low water contents had similar final viscosity as samples from the present study, which meant that less water led to more mechanical shear that dextrinized the starch, reducing final viscosity. Extrusion causes

mechanical disruption of molecular bonds within the starch granule, resulting in loss of crystallinity and gelatinization (Lai and Kokini, 1991; Gomez and Aguilera, 1983). The RVA method to measure cooked and raw starch should be employed more frequently in pet foods, but it is important that the same protocol is used so that results can be comparable across studies.

Extrudate temperature is a result of thermomechanical energy inputs during the process. Mechanical energy is largely affected by viscosity, which is affected by water content. The driving force for bubble expansion in the kibble can be represented as a function of water vapor pressure inside the air bubble and viscosity (specific volume of extrudate = P_{vs}/η, where P_{vs} is the water vapor pressure and η the molten viscosity; Kokini et al., 1992). In the present study both VEI and LEI had a high negative correlation with moisture content. This meant that the dough at 30% IBM likely had a lower viscosity compared to treatments produced at 35% IBM, which allowed for bubble expansion according to Kokini et al. (1992). Extrudates with high IBM won't absorb as much mechanical energy and therefore won't be as hot, so vapor pressure decreases (Fan et al., 1994), decreasing overall expansion. Moreover, a wetter extrudate takes a longer time to drop below glass transition temperature (Tg) and the shrinkage effect is greater (Fan et al., 1994).

Conclusion

This study was successful at describing the effect processing has on starch gelatinization and RS content in the kibble. Results suggested that a higher IBM, lower extruder shaft speed and larger particle size should contribute to the survival of RS during thermomechanical processing. Higher water content and lower extruder shaft speed lower the dough viscosity which directly affects SME. Resistant starch had large negative correlations with dough temperature

and SME, which means that extrusion should target a low thermomechanical to increase RS yield. This strengthens the RS prediction model built in this study since SME is a function of multiple factors such as viscosity, and treatment inputs extruder shaft speed, water content and particle size. The physical method RVA to characterize starch gelatinization was preferred over the enzymatic starch cook as it had a strong correlation with thermomechanical parameters. The model created to predict RS can be used as a platform for future studies. Future work should focus on improving this model by using a wider range of corn particle sizes.

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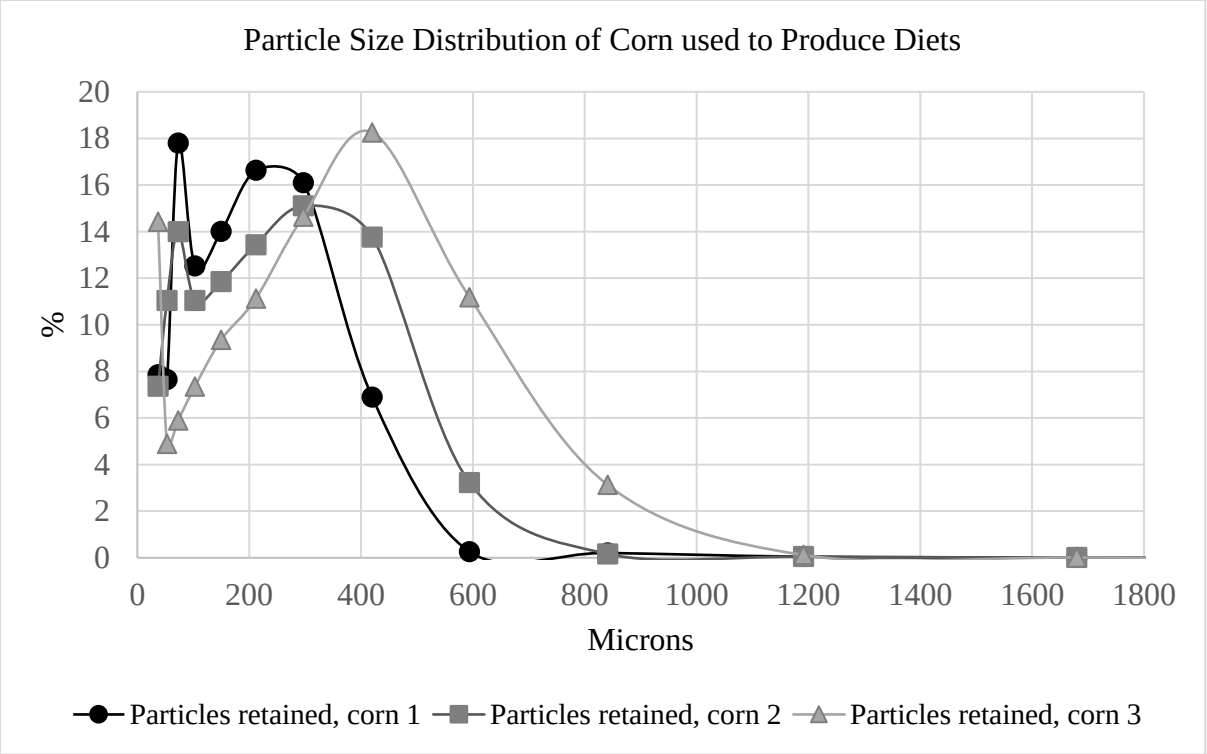

Figure 3.1

Particle Size Distribution of Corn Ground in a Hammermill with Sieve Sizes 0.79, 1.19 and 1.59 mm (Corn 1, 2 and 3, respectively).

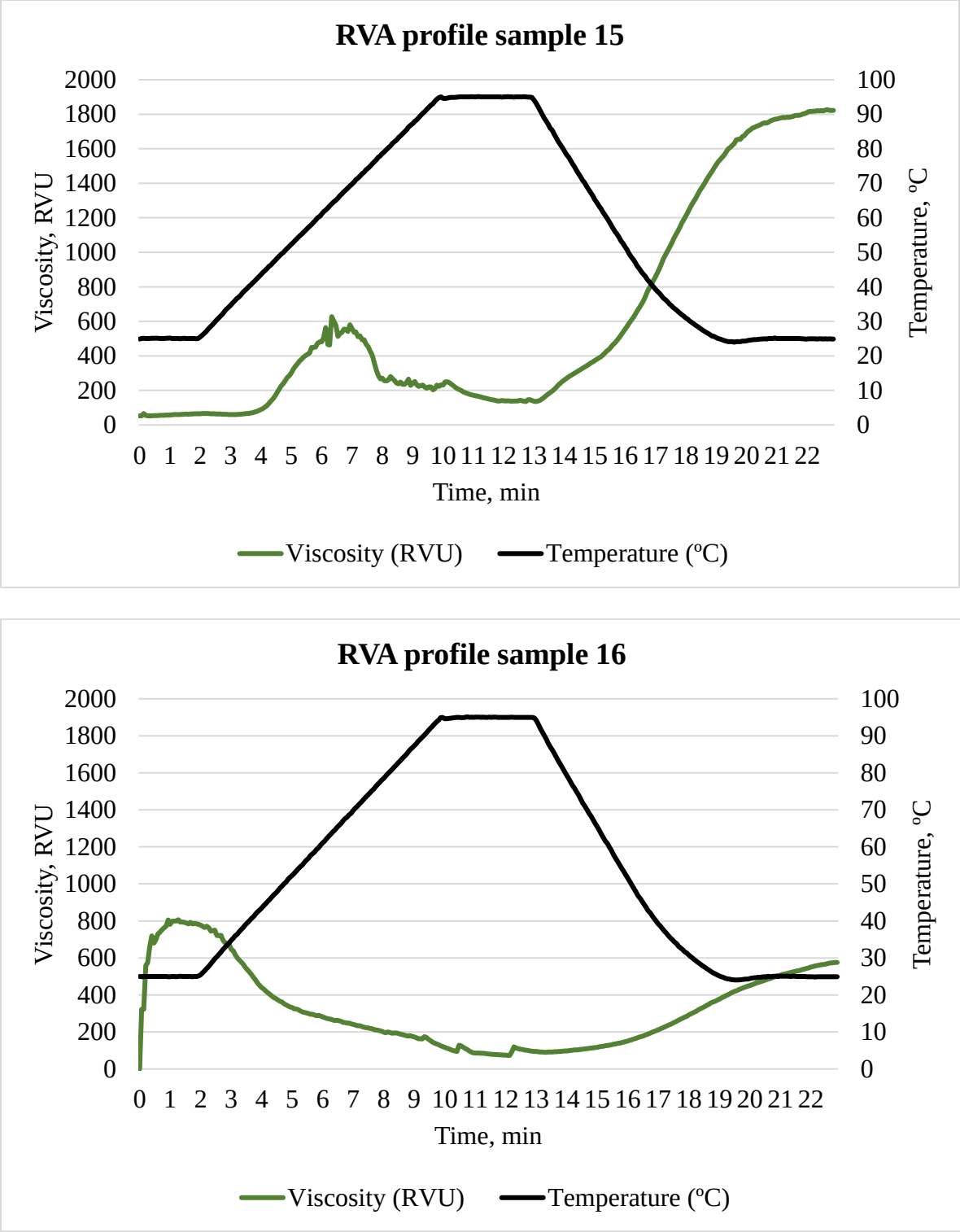

Figure 3.2

Viscosity Curves measured by RVA of Wet Kibbles of a Low (Sample 15) and a High (Sample 16) Thermomechanical Energy Process.

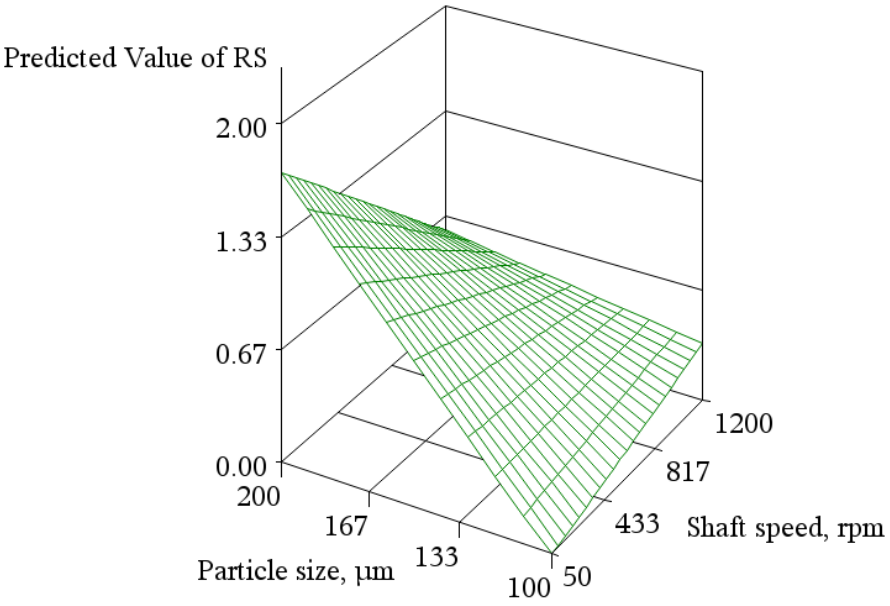

Figure 3.3

Surface Response Plot using Particle Size and Extruder Shaft Speed to predict Resistant Starch Levels in an Extruded Dog Food.

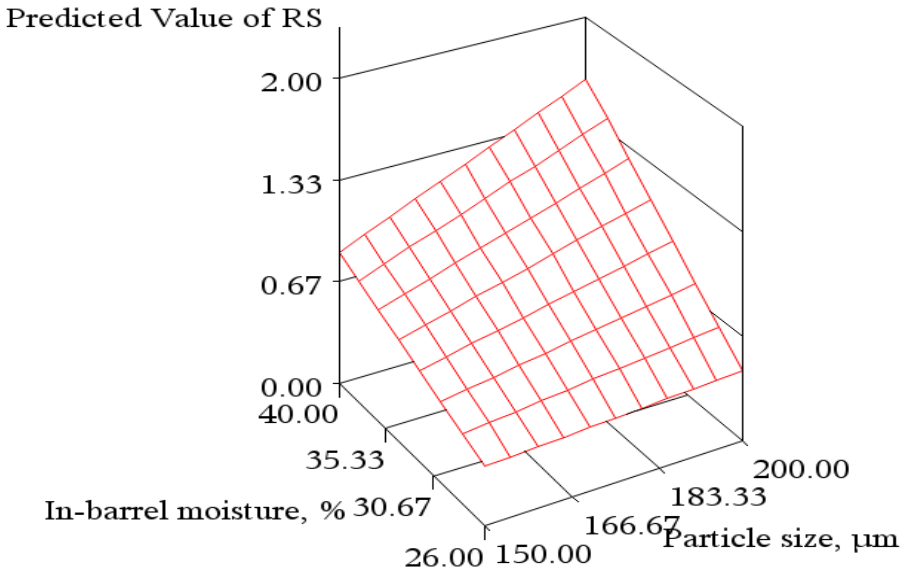

Figure 3.4

Surface Response Plot using In-Barrel Moisture and Particle Size to predict Resistant Starch Levels in an Extruded Dog Food.

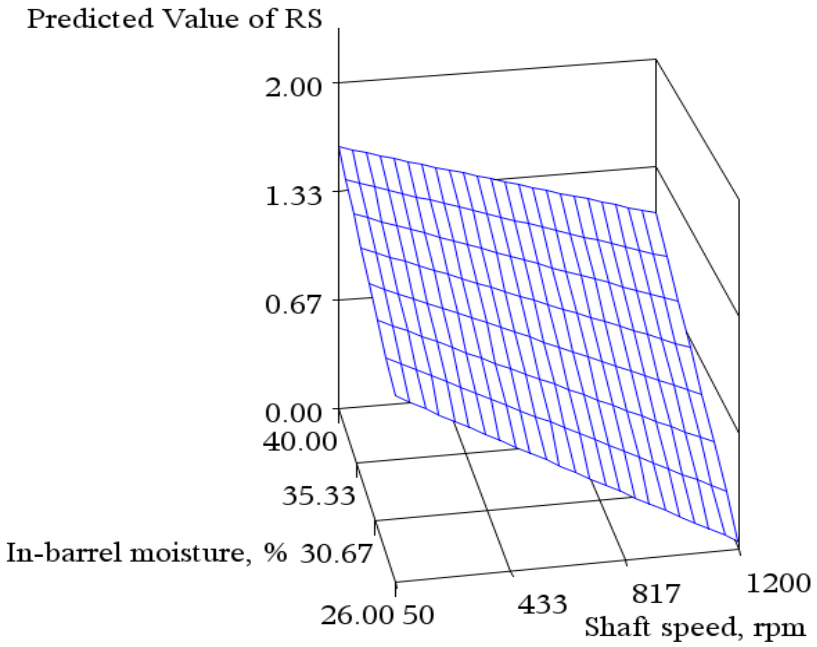

Figure 3.5

Surface Response Plot using In-Barrel Moisture and Extruder Shaft Speed to predict Resistant Starch Levels in an Extruded Dog Food.

Table 3.1.

Diet Formula with Ingredients added to the Grain Mix (as-is).

Ingredient	**%**
Whole yellow corn	75.84
Chicken meal	23.17
Potassium chloride	0.46
Vitamin premix	0.12
Lysine, hydrochloride	0.12
Sodium chloride, iodized	0.12
Taurine	0.06
Mineral premix	0.12

Table 3.2.
Input, Intermediate and Output Parameters recorded at the Evolum 25 Extrusion Panel.

Sample	Input parameters[1]			Intermediate[2] and output[3] parameters					
	PC water, kg/h	PC steam, kg/h	Feed Moisture, %	Motor Load, %	PC Temp., °C	IBM, %	SME, Wh/kg	Extrudate Temp. at the Die, °C	Extrudate Pressure at the Die, bar
1	3	4.5	11.05	8	80	32.4	32.5	118	11.0
2	3	4.5	11.05	8	81	32.4	31.6	116	11.0
3	3	4.5	11.05	8	80	32.4	31.2	117	11.0
4	3	4.5	11.05	8	83	32.4	30.5	118	11.0
5	3	4.5	11.05	8	85	32.4	30.3	116	11.0
6	3	4.5	11.05	8	83	32.4	30.2	117	11.0
13	3	4.3	11.05	8	87	32.1	30.0	116	11.0
12	1.5	4.3	10.93	8	88	29.3	51.8	128	10.5
11	1.5	4.1	10.93	11	88	28.9	22.6	118	17.0
14	4.5	4.5	10.93	6	86.5	34.8	34.9	116	8.0
15	4.5	4.4	10.93	8	88	34.7	11.6	108	11.0
7	3	4.45	10.93	8.5	85	32.3	51.2	127	10.5
8	3	4.5	11.05	9.5	87	32.4	15.7	113	13.5
9	4.5	4.5	11.05	6	86	34.9	22.3	114	9.0
10	1.5	4.2	11.05	8.5	88.5	29.2	34.1	117	12.0
16	1.5	4.9	11.13	8	85.5	30.6	52.0	130	10.5
17	1.5	5	11.13	8	86	30.8	20.3	118	17.0
18	3	4.9	11.13	8	86	33.2	32.3	119	12.0
19	4.5	5.3	11.13	8	86	36.2	35.0	118	8.0
20	4.5	5.1	11.13	8	85.5	35.9	12.8	108	11.5

[1]The following parameters were kept constant across all treatments: PC shaft speed set at 100 kg/h; feed rate at 30 kg/h; extruder water and steam at 2 and 0 kg/h, respectively, and knife speed at 900 rpm.
[2]Intermediate parameters: IBM and SME.
[3]Output parameters: motor load, PC temperature, extrudate temperature at the die and Extrudate pressure at the die.

Table 3.3.

Starch Analyses of Samples produced through the Small-Scale Twin-Screw Extruder (Evolum 25).

Sample	Total Starch[1], %	Starch Cook, %	RVA Cooked Starch AUC[2]	RVA Raw Starch AUC[2]	RVA Setback AUC[2]	RVA Raw:cooked[4]	Resistant Starch[3], %
1	57.9	83.3	967	2,340	8,182	2.42	0.821
2	61.2	89.4	1,078	2,084	7,302	1.93	0.731
3	59.3	92.9	1,055	1,816	5,963	1.72	0.737
4	61.6	87.0	928	2,044	6,508	2.20	0.820
5	59.5	88.3	2,451	1,864	6,984	0.76	0.928
6	55.3	92.9	1,035	2,307	6,953	2.23	0.860
7	54.6	93.2	1,089	2,279	7,422	2.09	0.240
8	51.1	90.7	1,186	2,137	8,345	1.80	1.057
9	55.0	83.6	1,183	1,992	9,492	1.68	0.952
10	48.8	99.2	1,189	2,051	5,575	1.72	0.751
11	52.5	93.6	1,228	2,443	8,170	1.99	0.721
12	61.7	99.7	1,748	1,336	2,590	0.76	0.326
13	65.9	90.1	2,479	2,233	6,060	0.90	0.573
14	60.3	88.6	1,087	2,497	7,614	2.30	0.719
15	52.7	94.0	759	2,131	10,558	2.80	0.903
16	59.9	99.6	3,239	1,199	2,915	0.37	0.350
17	59.0	84.6	1,037	1,995	7,962	1.92	1.056
18	57.2	98.8	1,243	1,789	6,227	1.44	0.907
19	61.1	92.9	1,364	1,740	4,560	1.28	0.801
20	59.3	88.0	850	2,487	9,392	2.93	1.480

Viscosity (RVA) measurements were determined on wet kibbles (out of the extruder), and resistant starch, total starch and starch cook were determined on dried kibbles.

[1]Starch cook of Grain mix 1, 2 and 3 were 11.0, 10.7 and 9.5%, respectively.

[2]Expressed as relative viscosity units (RVU)

[3]Resistant starch of Grain mix 1, 2 and 3 were 1.20, 1.05 and 2.28%, respectively.

Table 3.4.
Pearson Correlation between Chemical and Physical Methods of Starch Analyses.

Item		Resistant Starch	% Starch Cook
% Cook	r	-0.498	-
	P	*0.026*	-
RVA cooked starch AUC	r	-0.452	0.324
	P	*0.046*	*0.164*
RVA raw starch AUC	r	0.418	-0.525
	P	*0.066*	*0.017*
RVA Raw: cooked ratio	r	0.519	-0.420
	P	*0.019*	*0.065*
RVA Setback viscosity AUC	r	0.632	-0.624
	P	*0.003*	*0.003*

Table 3.5.

Pearson Correlation between Kibble Endpoints and Extrusion Parameters.

Item		VEI,	LEI	SEI	RS	% Starch Cook	RVA, Cooked AUC	RVA, Raw AUC	Raw: Cooked ratio	Setback viscosity AUC
Dough T at end	r	0.396	0.610	-0.179	-0.829	0.475	0.507	-0.603	-0.615	-0.772
of barrel, ºC	*P*	*0.084*	*0.004*	*0.450*	*<.0001*	*0.035*	*0.023*	*0.005*	*0.004*	*<.0001*
Shaft speed, rpm	r	-0.076	0.162	-0.311	-0.724	0.327	0.394	-0.448	-0.494	-0.694
	P	*0.749*	*0.495*	*0.182*	*0.000*	*0.160*	*0.086*	*0.048*	*0.027*	*0.001*
IBM, %	r	-0.890	-0.733	-0.350	0.482	-0.408	-0.305	0.266	0.384	0.433
	P	*<.0001*	*0.000*	*0.130*	*0.031*	*0.075*	*0.191*	*0.257*	*0.094*	*0.056*
SME, Wh/kg	r	0.302	0.476	-0.159	-0.862	0.486	0.485	-0.538	-0.580	-0.800
	P	*0.196*	*0.034*	*0.504*	*<.0001*	*0.030*	0.030	0.014	*0.007*	*<.0001*
PS, Dgw	r	-0.232	-0.046	-0.241	0.342	0.039	0.101	-0.327	-0.117	-0.177
	P	*0.324*	*0.847*	*0.306*	*0.140*	*0.870*	0.671	0.159	0.623	0.456

VEI= volumetric expansion index; LEI= longitudinal expansion index; SEI= sectional expansion index; RS= resistant starch; IBM= in-barrel moisture; SME= specific mechanical energy; PS= particle size.

Chapter 4 - **Extrusion processing modifications of a dog kibble at large scale alter levels of starch available to animal enzymatic digestion**

Abstract

Results from a preliminary extrusion work indicated that an increased extrusion in-barrel moisture (IBM) and decreased extruder shaft speed (SS) favored resistant starch (RS) retention in a dog kibble. Resistant starches have gained attention due to their prebiotic function that benefits colonic health of monogastric animals. In the present work, single recipe experimental foods to test *in vivo* with dogs were produced on a single screw extruder (model X-115, Wenger Mfg., Sabetha, KS, U.S.A.) in a completely randomized design with 3 replicates per treatment. The low shear (LS), medium shear (MS), high shear (HS) foods were produced with a SS of 400, 800 and 1,200 rpm, respectively. Post-production, kibble density, expansion, and texture analysis were determined. Starch transformations were measured as starch cook (glucoamylase procedure), RS, rapidly (RDS), slowly (SDS) and total (TDS) digestible starch (K-DSTRS kit; Megazyme Int.), and RVA. Single degree of freedom orthogonal contrasts for extrusion outputs, starch analyses and viscosity (RVA) were performed using the generalized linear mixed model (GLIMMIX) procedure from SAS (v. 9.4). Analysis of variance of kibble measurements and expansion indices were performed by the GLIMMIX procedure (SAS v 9.4; Cary NC) with replicate nested within diet. LS means were considered significantly different at a $P < 0.05$. In-barrel moisture had a quadratic ($Q < 0.0001$) increase from HS to MS and LS foods. Wet MS and LS kibbles were denser and less expanded ($P < 0.05$) than the HS treatment, but after drying these differences were minimal. Resistant starch, starch cook and raw: cooked starch RVA AUC

increased linearly from high (HS) to medium (MS) and low shear (LS) foods, while SDS was greater in the LS treatment. Results confirmed that lower thermomechanical energy process led to decreased starch gelatinization and greater retention of RS *in vitro*.

Keywords: extrusion, pet food, starch, gelatinization, resistant starch.

Introduction

Extrusion cooking is the most common process used to produce pet foods worldwide (Research and Markets, 2017). Prior to extrusion, ingredients are ground and mixed. There is a preconditioning stage before the dry mix enters the extruder barrel, where it is hydrated, steam heated and further mixed to allow for hydration time. At the extruder barrel, thermal and mechanical energies are transferred to the dough through water and steam additions, and mechanical shear forces from screw components generate mechanical energy, which occurs under pressure for a short time. Starches and other functional ingredients go through molecular changes that contribute to the formation of a viscoelastic material (Lai & Kokini, 1991). Under a moist environment and thermomechanical energy, starches gelatinize, paste, melt and may fragment (Lai & Kokini, 1991).

Although starch molecular transformations are important for matrix development and kibble expansion, these become readily available for enzymatic digestion in the animal's small intestine, which in their extreme may not be desired nutritionally. Rapidly digestible starches may contribute to a sharp increase in blood glucose (Kimura, 2013), whereas less cooked starches partially maintain their ordered structure and are more resistant to digestion (Ribeiro et al., 2019; Peixoto et al., 2018). Moreover, resistant starches (RS) are desirable because these bypass small intestine digestion and are fermented by saccharolytic bacteria in the colon, which

promote gut health (Jackson, Waldy, Cochrane, et al., 2020; Ribeiro et al., 2019; M. C. Peixoto et al., 2018). In previous work we explored the effects of changing processing parameters to create a surface response model to predict RS content in a dog food using a small-scale twin-screw extruder. The treatment with the lowest extruder shaft speed (SS), highest in-barrel moisture (IBM) and greatest particle size yielded the greater RS. Thus, the objective of the present study was to confirm these process parameters would produce diets with three levels of RS by modifying extruder SS and IBM for validation in an animal study.

Materials and methods

Treatments and extruder settings

A single diet was formulated (Concept5©; CFC Tech Services Inc., Pierz, MN, U.S.A.) to meet the AAFCO (2019) nutrient requirements for adult dogs at maintenance, without any fiber sources and corn as its sole starch ingredient (Table 4.1.). Before extrusion corn was ground in a Jacobson 120-D portable hammermill (Carter Day International Inc., Minneapolis, MN) using a 1.586 mm screen size, then mixed with the remainder of the ground dry recipe then ground again through the same screen size into three batches of 1,904.2 kg dry mix each,. Although the same basal recipe was used to produce treatments, each differed in SS and IBM (Table 4.2.) in order to target diets with 3 levels of RS and starch cook (low, medium and high).

Each treatment was extruded in triplicate in a completely randomized design (CRD) experiment. Ration was preconditioned (Wenger Model 7 Dual Differential Conditioner DDC; Wenger Mfg., Sabetha, KS, U.S.A.) and extruded in a single screw extruder (Model X-115 Wenger Mfg., Sabetha, KS, U.S.A.) equipped with a 100 hp drive motor and an integrated operating system which provided real time calculations for SME, STE and measurement of

system temperatures and pressure readings. The extruder barrel was approximately 1.495 m long (13:1 L/D) with solid flight conveying screws and spiral liners, and a low shear configuration screw profile composed of 7 heads: head 1 was the inlet head with tapered single flight, heads 2-5 were single flight conveying screws, head 6 was an intermediary screw, and 7 was a cone screw. The extruder die plate had 4 die holes of 0.82 cm diameter each. The treatments SS were set at either 250, 385 or 525 rpm, and IBM was targeted to for moisture addition to be considered low, medium and high levels in order with the expectation to produce diets with three levels of RS (Table 4.2.) at high, medium and low extruder shear. Fixed processing inputs included preconditioner (PC) parameters, dry feed rate and die knife speed (Table 4.3.).

Post extrusion, kibbles from each replicate were dried in a forced convection dryer (Binder; Model FP240; Bohemia, NY), which is equipped with load cells and programmed to track loss in weight for moisture control. In sequence, dry kibbles of each replicate were enrobed (Dinnissen liquid dosing system; Dinnissen Process Technology, The Netherlands) separately with fat and dry topical flavor, and coated samples were collected then composited, mixed, and packaged for a subsequent feeding study.

Data collection

Out of the extruder once the process achieved steady state for moisture and extrudate temperature inside the die, samples were collected at 3 times during each replicate production (at 0, 10 and 20 minutes). Each treatment transition lasted approximately 20 minutes. Photographs of the extruder panel were taken at the same time of sample collection, and data were recorded as averages in order to provide representative processing parameters for each replicate. Bulk density (g/L; off the extruder and off the dryer) and flow rate (kg/s) were measured at the same

time as sample collection. Post extrusion, a fraction of each subsample was weighed, combined, mixed and ground prior to laboratory analysis. This allowed for representative data per replicate.

Sample analyses

Viscosity of wet finished kibbles (off the extruder, before drying) was determined by rapid visco-analyzer (RVA) as described in Chapter 3. Data were reported as the area under the curve (AUC) of cooked starch (0.4-6.0 min), raw starch (6.1-14.0 min) and high MW starch (setback viscosity; 14.1-23 min). Starch cook was analyzed by the glucoamylase procedure described by Mason et al. (1982). Resistant starch, as well as rapidly, slowly and total digestible starch were measured on the dry ground raw recipe (K-DSTRS kit Megazyme Inc., Ireland). Insoluble and soluble dietary fibers (IDF and SDF) were determined on extruded replicates using an enzymatic kit (K-RINTDF; Megazyme Inc., Ireland).

Texture analysis was performed on 20 dried kibbles per replicate using a 25 mm cylindrical compression probe equipped with a 50 kg load-cell (TA-XT2; Texture Technology Corp., Scarsdale, NJ, U.S.A.) at 50% strain level. Prior to the analysis, kibbles were equilibrated in an oven at 40ºC overnight. The endpoints measured were kibble toughness (kg×mm) and hardness (kg) which was considered the highest significant fracture force per compression. The diameter and length of 15 wet and dry kibbles were measured twice with a digital caliper and averaged, and then these were weighed on an analytical balance (Ohaus, Explorer: E1RW60, OHAUS, Parsippany, NJ) to calculate kibble density and expansion indices. Kibble volumetric expansion index (VEI) was calculated on wet kibbles according to Alvarez-Martinez et al. (1988):

Equation 4.1

$$VEI = \frac{\rho d \times (1 - Md)}{\rho e \times (1 - Me)}$$

Where: ρd = extrudate density inside the die; Md= moisture content of the extrudate in the die; ρe= apparent density of the wet kibble; and Me= moisture content of the wet kibble. Moisture content inside the die (Md) was estimated to equal IBM. Steam loss was estimated according to Levine (1997) and subtracted from IBM to calculate moisture content of the wet extrudate after exiting the die (Me). Density of the kibbles inside the die (ρd) were calculated using a spreadsheet (Singh, 2013). Sectional expansion index (SEI) was calculated as cm^2_e /cm^2_d, where cm^2_e is the squared kibble diameter, and cm^2_d is the squared die diameter. Lastly, longitudinal expansion index (LEI) was calculated as a function of VEI divided by SEI.

Statistical analysis

The experiment was conducted as a complete randomized design (CRD). Single degree of freedom orthogonal contrasts for extrusion outputs, starch analyses and viscosity (RVA) were performed using the generalized linear mixed model (GLIMMIX) procedure from statistical analysis software (SAS v 9.4; Cary, NC), and linear (L) and quadratic (Q) relationships were considered significant at a $P < 0.05$. Analysis of variance of kibble measurements and expansion indices were performed by the GLIMMIX procedure (SAS v 9.4; Cary NC) with replicate nested within diet, and means were considered significantly different at a $P < 0.05$. Multiple testing was adjusted by the Tukey-Kramer post-hoc test.

Results

Each experimental diet was produced three times on a single day in a randomized order as follows: medium shear (MS), high shear (HS), low shear (LS), HS, MS, LS, MS, HS, and LS. The HS diet was produced with the highest extruder shaft speed (SS), while the LS was extruded with the lowest SS, and MS was intermediate (Table 4.2.). Dry feed rate was initially set to be constant across treatments, but was slightly higher in the LS treatment (Table 4.2.). Pre-conditioner (PC) shaft speed and steam were kept constant throughout extrusion, and PC water was modified to be the lowest in the HS and highest in the LS treatment. However, when the operator attempted to increase PC water in the LS above 23%, the wet kibbles exiting the extruder started to agglomerate and the added water application had to be decreased. As a result, the PC moisture and in-barrel moisture (IBM) increased from the high to medium shear process and plateaued from medium to low shear ($P < .0001$; Table 4.3.).

Pre-conditioner temperature was targeted to remain constant across treatments at 88°C (Table 4.3.). Mass flow rate had a slight linear increase ($P < 0.05$) due to the PC water additions in the MS and LS treatments. Extrusion motor load and SME had a quadratic decrease ($P < 0.05$) from HS to LS driven by the decrease in SS and increase in IBM in each treatment level. Although specific thermal energy was not significant among extrudates, the sum of total specific energy (TSE) decreased in a linear fashion ($P < 0.05$). As a result of extrusion inputs, both kibble wet and dry bulk densities increased linearly ($P < 0.05$) and moisture lost at the drier was greater in the treatments with less intensive cooking (MS and LS; Table 4.3.).

The HS wet kibble was lighter ($P < 0.05$) and more expanded volumetrically (VEI) and longitudinally ($P < 0.05$) than the MS and LS treatments, which had similar expansion indices to each other (Table 4.4.). Upon drying, both volume and kibble density were not different among

treatments but the LS kibbles had a tendency ($P < 0.10$) to be harder and tougher than the other treatments (Table 4.4.).

During the production of diets through this production scale extruder (Wenger model X115) the extrudate temperature inside the die was not recorded due to a system failure. To estimate steam flash-off at the die in order to calculate kibble expansion die temperature was one of the factors needed. Hence, it was estimated by the following equation:

Equation 4.2

$$T_e = \left(\frac{Q_e}{m_e \times C_{pe}}\right) + T_{ref}$$

Where:

$Q_e = P_e \times (motor\ load, \%) \times\ 36 + Q_{se} + Q_{we} + Q_p$

T_e; temperature of product in the extruder just before the die (°C), Q_e; total mechanical and thermal energy rate inside the extruder (kJ/h), m_e; total mass flow of water and dry material inside the extruder (kg/h), C_{pe}; specific heat of the extrudate (kJ/kg°C), T_{ref}; the reference temperature (0°C), P_e; extruder motor power (kW), Q_{se}; steam energy (kJ/h), $Q_{we;}$ water energy (kJ/h), Q_p; preconditioner discharge energy (kJ/h), calculated by the weighted average of the mass fraction of carbohydrate, protein, ash, fat and moisture inside the barrel.

Processing diets at various levels of thermomechanical energy led to different starch transformations. The RVA curve of the HS showed a more pronounced increase in viscosity from 0.4- 6.0 minutes relative to the other treatments. This suggests that starch underwent more extensive chain scission and greater damage (Figure 4.1.). Conversely, both the MS and LS kibbles may have had little to no cold swelling (little mechanically sheared starch). Moreover,

the LS treatment had a greater final viscosity which preserved high MW starch that formed a gel upon cooling (Figure 4.1.). The AUC which represents cold swollen starch decreased in a quadratic fashion ($P < 0.05$; Table 4.5.) and the raw: cooked ratio (raw starch AUC divided by cold swollen starch AUC) increased linearly ($P < 0.05$) driven by the cold swollen starch AUC results.

Starch enzymatic assays confirmed the extent of starch that was available for digestion. Rapidly digested starch (RDS) had a tendency ($P < 0.1$) to decrease linearly from the HS to LS diet. Both the SDS and RS increased linearly ($P < 0.05$) among treatments (Table 4.5.). Conversely, cooked starch decreased linearly ($P < 0.05$) consistent with thermomechanical energy that each treatment received. Fiber analysis was not different among treatments in any of the fiber fractions (Table 4.5.).

Discussion

For a long time, pet food companies have targeted expanded, palatable and highly digestible kibbles. There is an industry need for aesthetically pleasing and consistent croquettes (kibbles). This is usually achieved by fully cooking the starch under a high thermomechanical process known as food extrusion. While effectively cooked kibbles are sufficiently expanded (Baller et al., 2018), durable (Q. D. Tran et al., 2011), aesthetically pleasing, and denature potential antinutritional compounds (Alonso et al., 2000), their overall nutritional value may be compromised. Pet foods produced under high thermal and mechanical energies have been reported to result in vitamin losses (Quang D. Tran et al., 2008), decreased availability of amino acids (Q. D. Tran et al., 2011; Williams et al., 2006; Rutherfurd & Moughan, 1997), among other nutrient changes.

Starches are primary ingredients in extruded foods due to their functional properties to bind particles, form a matrix and assist with kibble expansion upon exiting the extruder die. Since this nutrient is not considered to be a dietary essential, it has been deemphasized regarding nutrition benefit until recently. Less cooked starches may partially retain native starch granules with greater RS compared to their highly cooked forms, which provides substrate for beneficial saccharolytic bacteria in the large intestine and act as a prebiotic (Jackson, Waldy, Cochrane, et al., 2020; Ribeiro et al., 2019; M. C. Peixoto et al., 2018; Q. D. Tran et al., 2008; S. M. Murray et al., 2001). Thus, starch may provide more benefit than merely an economical energy source.

It was the goal of the present study to produce diets with 3 levels of RS. This was achieved, although with less RS than anticipated. The corn used to produce the diets was ground through a hammermill once using a 1.59 mm sieve size, and a second time when mixed with the other basal ingredients (Chapter 3). This smaller particle size contributed to an increase in corn surface area (relative to mass) and resulted in more starch gelatinization than what was targeted. Ribeiro et al. (2019) used a 2.00 mm sieve size to grind corn (once), and that along with decreased SME were able to obtain a diet with nearly 50% more RS than the LS food from the present study. Nevertheless, the present work was able to confirm that starch transformation measured with physical and enzymatic analyses differed according to processing parameters.

The modification of extrusion SME can have a large effect on starch gelatinization. Altering process settings can affect SME and consequently TSE. The preservation of partially gelatinized starches during mild extrusion process have been shown to retain more RS (Murray et al., 2001) that acts as a prebiotic in both dogs (Jackson, Waldy, Cochrane, et al., 2020) and cats (Jackson, Waldy, & Jewell, 2020). In a recent study Jackson et al. (2020) produced a high RS diet by decreasing extruder shaft speed and restricting die open area in order to increase the

amount of energy transfer to the dough. At the opposite extreme of processing parameters (high shaft speed and no die opening restriction) a low RS food was produced. They were able to produce kibbles with a greater separation in TSE compared to the present study; wherein, the low RS had a TSE of 83.2 Wh/kg, while the high RS treatment was produced with nearly half that energy. Other authors have successfully modified the extrudate SME by solely altering the total die open area (Ribeiro et al., 2019; Peixoto et al., 2018). In the study by Jackson et al. (2020), the SME used to produce both low and high shear kibbles were lower than that obtained in the present study. This was likely a result of different extruder hardware with smaller production capacity that generated less total energy during processing.

The HS food in the present study had a 22% greater TSE compared to the LS, and this difference led to changes in kibble expansion, density and extent of starch gelatinization. However, the change in total energy input was not equally distributed across treatments; wherein, TSE decreased by 15% from HS to MS, and only by 8% from MS to LS. Leading these last two treatments to be more closely related in starch cook levels and kibble characteristics. Kibbles produced at lower SME were denser and less expanded than the food produced under high shear conditions. In turn, Baller et al. (2018) also found that higher SME relative to thermal energy led to greater kibble expansion as well, the LS kibbles tended to be harder and tougher than others. This corroborates findings from (Brnčić et al., 2006) regarding a high starch dry mix that produced harder extrudates under low screw speed and high moisture content.

Besides altering kibble characteristics, the differences in extrusion parameters in the present study also modified starch digestion profile. The physical-viscosity method exemplified by the RVA methodology was performed to confirm starch transformations due to limitations in the RS and starch cook enzymatic assays. The main limitations of the RS and starch cook

procedures are, starch digestion *in vivo* is dependent on animal factors which are not accounted for *in vitro,* and the digested starch from the starch cook procedure includes some raw starch that can be digested due to the presence of pores and channels in corn and other cereals (Fannon et al., 1992, 1993). In the RVA procedure the addition of water while heating the starch-containing material disrupts amylose helices and crystallinity which allows starch to swell and paste (Remsen and Clark, 1978). The pasting phenomenon occurs due to amylose leaching, which increases the matrix viscosity and gels upon cooling, further increasing viscosity (Remsen and Clark, 1978). All of these stages are measured in the RVA profile of a sample with temperature changes. The limitation of kibble RVA is that the composition of pet foods is not solely starch. Other nutrients and ingredient physical characteristics may interfere with the analysis and interferes with peaks in each stage of the RVA curve. For this reason, total area under the curve corresponding to each starch transformation was reported rather than peak viscosities.

The RVA profile of the HS diet had a slight increase in cold temperature viscosity (below 25°C), due to the high mechanical shear profile used to produce this treatment. This increase in cold viscosity reflects the presence of dextrins and the interactions among them (Whalen, 2015). Conversely, treatments MS and LS that were cooked with less mechanical energy resulted in lower initial cold swelling which translates into a greater presence of raw starches (Whalen, 2015). Final viscosity would be expected to be greater in the presence of more native raw starch (Whalen, 2015). However, the MS treatment had mixed outcomes: it behaved similar to a low shear profile for cold swollen (cooked) starch and raw starch viscosities, but more like the high shear food in regard to setback viscosity. When compared to the study that preceded this (Chapter 3), the setback viscosity of the MS diet was closest to sample 16. This treatment was produced under the greatest SME amongst all the variables. Regardless, the present study

confirmed that the MS food had an intermediate level of raw: cooked starch relative to the other treatments. This was also confirmed by the starch cook and resistant starch assays.

The last method explored in the present study to detect RS in dog kibbles was the total dietary fiber procedure (TDF). According to Codex Alimentarius Commission (2010), dietary fiber includes carbohydrate polymers hat are not hydrolyzed by the endogenous enzymes of humans. This may be extrapolated to other monogastric animals. Resistant starches from enzymatic digestion as part of the TDF procedure may be captured in the insoluble fiber component (McCleary et al., 2012). In the present study, the low concentration of RS associated with high variability in the TDF procedure did not result in this starch fraction to create a treatment difference.

Conclusion

The modifications in extrusion processing mechanical and thermal energies had an impact on kibble characteristics and starch transformation. The low shear (LS) and medium shear (MS) were more dense and less expanded than the high shear food (HS), and were more closely related due to their common IBM with little difference in the total specific energy imparted to the product. Physical and chemical starch analyses complemented and strengthened one another. Starch RVA profile, starch cook and resistant starch assays confirmed that there was a low, medium and high level of starch gelatinized in the low, medium and high shear foods. The effects these diets have on overall dog health will be determined on a future study.

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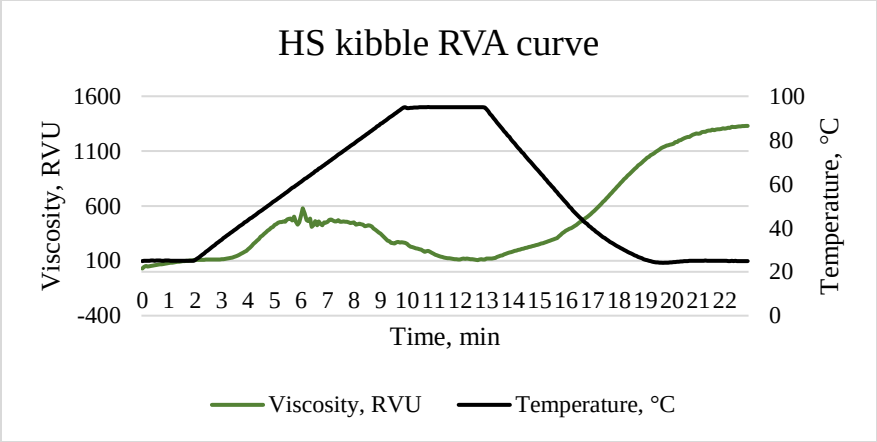

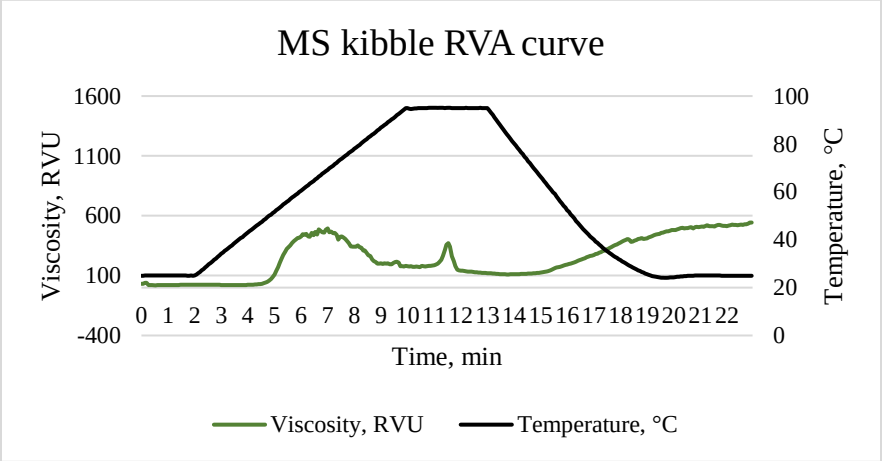

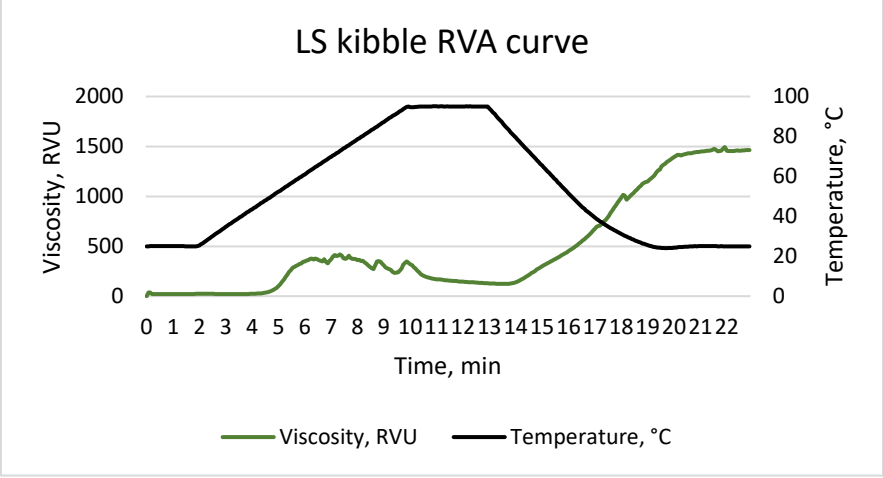

Figure 4.1

Rapid Visco Analysis (RVA) Curve of the Low, Medium and High RS Diets (HS, MS and LS, respectively; Average of 3 Replicates each). Cooked Starch AUC was considered from Min 0.4-6.0, Raw Starch AUC was Considered from Min 6.1-14.0, and High Mw Starch (Setback Viscosity) from 14.1-23 Min.

Table 4.1.

Ingredient composition of the Low, Medium and High Resistant Starch (RS) Experimental Treatments, as-is basis.

Ingredient	Inclusion, %
Grain mix	
Whole yellow corn	65.4
Chicken meal	20.0
Potassium chloride	0.400
Vitamin premix	0.100
Lysine	0.100
Sodium chloride	0.100
Taurine	0.050
Mineral premix	0.100
Preconditioner	
Choline chloride, liquid, 70%	0.200
Lactic acid, blend 84%	1.50
Coating	
Choice white grease	8.40
Chicken, viscera and liver digest	3.00
Pork liver digest	0.500
Vitamin E, oil, 29%	0.100
Mineral premix	0.042

Table 4.2.

Input Parameters (Average ± Standard Error) of Pilot Extrusion Process (X115) used to produce Diets with High Shear (HS), Medium Shear (MS), and Low Shear (LS).[1]

Item	HS	MS	LS
Shaft Speed, rpm	525	385	250
Grain Mix, g/L	668	668	668
Grain Mix Moisture, %	11.6	11.6	11.6
Dry Feed Rate, kg/h	871 ± 47.1	871 ± 47.1	898.1 ± 0.00
PC[2] Shaft Speed, rpm	338 ± 0.7	338 ± 0.5	338 ± 0.5
PC Water, kg/h	117.5 ± 6.35	197.6 ± 15.59	206.6 ± 0.00
PC Water, %	13.5 ± 0.00	22.67 ± 0.58	23.0 ± 0.00
PC Steam, kg/h	48.9 ± 1.18	50.0 ± 0.78	50.2 ± 0.12
PC Steam, %	5.6 ± 0.00	5.67 ± 0.12	5.6 ± 0.00
Extruder Shaft Speed, rpm	525	385	250
Die Knife Speed, rpm	700	700	700

[1]Extruder water and steam were kept at 0% during all the runs.
[2]PC= preconditioner

Table 4.3.

Least Square Means and Contrasts [Linear (L); Quadratic (Q)] for Outputs from Extrusion Processing used to produce Diets with High Shear (HS), Medium Shear (MS), and Low Shear (LS).

Item	HS	MS	LS	SEM	*L*	*Q*
[1]PC Load, %	33.8	32.7	32.3	0.47	0.0729	0.5996
PC Moisture, %	20.0	25.2	25.3	0.06	<.0001	<.0001
PC Temperature, °C	88.9	88.0	87.2	0.35	0.0244	0.7549
Flow Rate, kg/h	1,184	1,266	1,308	31.7	0.0326	0.6142
Motor Load, %	62.8	47.2	41.3	0.61	<.0001	0.0017
[2]SME, Wh/kg	39.5	27.9	23.6	0.85	<.0001	0.0128
[3]STE, Wh/kg	32.8	33.5	32.7	0.60	0.9748	0.3669
[4]TSE, Wh/kg	72.2	61.3	56.3	1.36	0.0002	0.1194
[5]IBM, %	25.8	31.2	31.3	0.09	<.0001	<.0001
Wet Bulk Density, g/L	386	428	435	7.6	0.0039	0.1117
Dry Bulk Density, g/L	296	324	338	6.2	0.0029	0.3615
Dry Flow Rate, kg/h	1,101	1,101	1,136	28.1	0.4198	0.6349
Moisture Loss at Drier, %	6.98	13.04	13.16	0.078	<.0001	<.0001

[1]PC= pre-conditioner.

Extruder moisture was the same as PC moisture because no water or steam were added to the extrusion barrel.

[2]SME= specific mechanical energy.

[3]STE = specific thermal energy.

[4]TSE = total specific energy.

[5]IBM = in-barrel moisture, calculated.

Table 4.4

Kibble Parameters and Texture Analysis of High, Medium and Low Shear Diets (HS, MS and LS, Respectively).

Item	**HS**	**MS**	**LS**	**SEM**	***P* (F)**
Wet kibble					
Volume, cm^3	1.541	1.257	1.260	0.0718	0.0497
Density, g/cm^3	0.683^b	0.866^a	0.848^a	0.0263	0.0049
[1]VEI, $cm^3_{kibble}/ cm^3_{die}$	1.097^a	0.720^b	0.728^b	0.0291	0.0001
[2]LEI, cm_{kibble}/ cm_{die}	0.823^a	0.611^b	0.601^b	0.0304	0.0034
[3]SEI, $cm^2_{kibble}/ cm^2_{die}$	1.353	1.188	1.232	0.0739	0.3336
Dry kibble					
Volume, cm^3	1.56	1.57	1.55	0.061	0.9691
Density, g/cm^3	0.527	0.540	0.554	0.0109	0.2770
Hardness, kg	8.37	8.13	9.96	0.534	0.0996
Toughness, kg×mm	1,063	1,101	1,449	105.2	0.0758

[1]Volumetric expansion index.
[2]Longitudinal expansion index.
[3]Sectional expansion index.

Table 4.5.

Least Square Means and Contrasts [High vs Medium and Low Shear (T); Linear (L); Quadratic (Q)] for Starch Analyses from Diets containing Three Levels of Cooking.

Item	HS	MS	LS	SEM	*L*	*Q*
Viscosity (RVA)						
Cold Swollen Starch AUC, RVU	1,120	402	330	72.61	0.0003	0.0109
Raw Starch AUC, RVU	2,206	1,988	1,996	110.2	0.2269	0.4327
[1]Raw:Cooked Ratio	2.02	4.98	6.24	0.485	0.0009	0.2029
High M_w Starch AUC, RVU	7,281	3,170	8,411	1,182.7	0.5243	0.0179
Starch Analyses						
Rapidly Digested Starch, %	45.9	42.2	41.1	1.53	0.0686	0.5079
Slowly Digested Starch, %	2.02	2.98	6.41	1.072	0.0276	0.3832
Total Digested Starch, %	53.7	51.7	51.1	0.898	0.0909	0.5417
Resistant Starch, %	0.650	0.940	1.057	0.0926	0.0210	0.4739
Total Starch, %	54.3	52.6	52.2	0.94	0.1567	0.6050
Cooked Starch, %	99.6	91.9	88.8	1.168	0.0006	0.1615
Fiber Analysis						
TDF, %	2.51	2.64	3.03	0.159	0.0621	0.5410
IDF, %	1.46	1.67	1.70	0.197	0.4227	0.7102
SDFP, %	1.052	0.969	1.326	0.1211	0.1598	0.1885

[1]Calculated by dividing the area under the curve (AUC) between the raw starch and cold swollen starch RVA.

Chapter 5 - **A low to medium shear extruded kibble with greater resistant starch leads to increased fecal oligosaccharides, butyrate, and other saccharolytic fermentation by-products in dogs**

Abstract

Resistant starches (RS) comprise starch or starch derivatives that escape small intestine (SI) digestion and reach the colon, where fermentation by saccharolytic bacteria produce beneficial postbiotics. These RS can be retained in the food upon mild extrusion processing. The objectives of this study were to assess whether diets with increased RS had a positive effect on the colonic health of dogs. Three identical recipe diets were produced with three levels of mechanical energy: high, medium and low shear (HS, MS and LS), and were fed to 24 adult dogs in a replicated 3x3 William's Latin square design (n=24) with 28-day periods. Fasting blood and fresh feces were collected during the last week of each period. Serum and plasma were separated and stored at -70°C until analyses. Fecal quality was maintained among treatments, assessed by a subjective scale. Gut integrity markers fecal IgA, serum lipoteichoic acid and lipopolysaccharide, and plasma satiety hormones were measured using ELISA procedures. Fecal short-chain fatty acids (SCFAs) were measured by liquid Chromatography with tandem mass spectrometry (LC MS/MS). In addition, the microbiome of dogs was determined on fresh feces by high throughput sequencing of the 16s rRNA gene and analyzed at the genus level. Untargeted metabolomics of both feces and serum were determined by UPLC. Data were analyzed using mixed models. The number of tests conducted for the microbiome and metabolomics data were also corrected for false discovery rate. There was no evidence for treatment effects on satiety hormones at fast, or in gut integrity markers. Dogs who were fed LS or MS diets had a marginal evidence (P = 0.07)

for decreased fecal pH relative to HS, with a higher concentration of fecal oligosaccharides, succinate and lactate, indicating greater fermentation in these treatments compared to HS. Also, dogs fed the MS or LS diets showed a shift towards more saccharolytic bacteria, with decreased counts of the non-saccharolytic genera. An increase in fecal butyrate in dogs fed the LS diet relative to the HS diet was considered an additional indicator of improved colonic health of dogs fed the mildly processed foods.

Keywords: dog, microbiome, resistant starch, extrusion, corn, metabolomics.

Introduction

Pet food companies are in constant search of innovation. This is frequently accomplished by exploring novel or exotic ingredients in their dietary formulas. Another sustainable approach is to modify ingredients already present in the food. Such modifications could affect nutrient availability in value-based ingredients, such as starches in grains. Starch ingredients that are extruded with low mechanical energy retain some natural resistant starch (RS) both *in vitro* (Murray *et al.*, 2001) and *in vivo* (Peixoto *et al.*, 2018; Ribeiro *et al.*, 2019; Jackson *et al.*, 2020). Resistant starches (RS) are starch derivatives that escape small intestinal digestion and absorption, and reach the colon, where their prebiotic function may provide health benefits (Peixoto *et al.*, 2018; Ribeiro *et al.*, 2019; Jackson *et al.*, 2020). To date, extruded pet foods produced in the US have little to no RS due to extensive starch gelatinization caused by the thermomechanical process (Corsato Alvarenga and Aldrich, 2020). Previous studies have explored health implications for dogs fed a low and high shear extrusion processes focused on local colonic effects (Peixoto *et al.*, 2018; Ribeiro *et al.*, 2019; Jackson *et al.*, 2020). In this study, we explore both systemic and local endpoints related to dogs consuming diets produced at

low and high shear, as well as an intermediate level of extrusion cooking. This work also aimed to characterize microbiome changes in the colon related to consumption of these foods.

We hypothesized that diets produced with less thermomechanical energy (i.e. low shear; LS) would promote an increase in short-chain fatty acids (SCFA) production in the colon of dogs, as well as increased satiety, fecal IgA (i.e. improved local immunity), and decreased serum LPS and LTA (i.e. improved local inflammation), relative to the low RS treatment (HS). Due to more extensive fermentation, we further hypothesized a decrease in colonic pH and an increase in lactate in dogs fed the LS treatment compared to the HS diet. The medium shear (MS) treatment was anticipated to present intermediate effects of both extremes.

Materials and methods

Dietary treatments

A single diet was formulated to meet the nutrient requirements of adult dogs (AAFCO, 2019), with whole yellow corn included at 65% as the sole starch source. The remainder of the formula included 20% chicken meal, 8.4% choice white grease, as well as minerals, vitamins and palatants. This ingredient blend was extruded at 3 levels of total specific energy, as described in Chapter 4, and stored in heat-sealed polypropylene bags. For each dietary treatment, nutrients were analyzed at a commercial laboratory (Eurofins; Des Moines, IA; Table C.1.), according to their respective analytical methods (Table C.2.). Treatments consisted of a high, medium and low shear foods (HS, MS and LS, respectively) that resulted in increasing concentrations of RS, respectively.

Digestibility & palatability assessment

For each dietary treatment, digestibility was estimated prior to the gut health study with three independent groups of dogs. This was a preliminary assessment for diet suitability for the following gut health study. Apparent total tract digestibility (ATTD) trials used 6 Beagle dogs over 10 days per diet, with 5 d adaptation and total feces collected during the last 5 days, according to official procedures (AAFCO, 2019). Samples of fresh feces were collected and frozen at -80°C until analysis. Proximate analyses were conducted on diets and on feces, and included dry matter (100% - moisture %; AOAC 930.15), ash (AOAC 942.05), organic matter (100% - moisture% - ash%), gross energy (measured as total heat of sample combustion by calorimetry), crude protein (AOAC 2001.11), true protein (feces only; crude protein in feces – estimated endogenous losses in feces), crude fat by acid hydrolysis (AOAC 954.02), and crude fiber (AOAC 962.09). Digestibility coefficients were calculated as (nutrient intake – nutrient fecal output)/ nutrient intake, on a dry matter basis. True protein digestibility was calculated based on an estimated factor (Kendall, Blaza and Holme, 1982; Golder, Weemhoff and Jewell, 2020). While crude protein accounts for all the protein measured in the feces, true protein considers the endogenous losses in the feces from host protein and microbial mass.

Fecal quality of dogs fed diets during the preliminary digestibility trial was determined according to a subjective scale from 1 to 5, recorded in 0.5 point increments; wherein, 1 = liquid feces (diarrhea), 2 = very soft feces, with little shape retention; 3= greater than 75% soft feces, less than 25% liquid feces, with some shape retention; 4= greater than 75% shape retention, with possible segmentation, and between 50-80% firm feces; and 5 = retained shape, above 80% firm feces, without segmentation. On this scale, scores 4 or 5 were considered ideal. Diet palatability was determined in 25 dogs with a two-bowl forced choice test over two days with bowl position

switched for each day to remove side-bias. Intake ratio of each animal was determined as the ratio of total consumption of food A and total consumption of foods A and B.

Gut health study

Animals and study design

Eighteen adult healthy Beagle dogs and 6 adult mixed breed dogs were recruited for the feeding study at the Hill's Pet Nutrition Center kennel (Topeka, KS). The experimental design was that of a replicated Williams Latin square design, whereby each dog received all three dietary treatments over three periods. Each dog was assigned to one of six feeding sequences arranged in two squares balanced for carry over effects with four dogs assigned to each sequence. Participating dogs consisted of two intact females, 9 neutered males, and 13 spayed females, with an average age (± standard deviation) of 5.1 years ± 3.20 (range 1.16 to 12.44 years old), and average BW (± standard deviation) of 10.6 kg ± 2.26 (range 6.8 to 15.8 kg). Dogs were weighed weekly to adjust food intake if necessary. The study was approved by the Institutional Animal Care and Use Committee (IACUC) at Hill's Pet Nutrition (protocol number 883.0.0.0).

Dogs were housed in one of three neighboring buildings and fed individually with an automated feeding system once (at 07:30) each day; fresh water was available at all times. Although dogs were housed in pairs, only one dog of each pair was included in the study. Dogs were allowed daily socializing time in a common playground area as part of the enrichment program. Before study initiation, dogs were fed a common baseline adult maintenance diet for 30 days to allow for a wash-out period from their previous diets. Then, dietary treatments were fed over three periods of 28 d each.

Sample collection and handling

Blood and fresh feces were collected at the beginning of the study (day 0), and at the end of each period; wherein, fresh feces were collected within 15 minutes of defecation within one hour after feeding (07:30 to 08:30) from day 24 to 26 of each period. Blood was collected on days 27 and 28 of each period. Dogs were not allowed access to the ballpark 2 days before and during the collection week to avoid ingestion of grass. One fresh feces was collected per dog per period and scored for consistency based on a subjective scale described above. Next, feces were homogenized with planetary mixing (model ARM-310; Thinky Corporation; Japan) at 2,000 rpm for 30 seconds. Then, 0.75 mL of fresh homogeneous feces were plunged into five cryotubes using a 60 mL syringe, and immediately placed in liquid nitrogen. These were kept in a -80 ºC freezer until analyses were performed.

Blood collection was performed prior to feeding at 07:00 of days 27 and 28 of each period. Approximately 9 mL of blood were collected through brachycephalic venipuncture with 5 mL placed in a 5-SST yellow-cap tube for serum separation (BD Vacutainer™ tube BD 367989), and 4 mL was equally divided into two Hemogard tubes (grey cap) with EDTA (BD Vacutainer™ tube BD 366450). Tubes were centrifuged for 10 minutes at 3,000 rpm (Beckman-Coulter Allegra X-15R) and kept on ice while processing. Plasma was separated for immediate complete blood count (Sysnex XT-2000i) and satiety panel analysis. A portion of the serum was used for blood chemistry (Roche Hitachi Cobas 6000) and the remainder stored in cryotubes at -70°C for LPS/LTA and metabolomics analyses.

Food intake and fecal analyses

For each animal, daily food intake was recorded and divided by the metabolic body weight (i.e. $BW^{0.75}$) of each animal for normalization. These were averaged per animal per period before statistical analyses. Resistant starch intake was calculated based on RS determinations on each food (Chapter 4).

Fecal parameters were determined on one stool defecation per dog per period. These fecal samples were representative because technicians were instructed to report any abnormal stools, and none were reported. Homogenized fresh feces from the feeding study were taken to the laboratory and fecal pH was measured immediately. Approximately 5 g of a thin layer of fresh feces were placed in an aluminum pan and dried at 104°C for 3 h to determine moisture (modified AOAC 935.29). Feces were ground using a vibratory micro mill (Pulverisette 0, Fritsch Milling and Sizing, Inc.; Pittsboro, NC). The remaining dried feces were stored in liquid nitrogen until mineral analysis. Another portion of the fresh feces was placed in a porcelain crucible and heated in a muffle furnace for approximately 2 hours at 600°C to determine ash content (AOAC 942.05). For mineral analysis, dried feces were ground in the vibratory micro mill, acid digested (modified EPA 200.2) and minerals were determined in an Agilent 5100 OES (Santa Clara, CA, U.S.A.). Fecal ammonia was determined as described by Chaney and Marbach (1962).

Satiety hormones

Satiety hormones were measured in plasma after a 20h fast. The satiety panel included ghrelin, gastric inhibitory polypeptide (GIP), glucagon-like peptide 1 (GLP-1), glucagon, insulin, leptin, pancreatic polypeptide (PP) and peptide YY (PYY). Samples were evaluated for satiety

hormones using an ELISA kit (Milliplex Map Canine Gut Hormone Magnetic Bead Panel - Endocrine Multiplex Assay; MilliporeSigma, Burlington, MA, U.S.A.), and measured on a Luminex 200 (Luminex Inc., Austin, TX, U.S.A.).

Microbiome: DNA extraction, 16S sequencing, PCR amplification and processing

Fecal samples to analyze for microbiome were divided into 2 runs of 48 single replicate samples each (baseline and 3 periods, N=24) which were balanced according to dog age (t-test; $P = 0.57$), gender (χ^2; $P = 0.86$), diet (χ^2; $P = 1$) and period (χ^2; $P = 1$). Fecal samples freshly collected from the gut health study were frozen, cryo-extracted, then total DNA was extracted using a commercial kit (ZymoResearch quick-DNA fecal/soil microbe 96 magbead, Irvine, CA, U.S.A.). This procedure allows for rapid isolation of inhibitor-free PCR-quality DNA. The sample lysis step was performed without beta-mercaptoethanol using the ZR BashingBead™ Lysis Rack through a series of steps. Next, DNA was purified using a buffer with magnetic beads that bind to the DNA. Briefly, the beads with bound DNA formed a pellet over a magnetic stand, the supernatant was discarded, and beads washed with an ethanol solution. This step was repeated three times. Once the DNA bound to beads was clean and dry a DNA elution buffer was added to each well. The beads and DNA were mixed into solution and beads were regrouped on a magnetic plate. The supernatant with the DNA was carefully transferred to a clean 96-well plate and stored at -20°C overnight.

The next step was the creation of 16S Amplicon for microbiome sequencing. Eluted clean DNA was transferred to a 96-well plate along with a positive and negative control (normalized mock community and PCR grade water, respectively) to assure the procedure was free of contaminants and performed as expected. A V3V4 16S forward (347F) and V3V4 16S reverse

(803R) primers were added to each sample, as well as an Archeal forward (A349F) and reverse (A806R) primers (Life Technologies; Carlsbad, CA, U.S.A.). The PCR plate was placed in a thermocycler (Biorad C1000 touch thermal cycler; Hercules, CA, U.S.A.) for 16S Amplicon production using an enzyme (Roche Faststart High Fidelity PCR System; Roche 03553400001; Madison, WI, U.S.A.) and stored at -20°C overnight.

The last step before sequencing was the library preparation. A series of steps were once again performed to purify the 16S amplicons produced by PCR using magnetic beads (Agencourt AMPure XP; Beckman Coulter; Brea, CA, U.S.A.) and ethanol as described in the first cleaning step. Next, Index 1 primers were added horizontally to the plate and Index 2 primers were added vertically (Nextera XT Index kit; Nextera XT Index kit, FC-131-1001; Ilumina, San Diego, CA, U.S.A.) so that each sample would have a unique combination of both labels. The plate went through a thermocycle PCR. The 16S V3V4 library product from the PCR was cleaned and washed once again.

The 16S V3V4 library was quantified by fluorimetry on a plate reader (Promega GloMax® Discover Microplate Reader; Madison, WI, U.S.A.) using a kit (ThermoFisher Quant-iT™ dsDNA High-Sensitivity Assay Kit; Whaltam, MA, U.S.A.) and each well diluted (normalized) to an equal final concentration of 4 nM so that the entire library size was close to 539 bp. An aliquot of 5 µL from each well library was pooled and mixed in a microtube for one single MiSeq run. The pooled final library was denatured into single strand DNAs with freshly prepared sodium hydroxide solution and a hybridization buffer was added to result in a 1 mL 20 pM denatured library. A control denatured Phix library (Ilumina; San Diego, CA, U.S.A.) was prepared similarly to the amplicon library, and diluted to the same final concentration. These libraries were combined, heat denatured, and loaded into the MiSeq V3 reagent cartridge

(Ilumina; San Diego, CA, U.S.A.) which was thawed and prepared simultaneously to the pooled DNA library preparation. The MiSeq V3 reagent cartridge and Miseq flow cell were both loaded in the MiSeq system (Ilumina, San Diego, CA, U.S.A.) for sequencing. During sequencing, the final DNA library was pooled from the cartridge into the flow cell, where clusters were positioned, and a template generated. Colored nucleotides were aligned with the template, and cycles of imaging were captured and interpreted by the Miseq system. A total of 251 cycles were run twice with the Miseq instrument.

Output FASTQ files from the Miseq instrument were pre-processed to contigs, chimeras were removed, and the highly variable V3V4 region of the 16S gene were filtered by the Mothur software (Kozich, 2013). Microbial taxa data sequences at the genus level were mapped with GreenGenes (V 13.5) (DeSantis *et al.*, 2006). Bacterial OTUs were normalized to copy number and further processed for functional profile using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt; Langille *et al*., 2013).

The 16S RNA sequencing was divided into 2 runs. Cluster density was 772 and 640 K/mm^2 for the first and second runs, respectively, with 87% and 90% clusters passing the first filter. There were 231 total OTUs identified after high-throughput sequencing and copy number correction. The entire raw OTU counts data was used to calculate alpha diversity indices following from Hill (1973), namely richness, inverse Simpson (invSimpson) and exponent Shannon (expShannon) (Hill, 1973; Chao, Chiu and Jost, 2010). Specifically, richness refers to the number of different OTUs present in each fecal sample, while invSimpson considers the diversity of common OTUs among samples, and expShannon focuses on the diversity of the most abundant OTUs. Pielou's evenness (J) was calculated as a fourth alpha diversity index (Hill, 1973). Evenness is a value constrained between 0 and 1 and indicates how close in number

are the different OTUs detected in a sample of dog gut microbiome. When the counts of OTUs are similar, evenness is close to one.

Fecal short-chain fatty acids

Fecal SCFA acetic, propionic, butyric, isobutyric, valeric, isovaleric, 2-methyl butyric and hexanoic (caproic) acids were analyzed by a commercial laboratory (Metabolon, Morrisville, NC). Briefly, fecal samples were spiked with a solution of eight stable labelled internal standards, homogenized, and subjected to protein precipitation. After centrifugation, an aliquot of the supernatant was derivatized. The reaction mixture was analyzed by LC MS/MS (Agilent 1290/AB Sciex 5500 system; Agilent Technologies Inc., Santa Clara, CA, U.S.A.).

Immunological assays

Dog fecal immunoglobulin A (IgA) was quantified using a dog IgA ELISA quantitation set (Bethyl Laboratories; Montgomery, TX, U.S.A.). Gram-positive bacteria cell wall component lipoteichoic acid (LTA) was measured on dog serum by sandwich ELISA using a human lipoteichoic acid ELISA kit (Novateinbio; Woburn, MA, U.S.A.), and gram-negative bacterial endotoxin and membrane constituent lipopolysaccharide (LPS) was measured on dog serum by a turbidimetric assay using limulus amebocyte lysate (LAL) extract that reacts with the bacterial proteins (Pyrogent-5000 bulk kinetic turbidimetric LAL assay; Lonza, Mapleton, IL, U.S.A.). All immunological assays were performed at a commercial laboratory (MD Biosciences; Oakdale, MN).

Serum and fecal metabolomics

Analysis of plasma and fecal metabolites derived from metabolism of carbohydrates, protein, fat, vitamins, nucleotides, among others, were performed by a commercial laboratory (Metabolon, Morrisville, NC). The first step was sample preparation with methanol extraction under vigorous shaking and centrifugation to recover chemically diverse compounds. Metabolites were measured by four different methods using a ultra-performance liquid chromatography (ACQUITY UPLC; Waters, Milford, MA, U.S.A.) and a high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II, Thermo Scientific Q-Exactive) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried and reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. The first and second aliquots (hydrophilic and hydrophobic) were analyzed using acidic positive ion conditions. The hydrophilic compounds were eluted using a water and methanol gradient-eluted C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm) with 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). The hydrophobic molecules were also eluted with a C18 column, but using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA. The third aliquot was analyzed using basic negative ion optimized conditions and eluted on a C18 gradient-eluted column using methanol and water with 6.5mM ammonium bicarbonate at pH 8. Finally, the fourth aliquot was analyzed via negative ionization following elution from a hydrophilic interaction liquid chromatography (HILIC) column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10mM ammonium formate at pH 10.8. Raw data ions were detected and processed using the laboratory library of standards for metabolite identification and

for metabolite quantitation by peak area integration. Missing values were imputed with the observed minimum for each individual compound. Furthermore, quality control of internal standards and endogenous metabolites ensured that results met the process specifications.

Statistical analyses

Digestibility study

As part of the preliminary assessment, the digestibility coefficients of the HS and LS were performed on the same group of 6 dogs, and those of the MS were obtained from a separate group of 6 animals. Because this assessment did not have an experimental design, an unpaired t-test was conducted for HS vs. MS and MS vs. LS, and a paired t-test was conducted for the HS vs. LS treatments, using the TTEST procedure from Statistical Analysis Software (SAS v 9.4; Cary, NC, U.S.A.).

Gut health study response variables

Response variables from the gut health study included food intake, fecal parameters, blood chemistry and CBC, satiety hormones, fecal SCFA, gut integrity hormones, and alpha diversity indices generated from the microbiome data. For each response, a general linear mixed model fitted to reflect the data generation process of a replicated Williams Latin Square design. The linear predictor included the fixed effects of diet (LS, MS, HS), treatment sequence (1 to 6), period (1 to 3), and carryover effects specified with a sum-to-zero restriction, and the random effect of housing location as an overarching blocking structure. A compound symmetry covariance structure was specified at the residual level for observations collected within a dog. Variance components were estimated using restricted maximum likelihood. Model assumptions

were evaluated using externally studentized residuals and were considered to be reasonably met. A Kenward-Rogers approach was used to estimate degrees of freedom and make corrections to estimated standard errors.

Statistical models were fitted using the GLIMMIX procedure of SAS (Version 9.4, SAS Institute, Cary, NC). Estimated least square means, corresponding standard errors and 95% confidence interval are presented in the original scale (OS). Pairwise treatment comparisons were conducted using a Tukey-Kramer approach to avoid inflation of Type I error rate due to multiple comparisons. Differences were considered significant at $P < 0.05$, and marginally significant at $0.05 < P < 0.10$. Marginal significance was also referred as a trend or tendency. Responses fecal acetic acid, valeric acid, 2-methylbutyric acid, isobutyric acid, isovaleric acid, hexanoic acid, total BCFA, ash, ammonium, as well as serum LTA, glucagon and leptin, and microbiome inverse Simpson, richness and Pileou's evenness, and RS intake required specification of heterogeneous residual variances, by diet or period. The covariance structure for each analyte was selected according to the Bayesian Information Criteria for best fitting. Responses fecal ash, IgA, hexanoic acid, valeric acid and butyric acid, as well as plasma GIP, Ghrelin, Insulin, PP, leptin, microbiome Pileou's evenness and exponent Shannon, and intake (in both g and kcal per $BW^{0.75}$)required a variance stabilizing transformation to meet model assumptions.

Fecal scores of dogs were analyzed as multinomial data assuming a cumulative logit distribution by the GLIMMIX procedure, and score frequency was computed by the FREQ procedure (SAS, v 9.4). Finally, a Spearman Rho's correlation was conducted between the significant microbiome OTUs (on the centered log-ratio scale) and markers of carbohydrate fermentation, including SCFAs, fecal pH, fecal lactate and succinate, and fecal oligosaccharides.

Gut health study metabolomics and microbiome

Metabolomics and microbiome OTU count data were filtered and transformed prior to analyses. Specifically, metabolomics raw data was obtained as intensities of each analyte peak and were first median centered by dividing the datapoints by the median of each analyte, then converted to logarithm at base 2 to normalize residuals. Only analytes detected in over 80% of samples within at least one dietary group were included in the statistical analysis. The microbiome OTU data was specified at the family and genus levels. Similar to metabolomics, the OTU data was filtered so that all OTUs analyzed had counts above zero in more than 80% of fecal samples within at least one dietary group. Next, zero OTU counts were inflated using the Bayesian multiplicative treatment (Martín-Fernández *et al.*, 2015) and converted to centered log-ratio (CLR) to normalize the arbitrary counts determined by the DNA sequencer:

Equation 5.1

$$CLR(OTU_X) = Ln\left(\frac{OTU_X}{G_{OTU}}\right)$$

Where OTU_X = counts of one OTU in the dataset; G_{OTU} = geometric mean of all OTU counts measured within a fecal sample. The robust outlier method correction Huber was applied to both OTU and Metabolomics datasets. The metabolomics serum and fecal data had 204 outliers removed altogether, and OTU data had 45.

The CLR transformed OTUs and logarithm at base 2 transformed serum and fecal metabolomics were analyzed using a mixed model with fixed effects for dietary treatment, period, treatment sequence and carryover (JMP Pro v. 15). A compound symmetry covariance

structure was specified with period as the repeated structure and animal nested within sequence as the subject to allow for intraclass correlation of dogs across periods. Tukey post-hoc test was applied to correct for multiple comparisons, and *P*-values of OTUs, serum and fecal metabolites were corrected for false discovery rate (FDR) (Storey, 2010) using the "qvalue" (R package; Storey et al., 2014). Treatment differences were considered significant when q-value < 0.10 and $P < 0.05$, and marginally significant when only *P* was less than 0.05. Sequential to statistical analysis, metabolites of both serum and feces were grouped according to the following classes: saccharolytic, proteolytic, energy metabolism, lipolysis, and bile salt metabolism for presentation and discussion purposes.

Results

Digestibility and palatability assessment of experimental diets

As designed, the complete nutrient profile among dietary treatments were similar and met levels for adult dogs at maintenance (AAFCO, 2019; Table C.1.). The digestibility assessment was conducted separately and prior to feeding dogs for the microbiome/metabolomics evaluation. For this preliminary digestibility study, one group of 6 dogs was fed the MS diet, and a second group of 6 dogs was fed the HS and LS sequentially. Thus, data were compared as paired t-tests for MS vs LS, and an unpaired t-test for the other combinations. There did not seem to be any problems with fecal scores, with most scores of 5 (91.95%) and 4 (8.05%), though fecal samples were missing for three dogs on a single day throughout the collection period. Dry matter (DM), organic matter (OM) and gross energy (GE) apparent total tract digestibility (ATTD) were not different between dietary treatments, with an overall mean estimate of 85.3%, 88.7%, and 88.8%, respectively across diets (Table 5.1.). Crude protein ATTD was

approximately 2 percentage points greater for dogs fed LS relative to HS and MS dietary treatments, though there was no evidence for differences between the latter. This dietary difference was not observed for digestibility coefficients of true protein ATTD. Crude fat ATTD was not significantly different among dietary treatments ($P > 0.05$). Finally, estimated ash ATTD was almost two-fold greater in LS diets compared to MS and HS diets ($P < 0.05$), though the latter two were not significantly difference. For daily intake and fecal outputs, on both as-is and DM basis, there was no evidence for any differences between dietary treatments.

The palatability test was conducted only for the HS vs MS using 25 dogs. In a production attempt to increase the extruder moisture content of the LS, the kibbles agglomerated, and enough grain mix was lost so that not enough LS kibble was available to conduct the palatability test for this diet. The intake ratio of HS [HS intake/(HS + MS intake)x100] was 37%, with a 0.0346 standard error, T value of 3.82 and $P <.0001$. Thus, it was concluded that dogs preferred the MS diet.

Gut health study

Dogs maintained body weight (BW) throughout the study and there was no evidence for a difference in mean BW among treatments (% body change of 0.033, 0.191 and -0.791 when dogs were fed the HS, MS and LS foods at different periods; $P = 0.2297$). No major adverse effects of diets were observed, and no dog was dismissed from the study. It is noted, though, that two animals received anti-inflammatory and antibiotic treatment during the third period due to wounds unrelated to the study. Overall, blood chemistry evaluations and CBC did not suggest any problems with overall health of dogs (Tables C.3. and C.4.). As an exception, dogs fed the

LS dietary treatment showed increased blood urea nitrogen (BUN) and BUN:creatinine ($P < 0.05$) relative to the other treatments, yet were within normal range (Table C.3.).

Food intake and fecal parameters

Dogs in the LS dietary treatment had a lower caloric intake on a metabolic body weight ($BW^{0.75}$) basis in comparison to the other treatments *(*$P < 0.05$; Table 5.2.). Meanwhile, dogs fed MS and LS diets showed an increase in RS intake of approximately 45% and 60% RS ($P < 0.01$) relative to dogs fed HS, respectively (Table 5.2.).

Most fecal samples were firm and ideal (scores 4 and 5), and only a small proportion had a score 3 (softer) and no evidence for treatment differences was detected ($P = 0.29$; Figure 5.1.). Across periods, stool samples had an average moisture of 69.4% and average organic matter of 22.8% (Table 5.2.) with no evidence for treatment differences. Fecal pH ($P = 0.07$) had marginal evidence for a decrease in dogs fed LS dietary treatment; this may be indicative of more carbohydrate fermentation. There was no evidence for any treatment effect on fecal ammonium neither fecal ash excretion ($P = 0.099$).

Local inflammation and immunity

Total fecal SCFA, acetic, or propionic acid concentrations had no evidence for any effect of dietary treatment ($P > 0.10$; Table 5.3.). By contrast, dietary effects were apparent on on fecal butyric acid concentration ($P = 0.024$); whereby, dogs fed the LS diet had approximately 37% greater fecal butyric acid than those fed the HS diet. Meanwhile, fecal butyric acid concentrations under the MS dietary treatment were intermediate to the other two diets and not

significantly different from either. The approximate proportions of acetate:propionate:butyrate in the HS, MS and LS dietary treatments were 47:32:22, 44:29:28 and 43:29:28, respectively.

Branched-chain fatty acids may be attributed in part to protein putrefaction. In general, there was no evidence for any dietary effect on fecal production of BCFA, with the exception of hexanoic acid which showed increased concentration under the MS and LS dietary groups compared to HS ($P = 0.02$; Table 5.3.).

Gut wall integrity was assessed by fecal immunoglobulin A and serum concentrations of gram-positive and gram-negative bacteria cell wall components (LTA and LPS, respectively). There was no evidence of any dietary effect on fecal IgA or serum LTA (Table 5.3.), but LPS was marginally increased ($P = 0.08$) under the MS and LS dietary treatments compared to HS.

Satiety hormones

When satiety hormones were measured in dog plasma, there was one sample which had concentrations below the limit of detection for every hormone and was therefore excluded from analyses. So, the statistical analysis was performed on 23 plasma samples. For concentrations of ghrelin, leptin, GIP, glucagon, PP and PYY, there was no evidence for any dietary effect (Table 5.4.). There was a marginal significance for an effect on insulin ($P = 0.06$) concentration. Glucagon-like peptide 1 had 35 samples below the limit of detection, so this hormone was removed from the analysis.

Gut health study

Metabolomics

A total of 832 fecal and 858 serum metabolites were detected in the samples, and 80% fecal and 88% serum analytes passed the 80% filter described under Materials & Methods. The majority of differences were identified in feces rather than serum and were mostly driven by the MS treatment. For fecal glucose, maltose and maltotetraose, significant dietary effects were apparent ($P < 0.05$; $q < 0.10$; Figure 5.2.); wherein, dogs fed the LS and MS foods had more fecal maltose and maltotetraose than those fed the HS diet, and fecal glucose was greater in the LS than the HS group, with no evidence for a difference between MS and LS or HS. There was also more succinate in feces of dogs fed the LS relative to HS food ($P < 0.05$; $q < 0.10$; Figure 5.2.), and a marginal significance of more lactate in the LS vs HS group which may indicate greater saccharolytic activity.

Two fecal amino acids and two dipeptides tended to be greater in dogs fed the less processed foods (MS and LS) relative to HS ($P < 0.05$; $q > 0.10$; Tables C.6. and C.7.). Seven putrefactive fecal compounds were affected by dietary treatment ($P < 0.05$; $q < 0.10$). Specifically, 3-hydroxyindolin-2-one and indole decreased in the LS and MS treatments relative to HS; phenol sulfate and indolin-2-one decreased only in the MS treatment relative to HS; 3-indoleglyoxylic acid increased only in dogs fed the LS food; and finally, indolepropionate and 2-hydroxy-3-methylvalerate increased in dogs fed the MS relative to HS ($P < 0.05$; $q < 0.10$; S. Table C.8.). We also found evidence to support an increase in both fecal and serum pyrraline ($P < 0.05$; $q < 0.10$; Table C.9.) in dogs fed the MS and LS foods in comparison to HS. Pyrraline belongs to the group of advanced glycation end-products (AGEs). This finding was unexpected

as these foods were processed in a similar manner except for extruder shaft speed and in-barrel moisture. The development of AGEs are associated with over-processed foods(Li and Yu, 2018).

Evidence for dietary effects was particularly apparent in components of lipid metabolism. For instance, fecal long chain mono-unsaturated and poly-unsaturated fatty acids (LCMUFA and LCPUFA) were overall higher in dogs fed the HS food (Figure 5.3. A). Two LCPUFAs, stearidonate (18:4n3) and eicosapentaenoate (EPA; 20:5n3), were higher in the HS relative to MS group ($P < 0.05$; $q < 0.10$), and arachidonate (20:4n6) had a tendency ($P < 0.05$; $q > 0.10$) to also be higher in the HS relative to LS group (Figure 5.3. A). Conversely, molecules from the intermediate fatty acid metabolism were greater overall in the MS dietary treatment (Figure 5.3. B). More specifically, diacylglycerols palmitoyl-linoleoyl-glycerol (16:0/18:2) and linoleoyl-linoleoyl-glycerol (18:2/18:2) were increased under the MS dietary treatment relative to HS and LS, and isomer linoleoyl-linoleoyl-glycerol (18:2/18:2) and diacylglycerol (16:1/18:2, 16:0/18:3) were greater in the MS dogs fed dietary treatment in comparison to LS, with no evidence for any differences compared to HS ($P < 0.05$; $q < 0.10$). There was also a marginal significance ($P < 0.05$; $q > 0.10$) favoring oleoyl-linoleoyl-glycerol (18:1/18:2) and palmitoyl-linoleoyl-glycerol (16:0/18:2) two diacylglycerols in dogs fed the MS food.

The primary bile salt taurocholate measured in dog feces was more abundant ($P < 0.05$; $q < 0.10$) when dogs were fed the MS and LS foods than the HS (Figure 5.3. C). Meanwhile, three products of secondary bile salt metabolism dehydrolithocholate, lithocholate and taurodeoxycholate were greater in feces of dogs fed the HS food relative to MS and LS ($P < 0.05$; $q < 0.10$), and tended to also be more abundant in dogs fed the HS treatment ($P < 0.05$; $q < 0.10$). Taken together, these findings seem to suggest that dogs fed the more processed food (HS) had more bacterial metabolism of bile salts.

Serum saturated, mono- and poly-unsaturated long-chain fatty acids had a pattern of being lower in dogs fed the MS food and 11 of these were tendencies ($P < 0.05$; $q > 0.10$; Figure 5.4.) toward a dietary effect. This was expected, as dogs fed the MS food also had lower concentrations of these fatty acids in feces. Findings regarding lipid metabolomics indicate that dogs fed the HS food had a higher lipid metabolism than dogs fed the MS, and those fed the LS food met an intermediary point.

Serum energy metabolites, non-esterified fatty acids and bile salts were not signicantly affected by dietary treatment ($P > 0.05$; $q > 0.10$). For some serum amino acids, putrefactive compounds and pyrraline (an AGE), the evidence for dietary treatment effects was only marginally significant ($P < 0.05$; $q > 0.10$).

Microbiome

Alpha diversity

There was no evidence for any dietary effect on alpha diversity richness, inverse Simpson, exponent Shannon or and Pileou's J ($P > 0.74$ in all cases; Table 5.5.). This would indicate that the number of bacterial OTUs identified, their abundance, and most prevalent OTUs, as well as the spread of OTUs within the microbiome of dogs from each dietary group had no evidence for a treatment difference.

OTU centered log-ratio (CLR) transformed

Ninety OTUs passed the 80% filter before conducting statistical analysis of OTUs with a centered log-ratio (CLR) transformation technique. Only OTUs that presented a $P < 0.05$ were reported on Table 5.6. for purposes of discussion, sorted primarily by Phylum and secondarily by their decreasing order of abundance. Table 5.6. reports estimated OTU counts expressed in the

CLR scale at the family and genus levels. When genus was unclassified, it was inconclusive (ambiguous OTU assignment) and could be of any genus of that given family. Whereas, a missing genus indicated that there was no further annotation available.

Overall, OTU abundance in feces of dogs fed the MS food had greater separation from those fed the HS diet, and LS was intermediate. It would be expected that the LS food would lead to a higher separation of OTU abundance relative to HS, but this only happened in one significant OTU.

Some of the most abundant OTUs for which there was no evidence of any dietary effect included Prevotellaceae Prevotella, Clostridiaceae Clostridium, Lactobacillaceae Lactobacillus, Alcaligenaceae Sutterella, Lachnospiraceae Ruminococcus and Fusobacteriaceae Fusobacterium.

The most abundant significant OTUs in the dataset pertained to Phylum Firmicutes. Lachnospiraceae Blautia had a tendency ($P = 0.06$; $q > 0.10$) to be higher in the HS group than MS, and LS was intermediate and not statistically significant from MS or HS (Table 5.6.). The other Firmicutes tended to be affected by dietary treatment ($P < 0.05$; $q > 0.1$). Turicibacteraceae Turicibacter and Veillonellaceae (no further annotation for genera) were less abundant in the MS treatment than HS, and LS had no evidence for a statistical significance compared to MS and HS, while Lachnospiraceae Roseburia and Erysipelotrichaceae Catenibacterium were more abundant in the MS group than HS, and LS had again no evidence to be different to both (Table 5.6.).

Blautia and Turicibacter had negative correlations with butyric acid, total SCFA and fecal maltose (Table 5.7.). Veillonellaceae had a positive correlation with acetic acid, but correlated negatively with fecal maltotetraose and succinate. These OTU counts may indicate that there was no saccharolytic activity from these organisms. Conversely, Reseburia had a positive correlation with SCFA, influenced mostly by butyric and propionic acids (Table 5.7.). These results

combined with their negative correlation to fecal pH and positive correlation to fecal maltose would suggest saccharolytic activity. Catenibacterium had a positive correlation with fecal glucose but were not correlated with SCFAs (Table 5.7.) indicating limited fermentative capacity.

Coriobacteriaceae Slackia was the only representative of phylum Actinobacteria and was the third most abundant within marginally significant OTUs ($P < 0.05$) from the statistical analysis. Similar to Blautia and Turicibacter, Slackia counts were also greater in the HS group than MS, with LS not different from HS and MS ($P < 0.05$; $q > 0.10$) and negatively correlated with butyric acid, total SCFA and fecal maltose (Table 5.7.).

Bacteroidaceae Bacteroides abundance were above the average and were marginally significant ($P < 0.05$; $q > 0.1$). The MS treatment had a greater abundance of this OTU (number 102407) than HS, with LS in between and similar to both (Table 5.6.). Bacteroides had a strong and positive correlation to butyric acid which may have contributed to its positive correlation to total SCFA. The negative correlations to acetic and propionic acid suggest that this OTU had a high capacity to produce butyrate (Table 5.7.). It was also correlated negatively to glucose. Based on evidence from this study, the genus Bacteroides is considered saccharolytic. Porphyromonadaceae with unclassified genera (ambiguous classification) was the only OTU in the dataset with a dietary effect ($P < 0.05$; $q < 0.10$) and its abundance was above the average (0.0 on CLR scale). This OTU was more abundant in dogs fed the HS than those fed the MS and LS diets. Porphyromonadaceae unclassified was positively correlated with acetate and propionate and negatively correlated with butyrate and succinate (Table 5.7.). The low-abundance bacteria that belonged to phylum Bacteroidetes ($P < 0.05$; $q > 0.1$; Table 5.6.), Paraprevotellaceae (without genera annotation) and Prevotellaceae unclassified (with ambiguous

genera annotation) had lower concentration in the HS and LS treatments relative to MS, and both had a strong correlation with butyrate. This may have accounted for a large proportion of the positive correlation to total SCFA and the negative relationship with glucose (Table 5.7.). Paraprevotellaceae tended to have a negative correlation to fecal lactate and maltotetraose, whereas Prevotellaceae unclassified was positively correlated with succinate.

Discussion

The term resistant starch (RS) refers to the starch fraction that escapes digestion by mammalian enzymes in the small intestine and reaches the large intestine where it is fermented by commensal bacteria into beneficial energy substrates (Flint et al., 2012). These starches can be protected by cell walls, can be in a granular crystalline format, retrograde, or chemically cross-linked. This study proposed to extrude kibbles with low thermomechanical energy and more process moisture to retain some resistant and slowly digestible starches (SDS). The goal was to utilize less torque in the extrusion process to decrease starch cook and thereby retain more RS types II and III. This could potentially lower production costs by reducing processing energy expenditures, as well as improve animal health by means of modulating the gut microbiota towards saccharolytic bacteria.

Corn was chosen as the starch ingredient because it is an important cereal for the economy, with high production and availability to pet food companies. It has also lost market share over the years to non-cereal (grain-free) products due to perceptions that it has inferior nutritional value. Although cereals do not possess as much resistant starch type II relative to pulses or tubers (Bhattarai et al., 2017, 2018; Fuentes-Zaragoza et al., 2010) it can be a source of RS for animals (Jackson, Waldy, Cochrane, et al., 2020; Ribeiro et al., 2019; Peixoto et al.,

2018). The composition of monosaccharides that make up corn starch are mainly glucose, with a small proportion of fructose, little mannose (in some varieties), and trace to no arabinose (Knapp et al., 2010).

The amount of starch that escapes small intestinal (SI) digestion and reaches the colon can vary according to effects such as food intake, starch processing and cooking level, species mastication habits, food matrix composition, and transit time among others (Flint et al., 2012). Resistant starches that are not chemically modified can be digested given the right amount of enzymes relative to substrate and adequate environmental conditions (Dhital et al., 2017). To account for these individual variation among animals we selected a strong study design in which every dog was fed each diet once. Additionally, study noise or sources of variation were reduced by adding more fixed effects into the model that reach beyond a typical Latin square design.

Processing inputs to produce dietary treatments in this experiment were intentionally modified in order to produce increasing levels of SDS and RS as determined by an *in vitro* enzymatic procedure (Corsato Alvarenga et al., 2020; Chapter 4). While the intent was to alter RS, the more appropriate description of dietary treatments was high, medium and low shear. The high shear food was produced similar to most pet foods available in the market with the highest level of starch gelatinization and lowest amount of RS or SDS (Corsato Alvarenga & Aldrich, 2020); while, the low shear food was produced with less mechanical energy and retained more RS and SDS, and lower starch cook (Corsato Alvarenga et al., 2020; Chapter 4). The MS diet was intended to be an intermediate between both high and low, but it behaved more like the LS food. The net result of increasing RS is a bypassing of starch from the SI into the large intestine/colon for fermentation. Thus, this RS could be considered a prebiotic which is defined as “selectively fermented ingredients that allow specific changes, both in the composition and/or

activity in the GI microflora that confer benefits upon host wellbeing and health" (Gibson et al., 2010).

There was an indication that the two diets produced with less thermomechanical energy, MS and LS, resulted in many similar physiological and biochemical aspects when fed to the dogs. They had more fecal glucose and oligosaccharides which was consistent with the work of Jackson *et al.* (2020) for dogs fed a low shear food based on corn and rice. This confirmed that the treatments produced with lower thermomechanical energy were effective in retaining RS since glucose and glucose-based oligosaccharides are starch derivatives. The small intestine (SI) is sensitive to dietary changes and has a higher abundance of carbohydrate fermenting bacteria (Wernimont et al., 2020; Aidy et al., 2015) so, that feces of dogs fed the LS or MS foods had more oligosaccharides would indicate the presence of higher concentrations of these sugars in both the SI and proximal regions of the large intestine.

The gastro-intestinal tract (GI) harbors trillions of metabolically active bacterial species that compose the microbiome, as well as a small percentage of fungi, archaea, protozoa and virus (Gibson and Roberfroid, 1995; Wernimont *et al.*, 2020). The gut microbiota in mammals are known to be commensal, which in Latin means "sharing a dining table" (W. J. Lee & Hase, 2014). This definition was attributed to these bacteria because they live in symbiosis with their host. Gut commensal bacteria can utilize host endogenous molecules or dietary products that bypass upper digestive tract digestion and generate fermentation by-products or post-biotics that benefit host health. In the event that there is a stressor and the luminal environment is perturbed with overgrowth of pathological microbes, there may be dysbiosis that can negatively affect the body system (Staley et al., 2018). There was no case of dysbiosis in the present study based on the assessment of stool quality, blood testing, and alpha diversity. Rather, the increase in starch

fermentation byproducts indicated that both the LS and MS foods improved markers of gut health.

There are two main techniques for bacterial gene sequencing, shotgun and amplicon sequencing. Shotgun randomly sequences for the totality of the genetic material, while amplicon sequences a targeted gene, most commonly 16S rRNA (Odintsova et al., 2017). The 16S rRNA gene is a bacterial ribosomal RNA that is highly preserved in all bacteria. It also contains some hyper-variable regions such as V3-V4 that can be used to differentiate each bacterial group. The resulting "reads" are subjected to quality filtering and the taxonomical classification is performed according to a database. In the case of 16S RNA sequencing, the common type of feature is operational taxonomic unit (OTU) (Odintsova et al., 2017). Once the OTU counts of each sample are obtained and properly identified, they need to be analyzed as compositional data because they are restricted to a constant sum determined by the Ilumina MiSeq instrument (Fernandes et al., 2014). The method chosen for this study was to convert OTU counts to centered-log ratios (CLR). This transforms each OTU count into a ratio relative to the geometric mean of all OTUs within each fecal sample (Gloor et al., 2016). Some properties of CLR are that the sum of all OTUs within a sample converge to zero (the mean), the reads are normalized to a common sequencing depth, and the CLR transformed values are scale invariant. This means that the same ratio is expected to be obtained in a sample with few read counts as a cohort sample with many read counts (Gloor et al., 2016). Before CLR transformation, OTU zero counts need to be inflated so that geometric means can be calculated. The Bayesian multiplicative approach prevents the most rare OTUs from being the most significant (Gloor et al., 2016).

The most predominant phylum in the gut of healthy dogs are Bacteroidetes, Firmicutes, Proteobacteria, Fusobacteria, and Actinobacteria (Wernimont et al., 2020; Deng & Swanson,

2015; Swanson et al., 2011) and this was true in our study. Firmicutes were the most abundant phylum, with some bacteria exhibiting saccharolytic activity and others not. The growth of species belonging to the phylum Firmicutes were favored by a mildly acidic pH in the colon, and dogs fed the more RS (LS and MS) tended to have lower fecal pH (due to fermentation). Leitch et al. (2007) reported that there was a high proportion of Firmicutes (51%) attached to resistant starch in the human colonic microbiota. Within the phylum Firmicutes, anaerobic Gram-positive bacteria Roseburia was the genera which had the most saccharolytic activity. Roseburia has the ability to utilize starch and produce butyrate (Flint et al., 2012; Aminov et al., 2006). Although its correlation with butyrate was not significant, this fatty acid was the main influencer of the positive correlation to total SCFA. Genus Blautia was the most prevalent OTU in our dataset and correlated negatively to both total SCFA and butyrate. Studies in other species have reported Blautia to have a negative correlation to carbohydrates (Kashtanova et al., 2018; Hooda et al., 2013); whereas, Sun, Su and Zhu, (2016) observed an increase in Blautia in the cecum and colon of pigs fed a retrograded potato starch (RS source) relative to corn starch.

Bifidobacterium from phylum Actinobacteria has been the focus on human studies due to their effectiveness in utilizing starches as energy substrate (Macfarlane & Englyst, 1986). However, the only marginally significant representative of phylum Actinobacteria in our dataset was Slackia, and it had a negative correlation to both SCFA and fecal maltose. Although our data did not indicate saccharolytic activity, Slackia has been previously reported to be saccharolytic and to increase with fermentable fiber addition to the diet (Jackson & Jewell, 2019). The Bacteroides genus was found to have broad saccharolytic potential which agrees with the other research reports in the literature (Jackson & Jewell, 2019; Flint et al., 2012; Salyers et al., 1977) The present study confirmed that Bacteroides increased in the MS and LS foods (with more RS).

Most members of phylum Bacteroidetes had a high saccharolytic activity with butyrate producing capacity. Bartonella could possibly be pathogenic and had a tendency to decrease in the LS and MS foods. Jackson and Jewell (2019) reported that a high meat food supplemented with a fiber blend led to a decline in Bartonella. A second study from the same group reported an increase in Bartonella in dogs fed a low shear diet (elevated RS) (Jackson, Waldy, Cochrane, et al., 2020). The latter study also reported an increase in Yersinia in dogs fed the low shear food (Jackson, Waldy, Cochrane, et al., 2020) which agrees with our study.

One important tool to analyze microbial compositional changes is to calculate alpha diversity indices, which are mathematical measures of species diversity in a community that provide information about rarity or commonness of different species. Alpha diversity is characterized as the variation in the microbiota within each dog. There are no clear definitions between an ecosystem diversity and its health as their validity are limited within each system (Kimmins, 1997). For example, when studying forests one can find environments with low diversity measures, but that are productive, healthy and have integrity; whereas, others can be highly diverse but with low stability and productivity (Kimmins, 1997). This analogy can be translated to the dog microbiome. It is difficult to characterize a specific microbiota profile for health or disease, because there is large individual variation (Wernimont et al., 2020; Schloss, 2018; Handl et al., 2011). The functional profile of the microbiome is usually a better assessment of health as compared to the microbiome composition (Wernimont et al., 2020). One can determine the healthfulness of a microbiota by measuring biomarkers known to positively or negatively affect the animal. In the present study, there were no differences in alpha diversity, there were changes in a few individual OTUs, and the most relevant effect of low and medium shear foods was linked to the increase in fecal butyrate. Similar to our work, De Souza Nogueira

et al. (2019) also observed that the addition of prebiotics to a dog food did not cause shifts in alpha diversity in the gut (assessed in fecal samples), but some fermentation products including SCFAs increased and proved the prebiotics to be beneficial. In our study, in order to detect differences in alpha diversity it might have been necessary to extend the adaptation time (over 4 weeks), or to produce foods with lower mechanical energy during the extrusion process. There was an increase in species richness when dogs were fed a low shear food after 6 weeks of feeding compared to dogs fed a high shear food (Jackson *et al.*, 2020). In their work, the difference in mechanical energy between the low and high shear foods was 4-fold, while in our study this difference was only 1.7-fold.

Large polysaccharides that reach the colon are first hydrolyzed by primary polysaccharidases which release smaller oligosaccharides that serve as substrate for fermentation and ultimately production of SCFAs (Puertollano et al., 2014). A small proportion of SCFAs are also derived from protein fermentation (MacFarlane & MacFarlane, 2012). Bacterial enzymes that degrade starch comprise glycoside hydrolases (Cantarel et al., 2009). Starch α-1,4 linkages can be catalyzed by bacterial α-amylase or α-glucosidases, while branching-point α-1,6 linkages are hydrolyzed by a pullulanase (Flint et al., 2012). Bacteria also contain binding domains from different families that are responsible for their adhesion to starch molecules as the first step to the degradation process (Anderson & Salyers, 1989).

Once the microbiota hydrolyzes fermentable fiber or resistant starches into monosaccharides, these are fermented by bacterial enzymes in the anaerobic environment of the colon. The major bacterial metabolic route for six-carbon sugars like glucose is the Embden-Meyerhof-Parnas pathway (Miller & Wolin, 1996). Den Besten *et al.*, (2013) provided a good review on pathways to produce SCFA to further understand the metabolism involved in their

synthesis. In short, glucose is first fermented to pyruvate, which is reduced to lactate and ethanol. The production of SCFA is linked to the attempt of bacteria to decrease reducing agents in their environment. A major part of pyruvate can be converted to acetyl-CoA with the formation of H_2 and CO_2. Acetate can be formed from hydrolysis of acetyl CoA or from CO_2, while propionate can be produced from the reduction of lactate. There is another metabolic pathway to produce propionate from the pentose-phosphate pathway, which is most relevant in fermentation of fibers rather than RS. Butyrate synthesis starts by linking two acetyl-CoA molecules, which can derive from pyruvate or acetate (Den Besten et al., 2013). Butyrate can also be produced by lactate-utilizing bacteria (Duncan et al., 2004). The LS and MS foods in the present study favored higher production of fecal butyrate. Butyrate is the preferred energy source of colonocytes (Leonel & Alvarez-Leite, 2012) and has been the target SCFA of studies with prebiotics.

Short chain fatty acids are mainly used as colonocyte energetic substrate, but specific SCFA receptors in other tissues suggest their systemic anti-inflammatory effects (Lee & Hase, 2014) to ameliorate metabolic conditions like diabetes, obesity, and cardiovascular disease. Short-chain fatty acids play a role in colonic homeostasis by decreasing inflammation and improving immunity, gut motility, gene expression and hormonal regulation (Flint et al., 2012). Moreover, SCFA can regulate satiety in the long-term via hormonal regulation, and propionate has been reported to regulate appetite through its high affinity for the fatty acid receptors 2 and 3 (FFA2 and 3)(Sleeth et al., 2010). Gut hormones PYY and GLP-1 are also involved in appetite regulation through this pathway (Matey-Hernandez et al., 2018). In the present study, propionate levels were similar across treatments and there were no dietary effects on satiety long term. It is possible that there wasn't enough RS to promote satiety through SCFA production and FFA2 activation, and(or) that dogs were fasting for a longer time (> 20 h) which overrode changes in

satiety hormones. The MS and LS foods in the present study also did not improve markers of colonic immunity in fasted dogs relative to the HS treatment. Likewise, Peixoto *et al.* (2018) did not find increases in fecal IgA when dogs were fed a high RS diet from corn. However, Jackson *et al.* (2020) reported greater amounts of epithelial sugar and fecal IgA after feeding a low shear food to dogs which indicated a faster epithelial cell turn over and improved local immunity. These findings were again likely related to a longer feeding time and a lower mechanical energy to produce the food than in the present study (specific mechanical energy 9 vs 23.6 Wh/kg, respectively; Corsato Alvarenga et al., 2020, Chapter 4).

The main reduced fermentation byproducts include lactate, succinate, H_2, and butyrate (Macfarlane & Englyst, 1986). Dogs fed the LS or MS foods tended to increase lactate, succinate and butyrate in their feces, and decrease pH which were directly linked to starch fermentation. Peixoto *et al.* (2018) also reported an increase in fecal lactate, butyrate, and a decrease in pH associated with low shear (high RS) food consumption. Luminal pH declines from the ileum to the colon due to higher SCFA production (Den Besten et al., 2013). The drop in pH in the large intestine is important for shifting the microbiota to prevent overgrowth of pathogenic strains (Den Besten et al., 2013; Cherrington et al., 1991). The SCFAs are absorbed by colonocytes in exchange for bicarbonate (HCO_3-), which acts as a buffer and increases the pH again as digesta passes to the rectum (Den Besten et al., 2013). So, we would expect dog colonic pH to be lower than what was measured in the feces. A similar phenomenon occurs with SCFAs. The concentration of SCFAs was much higher in the proximal regions of the swine colon compared to distal portions (Haenen, Zhang, da Silva, et al., 2013), which would be closer in composition to the feces excreted. Only ~5% of total SCFA produced are present in the feces with most butyrate absorbed by the epithelial cells (MacFarlane & MacFarlane, 2012; Topping & Clifton,

2001). Although the butyrate difference in the present study seemed small, the actual production could be much greater. Moreover, when carbohydrate substrates become less available at the distant colon, there is a downward shift in butyrate producing bacteria and propionate and acetate producing strains become more prevalent (Walker et al., 2005). Other studies have also reported that dogs and cats fed an extruded food with corn produced with less mechanical shear had increased fecal butyrate (Jackson, Waldy, & Jewell, 2020; Jackson, Waldy, Cochrane, et al., 2020; Ribeiro et al., 2019; M. C. Peixoto et al., 2018).

Butyrate has an important role in suppressing colonic carcinogenesis and regulating gut immunological homeostasis (Lee & Hase, 2014). Haenen *et al.*, 2013 found that RS was completely degraded in the swine cecum and that butyrate was increased in the proximal portion of their colon. The digestive system of pigs is larger than canines and possesses a greater fermentation capacity. Butyrate administered orally also prevented mice fed a high fat diets to develop insulin resistance and obesity (Gao et al., 2009).

While there were significant changes in carbohydrate metabolism by the gut microbiota of dogs fed the LS and MS foods, similar outcomes were not observed in serum. Metabolomics detection of analytes are directly tied to the time of blood collection. Since dogs were fasting and blood glucose levels are constantly being controlled by insulin, glucagon and other hormones, we did not expect changes in either carbohydrate or Krebs cycle metabolites in serum.

A possible route for energetic metabolism that sustains long term energy supply is through fatty acid oxidation which is activated by SCFAs concurrent with inhibition of lipid synthesis from glucose (Matey-Hernandez et al., 2018). In the present study there were some significant changes in metabolomics of lipids. The increase in diacylglycerols in feces of dogs fed the MS food could be explained by the higher bypass of these lipids to the colon.

Interestingly, serum of these dogs had lower levels of some fatty acids than the other treatments. This suggests that bacterial breakdown of diacylglycerols into fatty acids and consequent absorption into the blood stream were less pronounced in the MS treatment. Lee, Yoo and Lee (2012) reported that mice fed corn diets supplemented with chemically modified RS had lower body weight gain, as well as lower levels of serum total lipids, triglycerides and cholesterol. Some studies have reported reduced serum triglyceride associated with RS type II consumption by healthy humans (Snelson et al., 2019). In the present study, dogs fed the MS food had lower levels of serum fatty acids, but there were no differences in serum triglycerides or cholesterol among treatments. Conversely, dogs fed the high shear food had more fecal long-chain polyunsaturated fatty acids (LCPUFA) than the other treatments, and LCPUFA serum concentrations were comparable to the LS treatment. Saccharolytic bacteria like Bifidobacteria, Roseburia and Lactobacillus are responsible for the breakdown of LCPUFAs into conjugated linoleic acid (CLA)(Matey-Hernandez et al., 2018) which would translate into the decreased fecal LCPUFAs observed in our study.

The high shear food favored the increase of both primary and secondary bile salts in dog feces. Bile salts need to be deconjugated by microbial bile salt hydrolase (BSH) in order to not be reabsorbed and bypass to the colon (Enright et al., 2018) The activity of this enzyme is greater in gut microbes residing in the terminal ileal and colon. There is large variation of BSH between bacterial species and these can be transmitted between microbes through plasmids (Enright et al., 2018). The increase in bile salt excretion contributes to a decrease in serum cholesterol as it needs to be used to replenish the lost liver bile salts. In parallel, the fraction of secondary bile salts that are reabsorbed by the host modulates the systemic lipid and glucose metabolism, contributing to an improved liver and pancreatic functions, as well as improved glucose tolerance

(Matey-Hernandez et al., 2018). Taurocholate was greater in feces of dogs fed the high shear (less RS) food of the present study. This agrees with observations in dogs reported by Jackson *et al.* (2020) . Some secondary bile salts also increased in feces of dogs fed the HS food, which may suggest a higher microbial metabolism of bile salts in this treatment. Conversely, Jackson *et al.* (2020) reported that four secondary bile salts increased in dogs that consumed the low shear foods, and six that increased for dogs fed a high shear treatment. The mechanism that led consumption of the high shear food to increase bile salt excretion is not easily explained.

Conclusion

This was the first work to our knowledge that explored in depth the differences in starch transformation during extrusion process at different levels of thermomechanical energy, as well as the systemic effects of these foods in the dog down to the level of the microbiome. Most positive changes in the metabolomics and microbiome resulted from dogs fed the LS and MS treatments relative to the HS food. Although there were no differences in bacterial alpha diversity in fecal microbial populations of dogs fed these treatments, there was evidence to support growth of some saccharolytic bacteria favored by consumption of the LS and MS foods. This also resulted in more fecal glucose and oligosaccharides than the HS treatment. By-products of carbohydrate fermentation succinate, butyrate, lactate and H_2 (measured as fecal pH) confirmed that carbohydrate metabolism was more extensive in dogs fed diets produced at lower levels of thermomechanical energy. The most relevant health biomarker that increased with the consumption of the LS and MS foods was fecal butyrate. It has been identified in previous research to be the major player in providing energy substrate for colonocytes, preventing colonic cancer and decreasing local and systemic inflammation in monogastric animals. Only the MS

food led to a trend of serum fatty acid decline in dogs, and it was also more palatable than the HS food. Unfortunately, palatability information for the LS diet was missing. Based on evidence from this work, we conclude that a low to medium shear food (elevated RS) might benefit gastrointestinal and systemic health of dogs.

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Table 5.1.

Means of Total Tract Digestibility, Food Intakes and Fecal Output of Dogs (N=6) fed a High, Medium and Low Shear Food (HS, MS and LS, respectively).

Item	**HS**	**MS**	**LS**	**HS vs LS**		**HS vs MS**		**MS vs LS**	
				SE	***P*(t)**	**SE**	***P*(t)**	**SE**	***P*(t)**
ATTD, %									
Dry Matter	84.6	85.5	85.9	0.81	0.186	0.79	0.279	0.61	0.596
Organic Matter	88.4	89.0	88.7	0.57	0.682	0.52	0.268	0.45	0.457
Gross Energy	88.3	89.3	88.9	0.57	0.328	0.496	0.101	0.3	0.344
Crude Protein	84.1	84.6	86.7	0.739	0.016	0.84	0.502	0.66	0.013
True Protein	91.4	91.6	92.9	1.05	0.199	1.23	0.844	0.74	0.110
Crude Fat	93.9	93.5	94.3	0.57	0.582	0.44	0.351	0.58	0.228
Ash	25.0	27.0	43.9	6.104	0.006	5.99	0.623	4.09	0.002
Parameters									
Daily Intake, g*day^{-1} (as-is)	149	169	165	18.0	0.416	22.3	0.403	22.46	0.878
Daily Intake, g*day^{-1} (DM)	136	152	148	16.3	0.509	20.2	0.452	20.2	0.836
Daily Intake, Kcal*day^{-1}	556	628	615	66.9	0.416	83.0	0.403	83.7	0.878
Fecal Output, g*day^{-1} (as-is)	64.3	70.9	66.0	7.53	0.827	10.0	0.522	9.63	0.622
Fecal Output, g*day^{-1} (DM)	21.1	21.9	20.7	2.83	0.880	3.33	0.819	2.49	0.631

Table 5.2.

Feeding Study Food Intake and Fecal Parameters (Least Squared Means; 95% Confidence Interval) of Dogs (N=24) fed Diets produced at High, Medium and Low Shear (HS, MS and LS, respectively).

Item	HS	MS	LS	*P*(F)
Intake				
Food, g/BW$^{0.75}$/day	33.2 [30.0, 36.7]	33.1 [30.0, 36.6]	31.8 [28.8, 35.1]	0.0317
Food, Kcal/BW$^{0.75}$/day	154.5^{a} [139.7, 170.7]	153.5^{a} [138.8, 169.7]	144.4^{b} [130.6, 159.7]	0.0007
Resistant Starch, g/BW$^{0.75}$/day	0.206^{c} [0.189, 0.224]	0.295^{b} [0.273, 0.318]	0.319^{a} [0.297, 0.342]	<.0001
Fecal Responses				
Moisture, %	69.7 [68.5, 70.9]	69.8 [68.6, 71.0]	68.7 [67.5, 69.9]	0.2200
Organic Matter, %	22.4 [21.8, 23.0]	22.7 [22.1, 23.3]	23.1 [22.4, 23.7]	0.2522
pH	5.82 [5.70, 5.93]	5.72 [5.61, 5.84]	5.67 [5.56, 5.78]	0.0719
Ammonium, mmol/g	0.034 [0.032, 0.037]	0.032 [0.028, 0.035]	0.031 [0.026, 0.035]	0.1987
Ash, %	7.66 [6.85, 8.56]	7.09 [6.34, 7.93]	8.15 [7.29, 9.11]	0.0986

^{abc}Letters indicate significant pairwise differences (P < 0.05).

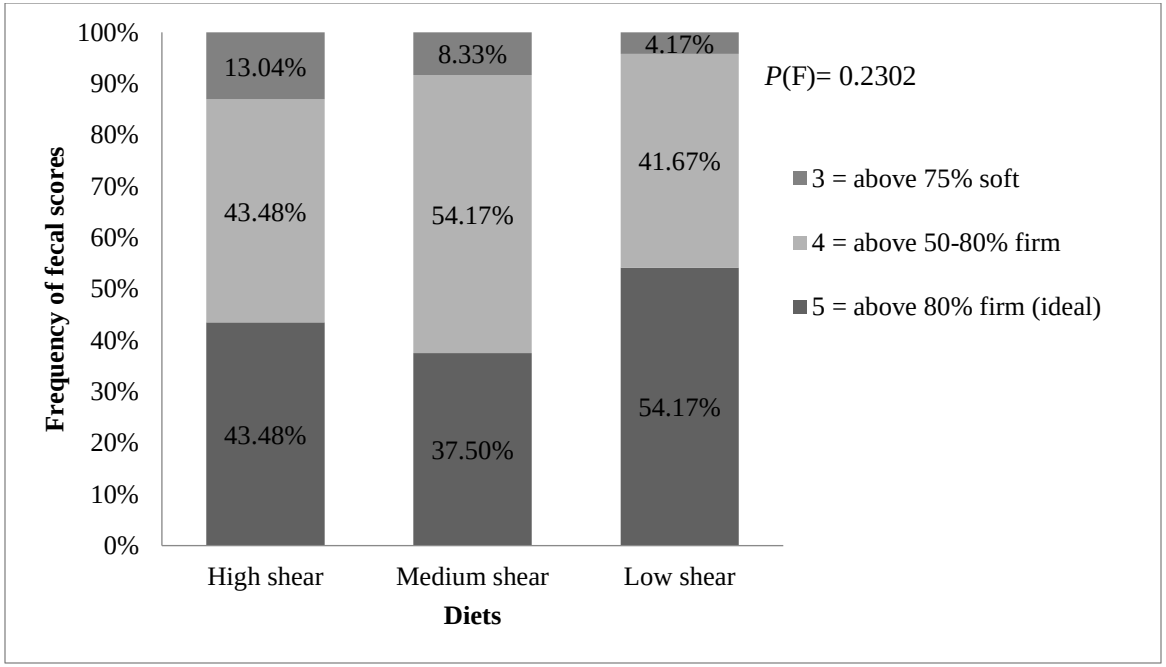

Figure 5.1.
Stool Score Frequency of Dogs fed a High, Medium and Low Shear Food (N= 23, 24 and 24 for High, Medium and Low Shear Treatments, respectively).

Table 5.3.

Fecal Short-Chain Fatty Acids (SCFA), Branched-Chain Fatty Acids (BCFA) and Immunoglobulin A, and Serum Gram-Positive and Gram-Negative Bacterial Cell Wall Constituents Lipoteichoic Acid (LTA) and Lipopolysaccharide (LPS), respectively, of Fasting Dogs (N=24) fed Diets produced at High, Medium and Low Shear (HS, MS and LS, respectively). Expressed as LS Means [95% Confidence Intervals].

Item	HS	MS	LS	*P*
Feces				
SCFA				
Total, mg/g	10.41 [9.26, 11.56]	10.20 [9.06, 11.35]	11.01 [9.86, 12.16]	0.1700
Acetic Acid, mg/g	4.73 [4.31, 5.14]	4.26 [3.87, 4.66]	4.59 [4.03, 5.15]	0.1243
Propionic Acid, mg/g	3.23 [2.80, 3.66]	2.77 [2.35, 3.20]	3.10 [2.67, 3.52]	0.1572
Butyric Acid, mg/g	2.19[b] [1.77, 2.72]	2.68[ab] [2.16, 3.32]	3.01[a] [2.43, 3.73]	0.0236
BCFA				
Total, mg/g	0.582 [0.386, 0.878]	0.667 [0.437, 1.019]	0.654 [0.406, 1.055]	0.5992
2-methylbutyric Acid, mg/g	0.108 [0.081, 0.134]	0.098 [0.071, 0.125]	0.103 [0.074, 0.133]	0.4744
Isobutyric Acid, mg/g	0.159 [0.130, 0.188]	0.144 [0.115, 0.172]	0.145 [0.116, 0.173]	0.5150
Isovaleric Acid, mg/g	0.200 [0.163, 0.238]	0.186 [0.148, 0.224]	0.190 [0.140, 0.240]	0.6484
Valeric Acid, mg/g	0.109 [0.052, 0.226]	0.171 [0.082, 0.354]	0.186 [0.089, 0.386]	0.3500
Hexanoic Acid, mg/g	3.69 [1.80, 7.55]	8.51 [3.88, 18.65]	8.19 [3.15, 21.24]	0.0214
IgA, mg/g	19.2 [11.0, 33.5]	23.7 [13.6, 41.3]	20.0 [11.5, 34.9]	0.6174
Serum				
LTA, ng/mL	4.96 [3.75, 6.17]	5.05 [3.84, 6.26]	5.27 [4.06, 6.48]	0.8384
LPS, Eu/mL	1.18 [0.95, 1.40]	1.35 [1.12, 1.57]	1.38 [1.15, 1.60]	0.0763

[ab]Letters indicate significant pairwise differences ($P < 0.05$).

Table 5.4.

Satiety Hormones (N=23; LS Means [95% Confidence Interval]) in Plasma of Fasting Dogs fed Diets produced at High, Medium and Low Shear (HS, MS and LS, respectively).

Satiety Hormone	**HS**	**MS**	**LS**	***P***
Ghrelin, pg/mL	105.3 [79.6, 139.4]	118.8 [89.9, 157.0]	112.7 [85.2, 149.1]	0.3853
Leptin, pg/mL	252.3 [159.2, 366.8]	312.1 [207.8, 437.6]	277.3 [179.5, 396.3]	0.1379
GIP, pg/mL	3.05 [2.30, 4.05]	2.49 [1.88, 3.29]	2.81 [2.12, 3.72]	0.1968
Glucagon, pg/mL	41.8 [29.0, 60.1]	42.5 [29.0, 62.3]	41.2 [28.2, 60.3]	0.7462
Insulin, pg/mL	41.6 [26.4, 65.7]	51.4 [32.7, 81.0]	61.1 [38.7, 96.3]	0.0648
PP, pg/mL	53.2 [41.2, 68.8]	53.1 [41.3, 68.3]	57.2 [44.4, 73.7]	0.8401
PYY, pg/mL	101.5 [89.8, 114.7]	106.9 [94.7, 120.7]	106.4 [94.2, 120.1]	0.4533

Origin	Starch derivative			Other sugar												Epithelial sugar			Energetic metabolism							
	1	2	3																					24		26
Metabolite	**	**	**	4	5	6	7	8	9	10	11	12*	13	14	15	16	17	18	19	20	21	22	23	**	25	*
HS																										
MS																										
LS																										

Figure 5.2.

Fecal Saccharides Metabolomics Heatmap of Dogs (N=24) fed Diets produced at High, Medium and Low Shear (HS, MS and LS, respectively), with each Individual Group of Analytes (Starch Derivatives, Other Sugars, Epithelial Sugars and Energetic Compounds) Conditionally Formatted on a Blue to Red Scale from Low to High Concentrations, respectively.

1, glucose; 2, maltose; 3, maltotetraose; 4, arabinose; 5, erythrose; 6, fructose; 7, fucose; 8, glucuronate; 9, glycerate; 10, maltol; 11, mannitol/sorbitol; 12, ribose; 13, ribulose/xylulose; 14, sedoheptulose; 15, xylose; 16, mannose; 17, N-acetylglucosamine/N-acetylgalactosamine; 18, N-acetylglucosaminylasparagine; 19, α-ketoglutarate; 20, citraconate/glutaconate; 21, fumarate; 22, malate; 23, phosphate; 24, succinate; 25, succinylcarnitine (C4-DC); 26, lactate.
***P* < 0.05; q < 0.10
* *P* < 0.05; q > 0.10

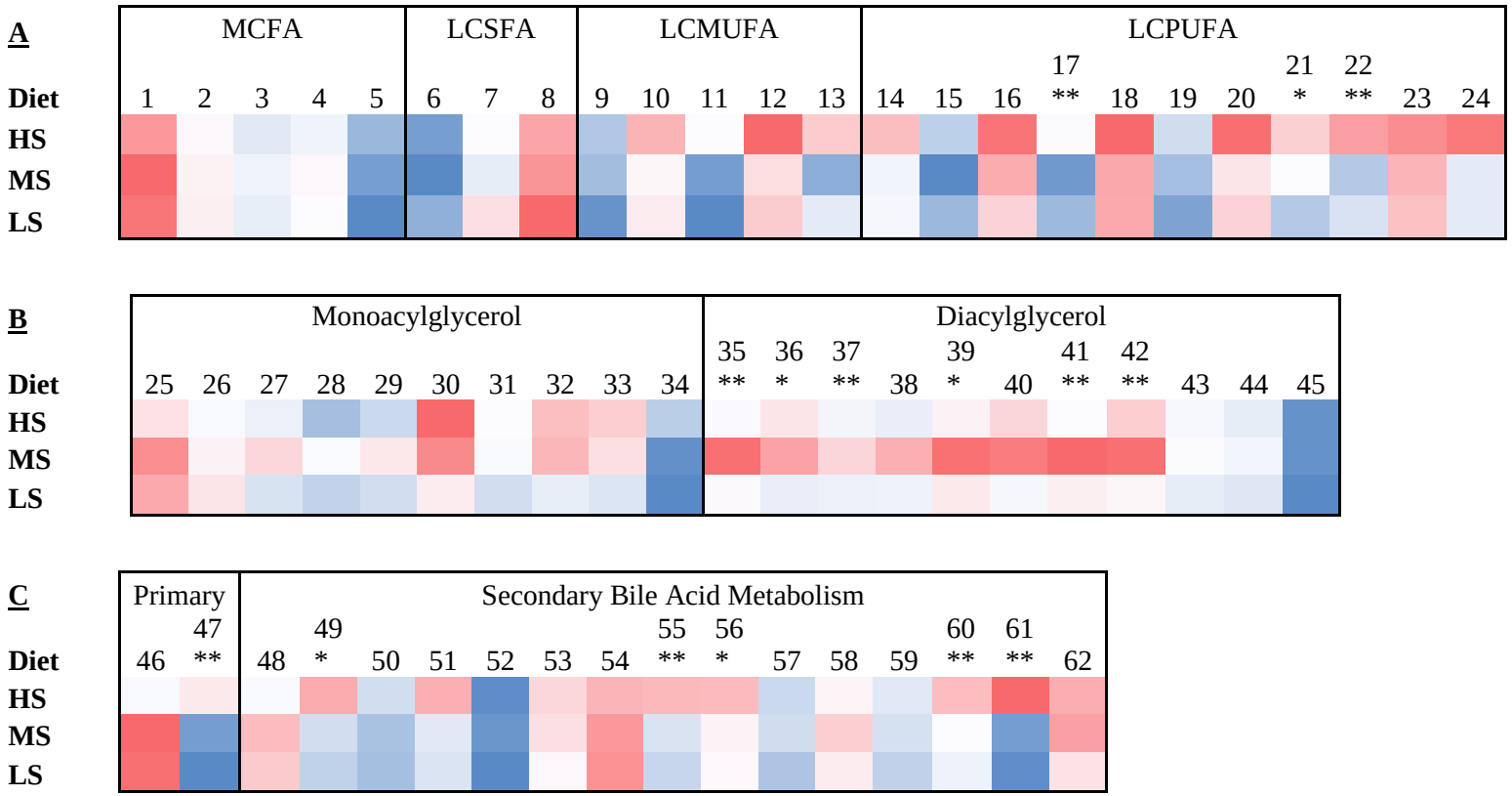

Figure 5.3.

Fecal Lipid Metabolism Analytes Heatmap of Dogs (N=24) fed Diets produced at High, Medium and Low Shear (HS, MS and LS, respectively), with each Individual Group of Analytes (A: MCFA, Medium-Chain Fatty Acids; LCSFA, Long-Chain Saturated Fatty Acids; LCMUFA, Long-Chain Mono-Unsaturated Fatty Acids; LCPUFA, Long-Chain Poly-Unsaturated Fatty Acids. B: Monoacylglycerols and Diacylglycerols. C: Primary and Secondary Components of Bile Salt Metabolism) Conditionally Formatted on a Blue to Red Scale from Low to High Concentrations, respectively.

A: 1, caproate (6:0); 2, heptanoate (7:0); 3, caprylate (8:0); 4, caprate (10:0); 5, 5-dodecenoate (12:1n7); 6, palmitate (16:0); 7, stearate (18:0); 8, arachidate (20:0); 9, palmitoleate (16:1n7); 10, 10-heptadecenoate (17:1n7); 11, oleate/vaccenate (18:1); 12, 10-nonadecenoate (19:1n9); 13,

erucate (22:1n9); 14, hexadecadienoate (16:2n6); 15, hexadecatrienoate (16:3n3); 16, linoleate (18:2n6); 17, stearidonate (18:4n3); 18, docosadienoate (22:2n6); 19, mead acid (20:3n9); 20, adrenate (22:4n6); 21, arachidonate (20:4n6); 22, eicosapentaenoate (EPA; 20:5n3); 23, docosapentaenoate (n6 DPA; 22:5n6); 24, docosahexaenoate (DHA; 22:6n3). **B**: 25, 1-palmitoylglycerol (16:0); 26, 2-palmitoylglycerol (16:0); 27, 1-palmitoleoylglycerol (16:1); 28, 2-palmitoleoylglycerol (16:1)*; 29, 1-heptadecenoylglycerol (17:1); 30, 1-oleoylglycerol (18:1); 31, 2-oleoylglycerol (18:1); 32, 1-linoleoylglycerol (18:2); 33, 2-linoleoylglycerol (18:2); 34, 1-linolenoylglycerol (18:3); 35, palmitoyl-linoleoyl-glycerol (16:0/18:2); 36, palmitoyl-linoleoyl-glycerol (16:0/18:2); 37, diacylglycerol (16:1/18:2, 16:0/18:3); 38, oleoyl-oleoyl-glycerol (18:1/18:1); 39, oleoyl-linoleoyl-glycerol (18:1/18:2); 40, oleoyl-linoleoyl-glycerol (18:1/18:2); 41, linoleoyl-linoleoyl-glycerol (18:2/18:2); 42, linoleoyl-linoleoyl-glycerol (18:2/18:2); 43, linoleoyl-linolenoyl-glycerol (18:2/18:3); 44, linoleoyl-linolenoyl-glycerol (18:2/18:3); 45, linolenoyl-linolenoyl-glycerol (18:3/18:3). **C**: 46, cholate; 47, taurocholate; 48, 12-dehydrocholate; 49, 12-ketolithocholate; 50, 3b-hydroxy-5-cholenoic acid; 51, 3-dehydrodeoxycholate; 52, 6-oxolithocholate; 53, 7alpha-hydroxycholestenone; 54, 7-ketodeoxycholate; 55, dehydrolithocholate; 56, deoxycholate; 57, deoxycholic acid 12-sulfate; 58, hyocholate; 59, isohyodeoxycholate; 60, lithocholate; 61, taurodeoxycholate; 62, ursocholate.

**$P < 0.05$; $q < 0.10$

* $P < 0.05$; $q > 0.10$

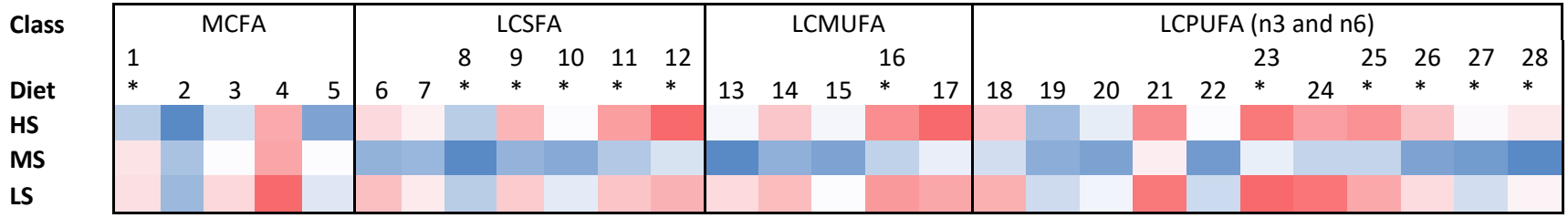

Figure 5.4.

Serum Non-Esterified Fatty Acids Heatmap of Dogs (N=24) fed Diets produced at High, Medium and Low Shear (HS, MS and LS, respectively), with each Individual Group of Fatty Acids (MCFA: Medium-Chain Fatty Acids; LCSFA: Long-Chain Saturated Fatty Acids; LCMUFA, Long-Chain Mono-Unsaturated Fatty Acids; LCPUFA, Long-Chain Poly-Unsaturated Fatty Acids) conditionally formatted on a Blue to Red Scale From Low to High Concentrations, respectively.

1, heptanoate (7:0); 2, caprylate (8:0); 3, caprate (10:0); 4, laurate (12:0); 5, 5-dodecenoate (12:1n7); 6, myristate (14:0); 7, pentadecanoate (15:0); 8, palmitate (16:0); 9, margarate (17:0); 10, stearate (18:0); 11, nonadecanoate (19:0); 12, arachidate (20:0); 13, palmitoleate (16:1n7); 14, 10-heptadecenoate (17:1n7); 15, oleate/vaccenate (18:1); 16, 10-nonadecenoate (19:1n9); 17, erucate (22:1n9); 18, hexadecadienoate (16:2n6); 19, hexadecatrienoate (16:3n3); 20, linoleate (18:2n6); 21, stearidonate (18:4n3); 22, arachidonate (20:4n6); 23, eicosapentaenoate (EPA; 20:5n3); 24, heneicosapentaenoate (21:5n3); 25, docosadienoate (22:2n6); 26, docosatrienoate (22:3n3); 27, docosapentaenoate (n6 DPA; 22:5n6); 28, docosahexaenoate (DHA; 22:6n3);
* $P < 0.05$; $q > 0.10$

Table 5.5.

Alpha Diversity Indices Richness, Inverse Simpson, Exponent Shannon and Pileou's Evenness (J) (LS Means [95% Confidence Interval]) of the Microbiome of Dogs (N=24) fed Diets produced at High, Medium and Low Shear (HS, MS and LS, respectively).

Indices	**HS**	**MS**	**LS**	***P***
Richness	75.0 [71.9, 78.0]	76.1 [73.0, 79.1]	76.6 [73.5, 79.6]	0.7438
InvSimpson	11.7 [10.0, 13.4]	11.9 [10.6, 13.3]	11.9 [10.2, 13.6]	0.9581
ExpShannon	18.4 [16.7, 19.9]	18.6 [17.0, 20.1]	18.4 [16.7, 19.9]	0.9644
Pileou's J	0.667 [0.645, 0.688]	0.666 [0.645, 0.685]	0.658 [0.629, 0.684]	0.7726

Table 5.6.

Operational Taxonomic Unit (OTU) Centered Log-Ratios Least Square Means (± Standard Error) of Dogs fed Diets produced at High, Medium and Low Shear (HS, MS and LS, respectively).

Phylum	OTU[1]	HS	MS	LS	*P*	q-value
Firmicutes	361186_Lachnospiraceae_Blautia	$5.25^{a} \pm 0.148$	$4.89^{b} \pm 0.148$	$5.05^{ab} \pm 0.148$	0.0618	0.2645
	99508_Turicibacteraceae_Turicibacter	$4.23^{a} \pm 0.171$	$3.72^{b} \pm 0.169$	$4.01^{ab} \pm 0.169$	0.0266	0.1966
	100212_Veillonellaceae_	$0.88^{a} \pm 0.258$	$0.18^{b} \pm 0.257$	$0.35^{ab} \pm 0.263$	0.0373	0.1966
	28914_Lachnospiraceae_Roseburia	$-0.59^{b} \pm 0.260$	$0.18^{a} \pm 0.260$	$-0.32^{ab} \pm 0.260$	0.0377	0.1966
	40839_Erysipelotrichaceae_Catenibacterium	$-0.81^{b} \pm 0.214$	$-0.19^{a} \pm 0.214$	$-0.58^{ab} \pm 0.214$	0.0305	0.1966
Actinobacteria	367139_Coriobacteriaceae_Slackia	$3.20^{a} \pm 0.229$	$2.40^{b} \pm 0.234$	$2.72^{ab} \pm 0.228$	0.0070	0.158
Bacteroidetes	102407_Bacteroidaceae_Bacteroides	$0.58^{b} \pm 0.396$	$1.75^{a} \pm 0.396$	$0.92^{ab} \pm 0.396$	0.0117	0.1584
	1000062_Porphyromonadaceae_unclassified	$1.23^{a} \pm 0.197$	$0.79^{b} \pm 0.200$	$0.47^{b} \pm 0.200$	0.0004	0.0272
	1105615_Paraprevotellaceae_	-2.31 ± 0.513	-1.33 ± 0.513	-2.29 ± 0.513	0.0555	0.2645
	1066621_Prevotellaceae_unclassified	$-2.42^{b} \pm 0.469$	$-1.31^{a} \pm 0.469$	$-2.38^{b} \pm 0.469$	0.0219	0.1966
Fusobacteria	298592_Fusobacteriaceae_	$1.24^{a} \pm 0.214$	$0.46^{b} \pm 0.211$	$0.99^{ab} \pm 0.214$	0.0039	0.1337
	838467_Fusobacteriaceae_Cetobacterium	$0.68^{a} \pm 0.225$	$0.00^{b} \pm 0.225$	$0.42^{ab} \pm 0.225$	0.0335	0.1966
Proteobacteria	4319416_Bartonellaceae_Bartonella	$0.733^{a} \pm 0.24$	$0.085^{b} \pm 0.24$	$0.241^{ab} \pm 0.24$	0.0110	0.1584
	10001_Enterobacteriaceae_unclassified	$-1.41^{a} \pm 0.141$	$-1.87^{b} \pm 0.141$	$-1.56^{ab} \pm 0.141$	0.0264	0.1966
	527323_Enterobacteriaceae_Yersinia	$-3.38^{b} \pm 0.192$	$-3.28^{ab} \pm 0.192$	$-2.79^{a} \pm 0.192$	0.0254	0.1966

[1]OTU expressed at the Family_Genera levels with a decreasing order of abundance.
[ab]Letters indicate significant pairwise differences ($P < 0.05$).

Table 5.7.

Spearman Rho's Correlations between Significant Operational Taxonomic Units (OTUs) (Centered Log-Ratio Transformed) and Fecal SCFA, Lactate, pH, Oligosaccharides and Succinate (measured by Metabolomics as Log_2).

OTU[1]	Total SCFA	Butyric Acid	Acetic Acid	Propionic Acid	Fecal Lactate	Fecal pH	Fecal Glucose	Fecal Maltose	Fecal Malto-Tetraose	Fecal Succinate
361186	-0.52**	-0.52**	0.05	-0.07	0.02	0.25**	0.06	-0.32**	-0.18	-0.19
99508	-0.35**	-0.26**	-0.02	-0.08	0.06	0.16	0.09	-0.18	-0.08	-0.07
100212	0.01	-0.08	0.36**	0.09	-0.13	0.2	-0.13	-0.08	-0.25**	-0.27**
28914	0.27**	0.17	-0.08	0.12	0.04	-0.21*	-0.01	0.35**	0.19	0.11
40839	-0.03	-0.19	0.03	0.06	0.00	-0.15	0.38**	0.21	0.17	-0.04
367139	-0.47**	-0.37**	-0.06	-0.10	0.09	0.13	0.17	-0.23*	0.04	-0.18
102407	0.38**	0.67**	-0.26**	-0.20*	-0.13	0.06	-0.30**	0.14	-0.07	0.16
1000062	-0.06	-0.32**	0.23*	0.27**	-0.12	-0.01	0.14	-0.05	-0.07	-0.34**
1105615	0.37**	0.55**	-0.12	-0.18	-0.21*	0.00	-0.36**	0.01	-0.21*	0.16
1066621	0.36**	0.59**	-0.18	-0.18	-0.14	-0.01	-0.37**	0.08	-0.16	0.25**
298592	-0.30**	-0.33**	0.06	0.08	0.16	0.08	0.20	-0.17	0.07	-0.11
838467	-0.09	-0.05	0.12	0.06	-0.24**	0.40**	0.04	-0.11	-0.13	-0.46**
4319416	0.17	-0.11	0.41**	0.25**	-0.19	0.09	-0.12	-0.24**	-0.34**	-0.35**
10001	-0.25**	-0.31**	0.08	0.05	-0.06	0.30**	-0.03	-0.30**	-0.19	-0.25**
527323	0.03	-0.03	-0.06	-0.05	0.29**	-0.36**	0.16	0.08	0.27**	0.34**

[1]361186_Lachnospiraceae_Blautia, 99508_Turicibacteraceae_Turicibacter, 100212_Veillonellaceae_, 28914_Lachnospiraceae_Roseburia, 40839_Erysipelotrichaceae_Catenibacterium, 367139_Coriobacteriaceae_Slackia, 102407_Bacteroidaceae_Bacteroides, 1000062_Porphyromonadaceae_unclassified, 1105615_Paraprevotellaceae_, 1066621_Prevotellaceae_unclassified, 298592_Fusobacteriaceae_, 838467_Fusobacteriaceae_Cetobacterium, 4319416_Bartonellaceae_Bartonella, 10001_Enterobacteriaceae_unclassified, 527323_Enterobacteriaceae_Yersinia.

**Correlations with a Spearman rho's $P < 0.05$.

* Correlations with a Spearman rho's $0.05 < P < 0.10$.

Appendix A - Supplementary Tables of Chapter 3

Length (mm)	12.5	25	12.5	18.75	6.25	25	25	18.75	18.75	18.75	18.75	18.75	31.25
Length (D)	0.5	1	0.5	0.75	0.25	1	1	0.75	0.75	0.75	0.75	0.75	1.25
Right Screw	Double Flight Cut Flight Compression	Double Flight Compression	Reverse Cut Flight	Double Flight	Transition Spacer	Double Flight Transport	Double Flight Transport	Right Hand Kneading Block (45°)	Double Flight Transport	Double Flight Transport	Double Flight Transport	Double Flight Transport	Single Flight Trapazoid Transport
Left Screw	Double Flight Cut Flight Compression	Double Flight/Compression	Reverse Cut Flight	Double Flight	Transition Spacer	Double Flight Transport	Double Flight Transport	Right Hand Kneading Block (45°)	Double Flight Transport	Double Flight Transport	Double Flight Transport	Double Flight Transport	Single Flight Trapazoid Transport

Figure A.1. *Right and Left Screw Profile of the Evolum 25 Extruder used during the Experiment. The Die would be situated on the Left Side, and the Preconditioner Feeder on the Right Side.*

Table A.1.

Central Composite Design of Evolum 25 Extrusion with Parameters selected within the same Block. Each Number under Screen Size, Extruder Screw Speed, and In-Barrel Moisture corresponds to Low (-1), Medium (0) and High (1) Settings.

Sample	Run order	Particle size[1]	Shaft speed[2]	In-barrel moisture[3]	Production day
1	1	0	0	0	1
2	2	0	0	0	1
3	3	0	0	0	1
4	4	0	0	0	1
5	5	0	0	0	1
6	6	0	0	0	1
13	7	-1	0	0	1
12	8	-1	1	-1	1
11	9	-1	-1	-1	1
14	10	-1	1	1	1
15	11	-1	-1	1	1
7	12	0	1	0	1
8	13	0	-1	0	1
9	14	0	0	1	1
10	15	0	0	-1	1
16	16	1	1	-1	2
17	17	1	-1	-1	2
18	18	1	0	0	2
19	19	1	1	1	2
20	20	1	-1	1	2

[1]Determined by particle size analysis: -1 = 161.5um; 0= 172.4 um; 1= 195.3 um.
[2]Measured during extrusion and averaged for each treatment: -1 = 29.8%; 0= 32.5%; 1= 35.3%.
[3]Shaft speed set during the process at: -1 = 400 rpm; 0= 800 rpm; 1= 1200 rpm.

Table A.2.

Particle Size Analysis determined by the Morphologi G3 Instrument and the Ro-Tap Procedure. [1]*Corn 1, 2 and 3 ground with a 0.793, 1.19 and 1.59 mm Screen Size, respectively.*

Analysis	**Corn 1**	**Corn 2**	**Corn 3**	**Grain mix 1**	**Grain mix 2**	**Grain mix 3**
Morphologi G3						
Number of particles analyzed	76,892	51,448	41,324	24,479	30,924	29,871
[1]CE diameter distribution (v, 0.5), μm	183.4	331.6	287.3	152.9	151.1	160.4
[2]HS circularity (n, 0.5)	0.879	0.901	0.913	0.781	0.798	0.808
Apect ratio number distribution (n, 0.5)	0.782	0.796	0.806	0.74	0.742	0.745
Elongation (n, 0.5)	0.215	0.201	0.191	0.258	0.256	0.253
Ro-tap procedure						
Mean geometric diamater (Dgw), μm	158.6	174.8	221.1	161.6	172.7	195.3
Standard deviation (Sgw)	2.05	2.24	2.6	1.99	2.08	2.24

Grain mix 1, 2 and 3 were the same diet recipe mixed with corn 1, 2 and 3, respectively.
[1]CE= circle equivalent; (v, 0.5) = median of the volume distribution.
[2]HS= high sensitivity; (n, 0.5) = median of the number distribution.

Table A.3.

Kibble Characteristics of Small-Scale Extrusion (Evolum 25)[1].

Sample	Volume, cm^3	density g/cm^3	SEI, cm^2_e/cm^2_d	VEI, cm^3_e/cm^3_d	LEI, cm_e/cm_d	Hardness, kg
1	1.054	0.479	1.668	1.194	0.716	6.46
2	1.328	0.427	1.962	1.337	0.681	16.41
3	1.265	0.431	1.913	1.328	0.694	11.44
4	1.270	0.425	1.919	1.349	0.703	9.14
5	1.274	0.428	1.925	1.333	0.692	7.28
6	1.237	0.441	1.886	1.298	0.688	9.87
7	1.429	0.445	1.670	1.308	0.783	12.16
8	1.226	0.439	1.705	1.297	0.760	6.62
9	1.125	0.732	1.868	0.719	0.385	8.31
10	1.372	0.377	2.082	1.679	0.806	6.37
11	1.291	0.408	1.790	1.567	0.875	7.34
12	1.290	0.412	1.614	1.550	0.961	8.65
13	1.256	0.412	1.845	1.401	0.760	12.95
14	1.015	0.547	1.391	0.966	0.695	10.01
15	1.102	0.478	1.599	1.100	0.688	10.35
16	1.108	0.470	1.411	1.310	0.928	12.46
17	1.113	0.456	1.641	1.325	0.808	11.22
18	1.326	0.423	1.964	1.323	0.673	9.31
19	0.995	0.543	1.328	0.931	0.701	10.77
20	1.056	0.493	1.583	1.023	0.646	15.80

[1]Kibble volume, density and SEI were measured on 20 kibbles per treatment and averaged. Hardness was measured on 30 kibbles and averaged.

Appendix B - Supplementary Table of Chapter 4

Table B.1.
Drier Settings used during the X115 Extrusion (Average ± Standard Deviation) of a High, Medium and Low Shear (HS, MS and LS, respectively) Dog Food.

Item	HS	MS	LS
Temp 1 before bed, °C	132 ± 8.5	141 ± 2.5	135 ± 12.1
Temp 1 after bed, °C	90.3 ± 5.64	97.6 ± 5.55	93.1 ± 8.20
Bed depth 1, inches	1.60 ± 0.327	1.24 ± 0.429	1.40 ± 0.362
[1]RT 1, min	0:00	3:55 ± 0:00	3:56 ± 0:01
Damper open 1, %	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Temp 2 before bed, °C	132 ± 10.1	142 ± 0.6	134 ± 10.9
Temp 2 after bed, °C	114 ± 7.1	119 ± 2.0	115 ± 7.0
Bed depth 2, inches	13.8 ± 4.71	16.3 ± 0.43	16.5 ± 0.00
RT 2, min	4:03 ± 0:07	4:02 ± 0:06	3:58 ± 0:01
Damper open 2, %	2.58 ± 0.037	2.58 ± 0.040	2.59 ± 0.031
Temp 3 before bed, °C	132 ± 10.4	142 ± 1.3	134 ± 11.2
Temp 3 after bed, °C	113 ± 8.5	118 ± 1.7	114 ± 8.7
Bed depth 3, in	1.10 ± 0.245	1.08 ± 0.189	1.10 ± 0.254
RT 3, min	3:56 ± 0:02	3:57 ± 0:03	3:58 ± 0:03
Damper open 3, %	1.67 ± 1.179	1.75 ± 1.146	2.49 ± 0.031
Total RT, min	11:54 ± 0:05	11:54 ± 0:03	11:52 ± 0.02
Dry flow rate	1101 ± 59.6	1101 ± 47.3	1136 ± 0.0
Moisture lost in drier	6.99 ± 0.080	13.05 ± 0.169	13.18 ± 0.037

[1]Retention time.

Appendix C - Supplementary Tables of Chapter 5

Table C.1.

Nutritional Composition of Equal Recipe Foods Extruded at High (HS), Medium (MS) and Low (LS) Shear.

Nutrient	HS	MS	LS
Moisture, %	8.69	9.87	10.40
Dry Matter, %	91.31	90.13	89.6
Organic Matter, %	86.03	85.15	84.1
Energy, kcal/kg	4652	4630	4541
Ash, %	5.28	4.98	5.50
Crude Protein, %	19.5	19.06	19.94
Fat Crude, %	15.04	14.94	14.28
Total Fatty Acids, %	13.95	13.75	13.08
Monounsaturated Fatty Acids, %	5.86	5.77	5.53
Polyunsaturated Fatty Acids, %	3.17	3.17	3.00
Saturated Fatty Acids, %	4.91	4.81	4.55
Total Fat as Triglycerides, %	14.60	14.38	13.68
Fiber Crude, %	1.30	1.2	1.10
Fiber Total Dietary, %	6.2	5.8	6.7
Fiber Insoluble, %	6.1	5.8	6.1
Fiber Soluble, %	< 0.2	< 0.2	0.6
Sucrose	1.03	1.07	0.83
Sugars - Total	1.67	1.07	0.83
Fructose	0.19	< 0.15	<0.15
Glucose	0.28	< 0.15	< 0.15
Lactose	0.17	< 0.15	<0.15
Maltose	<0.15	< 0.15	< 0.15
Starch	41.7	41.6	39.2
Resistant starch, %	0.650	0.940	1.057
Aminoacids			
Alanine, %	1.40	1.36	1.41
Arginine, %	1.13	1.07	1.14
Aspartic Acid, %	1.50	1.46	1.56
Glutamic Acid, %	2.85	2.76	2.92
Glycine, %	1.66	1.59	1.71
Histidine, %	0.46	0.44	0.47
Isoleucine, %	0.70	0.68	0.70
Leucine, %	1.63	1.6	1.59
Lysine, %	1.17	1.13	1.24
Phenylalanine, %	0.82	0.8	0.79
Proline, %	1.49	1.4	1.39

Serine, %	0.77	0.75	0.80
Threonine, %	0.70	0.68	0.74
Tyrosine, %	0.42	0.4	0.37
Valine, %	0.89	0.84	0.88
Cystine, %	0.19	0.2	0.19
Methionine, %	0.37	0.38	0.39
Tryptophan, %	0.17	0.17	0.18
Taurine, %	0.10	0.11	0.10
Hydroxyproline, %	0.52	0.46	0.50
Minerals			
Chloride, %	0.42	0.42	0.45
Calcium, %	1.160	1.11	1.230
Magnesium, %	0.098	0.095	0.098
Phosphorus, %	0.86	0.84	0.90
Potassium, %	0.59	0.57	0.61
Sodium, %	0.173	0.175	0.188
Protein Crude, %	19.50	19.06	19.94
Sulfur, %	.22	0.22	.23
Chromium, ppm	0.26	0.33	0.24
Copper, ppm	17	17	19
Iron, %	0.0178	0.0177	0.0189
Manganese, ppm	19	20	20
Zinc, ppm	309.000	309	340.000
Molybdenum, ppm	0.25	0.3	0.23
Selenium, ppm	0.68	0.66	0.70
Aluminum, ppm	< 10.0	< 10.0	< 10.0
Cobalt, ppm	< 0.75	< 0.75	< 0.75
Nitrate, mg / kg	<10	<10	<10
Vitamins			
Vitamin A, IU / 100 g	381.0	485	416.0
Vitamin B7 - Biotin, mg / 100 g	0.03	0.03	0.03
Choline, mg / 100 g	182	179	181
Vitamin B3 - Niacin, mg / 100 g	6.510	6.77	5.930
Vitamin B5 - Pantothenic Acid, mg / 100 g	1.240	1.15	1.200
Vitamin B9 - Folic Acid, mg / 100 g	0.141	0.117	0.108
Vitamin A - Beta Carotene, IU / 100 g	46.500	46.4	86.900
Vitamin A - Retinol, IU / 100 g	334.000	439	329.000
Vitamin B1 Mononitrate, mg / 100 g	2.81	2.61	2.73
Vitamin B1 Thiamin Base, mg / 100 g	2.58	2.4	2.51
VitaminB1-ThiamineHydrochloride, mg / 100 g	2.89	2.69	2.81
Vitamin B12 - Cobalamin, µg / 100 g	6.51	5.7	3.73
Vitamin B2 - Riboflavin, mg / 100 g	.62	0.619	.578
Vitamin B6 - Pyridoxine, mg / 100 g	0.8	0.8	0.9

Table C.2.

Analytical Methods of each Nutrient Analyzed in Equal Recipe Foods produced at High (HS), Medium (MS) and Low (LS) Shear, as well as Fecal Samples of Dogs Fed These Diets.

Analysis	Method
Acid Detergent Fiber QD002-Eurofins	ANKOM ADF for A2000 mod - Gravimetry
Aluminum by ICP	AOAC 984.27 mod,927.02 mod,985.01 mod,965.17 mod
Amino Acids Excluding Tryptophan	AOAC 982.30 mod.
Ash	AOAC 942.05
Biotin	Met. of Vitamin Assay,Interscience Publ.,Ch.12 - Nephelometry
Calories	Calorimetry
Chloride Soluble by Chloridometer	AOAC 2016.03, AOAC 971.27
Choline Total by Ion Chromatography	AOAC 2012.20 mod.
Chromium by ICP-MS - QD0K2	AOAC 2011.19 mod.
Cobalt by AAS	AOAC 965.17 / 968.08 modified
Cystine & Methionine	AOAC 994.12 mod.
Fat Crude by Acid Hydrolysis	AOAC 954.02
Fatty Acids-Omega 6 & 3 %W/W	AOCS Ce 2-66 mod., AOCS Ce 1b-89 mod.
Fiber Crude	AOAC 962.09; AOCS Ba 6-84
Fiber Dietary Complete	AOAC 991.43
Fiber Neutral Detergent	ANKOM NDF for A2000 mod. - Gravimetry
Fluoride	AOAC 975.08
Hydroxyproline - AOAC	AOAC 982.30 mod.
Macro Elements by ICP	AOAC 984.27 mod,927.02 mod,985.01 mod,965.17 mod
Minor Elements by ICP	AOAC 984.27 mod,927.02 mod,985.01 mod,965.17 mod
Moisture - Forced Draft Oven	AOAC 930.15
Molybdenum by ICP-MS - QD0K1	AOAC 2011.19 mod.
Niacin or Niacinamide - AOAC	AOAC 944.13 mod.
Nitrate by IC	Internal Method based on EN 12014-2, (QA02F) J. AOAC Int., 2005, 88(6), 1793-1796 - IC-EC
Nitrite as Nitrogen - AOAC 968.07	AOAC 968.07
Pantothenic Acid - AOAC	AOAC 945.74 (mod.)
Protein by Kjeltec (Kjeldahl Replacement)	AOAC 2001.11
Selenium by ICP-MS - QD0K0	AOAC 2011.19 mod.
Starch Total - AOAC	AOAC 996.11
Sugar Profile by HPLC - AOAC	AOAC 982.14, mod.
Sulfate - AOAC Gravimetric	AOAC 920.46
Sulfur by ICP	Internal Method - MET3289 - ICP-OES
Taurine Food	AOAC 982.30 mod.
Total Folate as Folic Acid	AOAC 992.05 mod.
Tryptophan - AOAC	AOAC 988.15 mod.
Vitamin A Total-AOAC	AOAC 974.29 Mod.

Vitamin B1	AOAC 942.23 mod.
Vitamin B12 - AOAC	AOAC 952.20 mod.
Vitamin B2	AOAC 970.65 mod.
Vitamin B6 -Pyridoxine by HPLC	J. AOAC 88, 30-37 (2005), mod.
Water Activity	AOAC 978.18 mod.

Table C.3.

Blood Chemistry of Dogs (N=24) fed Diets produced at High, Medium and Low Shear (HS, MS and LS, respectively). Reported either as LS Means; 95% CI, or LS Means ± Standard Error.

Parameter	**HS**	**MS**	**LS**	***P***	**Reference range**
ALT, U/L	24.8; 21.4-29.4	25.6; 22.0-30.6	26.8; 22.9-32.4	0.0822	17 – 55
Albumin, g/dL	3.34 ± 0.115	3.35 ± 0.110	3.36 ± 0.106	0.9098	2.8- 4.0
Alb:Glob	1.66 ± 0.100	1.62 ± 0.100	1.64 ± 0.100	0.7102	1.1- 2.4
ALP, U/L	60.3 ± 2.51	61.0 ± 2.51	55.3 ± 2.51	0.0580	17- 134
BUN, mg/dL	12.6; 11.0-14.9	12.8; 11.0-15.3	14.2; 12.1-17.2	0.0284	7.6-19.3
BUN:creat	14.8[b]; 13.2-16.6	15.0[b]; 13.4-16.8	16.4[a]; 14.7-18.4	0.0040	11.3- 26.4
Creatinine, mg/dL	0.864; 0.771-0.963	0.862; 0.768-0.962	0.875; 0.786-0.970	0.4778	0.5- 1.0
Calcium, mg/dL	9.95 ± 0.066	9.99 ± 0.066	10.02 ± 0.066	0.3718	9.0- 10.8
Chloride, mmol/L	115.8 ± 0.46	116.4 ± 0.38	116.4 ± 0.46	0.2253	108- 116
Cholesterol, mg/dL	191.3; 173.9-210.5	191.7; 174.2-210.9	188.7; 171.5-207.6	0.7233	127- 318
Globulin, g/dL	2.03 ± 0.091	2.06 ± 0.091	2.07 ± 0.091	0.7233	1.5- 2.6
Glucose, mg/dL	94.0 ± 1.55	93.7 ± 1.55	93.6 ± 1.55	0.9717	79- 116
Phosphorous, mg/dL	3.42 ± 0.091	3.39 ± 0.091	3.45 ± 0.091	0.8077	2.3- 4.7
Magnesium, mg/dL	1.83 ± 0.039	1.82 ± 0.044	1.85 ± 0.032	0.7026	1.7- 2.2
Potassium, mmol/L	4.68 ± 0.046	4.67 ± 0.046	4.75 ± 0.046	0.2153	3.7- 5.1
Sodium, mmol/L	147.5 ± 0.30	147.8 ± 0.30	148.0 ± 0.23	0.2317	145- 150
Na:K	31.5 ± 0.31	31.7 ± 0.31	31.2 ± 0.31	0.3068	29- 40
Total protein, g/dL	5.43; 5.29-5.56	5.46; 5.29-5.64	5.48; 5.33-5.63	0.5898	4.8- 6.1
Triglycerides, mg/dL	49.7; 39.5-62.4	50.5; 40.2-63.5	49.0; 39.0-61.6	0.8934	19- 119

[ab]LS means with unlike superscripts differ.

Table C.4.

Complete Blood Count (CBC) of Dogs (N=24) fed Diets produced at High, Medium and Low Shear (HS, MS and LS, respectively). Reported either as LS Means; 95% CI, or LS Means ± Standard Error.

Parameter	HS	MS	LS	*P*	Reference range
IRF, %	17.4 ± 1.93	18.5 ± 1.93	19.9 ± 1.93	0.2455	5.2- 32.7
MCH, pg	23.1 ± 0.17	23.1 ± 0.17	23.2 ± 0.18	0.8617	21.6- 24.6
MCHC, g/dL	34.8 ± 0.08	34.8 ± 0.08	34.9 ± 0.08	0.4726	32.4- 36.0
MCV, fL	66.7 ± 0.51	66.7 ± 0.51	66.6 ± 0.51	0.8014	63.6- 72.9
Platelets, k/µL	316.0; 269.5-370.5	309.2; 263.7-362.5	306.3; 261.2-359.0	0.4954	131- 429
HCT, %	48.1 ± 0.68	47.9 ± 0.68	48.8 ± 0.68	0.2894	35.2- 54.2
Red Blood Cells, M/µL	7.24 ± 0.129	7.20 ± 0.129	7.35 ± 0.129	0.2690	5.16- 8.24
RDW, fL	34.0 ± 0.28	34.0 ± 0.28	33.9 ± 0.28	0.8014	31.4- 37.0
HGB, g/dL	16.8 ± 0.23	16.7 ± 0.23	17.0 ± 0.23	0.2197	11.9- 18.5
Reticulocytes, %	0.695 ± 0.0643	0.677 ± 0.0501	0.704 ± 0.0703	0.9226	0.23- 1.30
Basophils, %	0.206 ± 0.0210	0.217 ± 0.0217	0.214 ± 0.0319	0.9185	-
Eosinophils, %	3.41; 2.62-4.45	3.14; 2.41-4.10	3.12; 2.39-4.07	0.4514	-
Lymphocytes, %	30.8 ± 1.43	30.2 ± 1.43	30.0 ± 1.43	0.8562	-
Monocytes, %	5.07 ± 0.279	4.92 ± 0.279	5.56 ± 0.279	0.0642	-
Neutrophils, %	60.2 ± 2.22	61.1 ± 2.22	60.7 ± 2.22	0.8202	-
Reticulocytes, M/µL	0.065 ± 0.0052	0.060 ± 0.0052	0.062 ± 0.0052	0.4785	-
Eosinophils, k/µL	0.215; 0.141-0.326	0.198; 0.138-0.285	0.198; 0.135-0.292	0.5285	0.06- 0.62
Lymphocytes, k/µL	1.90 ± 0.15	1.87 ± 0.16	1.88 ± 0.17	0.8059	0.87- 3.04
Monocytes, k/µL	0.354 ± 0.0240	0.346 ± 0.0236	0.319 ± 0.0235	0.2912	0.14- 0.69
Neutrophils, k/µL	3.84; 3.45-4.27	3.86; 3.47-4.29	3.77; 3.40-4.19	0.9317	2.09- 7.32
Basophils, k/µL	0.012; 0.005-0.0189	0.016; 0.009-0.0226	0.013; 0.0053-0.0198	0.7036	0.00- 0.04
White Blood Cells, k/µL	6.65 ± 0.349	6.53 ± 0.342	6.40 ± 0.343	0.7463	3.3- 11.3

Table C.5.

Fecal Minerals of Dogs (N=24) fed Diets produced at High, Medium and Low Shear (HS, MS and LS, respectively). Reported either as LS Means; 95% CI, or LS Means ± Standard Error.

Fecal mineral	**HS**	**MS**	**LS**	***P***
Calcium, %	7.58^{ab} ± 0.280	7.17^{b} ± 0.280	8.01^{a} ± 0.280	0.0622
Phosphorous, %	4.21 ± 0.173	4.21 ± 0.153	4.49 ± 0.222	0.4365
Potassium, %	0.362 ± 00286	0.370 ± 0.0305	0.359 ± 0.0297	0.9235
Magnesium, %	0.537 ± 0.0267	0.530 ± 0.0267	0.542 ± 0.0267	0.9204
Sodium, %	0.257^{a}; 0.213-0.310	0.208^{ab}; 0.172-0.252	0.182^{b}; 0.154-0.215	0.0123
Zinc, ppm	2,040; 1,897-2,183	2,008; 1,865-2,152	2,160; 2,016-2,303	0.1934
Copper, ppm	99.9; 92.6-108.5	95.7; 88.9-103.5	101.8; 94.2-110.7	0.3925
Iron, ppm	1,226; 1,110-1,369	1,190; 1,122-1,266	1,259; 1,181-1,349	0.4399
Manganese, ppm	146.5 ± 8.40	138.8 ± 6.31	160.6 ± 8.93	0.1178

Table C.6.

Fecal Aminoacids Metabolomics of Dogs (N=24) fed Diets produced at High, Medium and Low Shear (HS, MS and LS, respectively).

Analyte	Estimate HS	Estimate MS	Estimate LS	Std Error HS	Std Error MS	Std Error LS	*P*	q-value
alanine	-0.134	0.016	-0.091	0.2106	0.2106	0.2216	0.612	0.518
arginine	-0.087	0.370	-0.094	0.5565	0.5565	0.5798	0.352	0.411
asparagine	0.358	0.982	0.588	0.7919	0.7919	0.8262	0.494	0.477
aspartate	-0.209	-0.040	-0.519	0.4044	0.4044	0.4213	0.197	0.316
cysteine	-0.020	0.116	-0.005	0.1613	0.1625	0.1697	0.219	0.330
glutamate	-0.420[b]	0.055[a]	-0.269[ab]	0.3304	0.3307	0.3436	0.041	0.135
glutamine	0.073	0.213	0.217	0.3285	0.3289	0.3434	0.717	0.560
glycine	0.086[b]	0.470[a]	0.303[ab]	0.2516	0.2519	0.2630	0.051	0.142
histidine	-0.014	0.453	0.047	0.3612	0.3612	0.3753	0.097	0.217
isoleucine	-0.078	0.144	-0.115	0.2812	0.2812	0.2939	0.348	0.410
leucine	-0.164	0.050	-0.177	0.2832	0.2832	0.2962	0.428	0.440
lysine	-0.224	-0.064	-0.224	0.2531	0.2531	0.2637	0.543	0.487
methionine	0.061	0.164	0.023	0.2062	0.2062	0.2158	0.597	0.514
phenylalanine	-0.153	-0.068	-0.185	0.2875	0.2875	0.2978	0.783	0.581
proline	-0.098	0.184	-0.012	0.2248	0.2251	0.2369	0.158	0.286
serine	-0.081	0.104	-0.275	0.3433	0.3433	0.3563	0.220	0.330
taurine	0.004	-0.264	-0.056	0.8388	0.8388	0.8522	0.707	0.558
threonine	-0.173[b]	0.234[a]	0.006[ab]	0.2421	0.2424	0.2551	0.047	0.142
tryptophan	-0.347	-0.202	-0.196	0.3363	0.3363	0.3527	0.774	0.581
tyrosine	-0.132	-0.134	-0.340	0.3091	0.3091	0.3236	0.539	0.485
valine	-0.210	0.036	-0.258	0.3181	0.3181	0.3335	0.380	0.417

Table C.7.
Fecal Di- and Tripeptides Metabolomics of Dogs (N=24) fed Diets produced at High, Medium and Low Shear (HS, MS and LS, respectively).

Analyte	Estimate HS	Estimate MS	Estimate LS	Std Error HS	Std Error MS	Std Error LS	*P*	q-value
ala-ile-ala	-0.221	0.175	0.089	0.3322	0.3322	0.3505	0.262	0.367
ala-leu-ala	-0.275	0.142	0.084	0.3328	0.3328	0.3532	0.245	0.352
alanylleucine	-0.006	0.044	0.177	0.2777	0.2777	0.2932	0.670	0.545
glycylisoleucine	0.123	0.157	-0.052	0.3494	0.3469	0.3650	0.682	0.551
glycylleucine	-0.109	-0.103	-0.082	0.2561	0.2546	0.2650	0.986	0.644
glycylvaline	-0.108	-0.032	-0.104	0.2611	0.2611	0.2744	0.901	0.623
histidylalanine	0.136	0.187	0.019	0.3402	0.3402	0.3578	0.786	0.582
isoleucylglycine	0.259	0.013	-0.053	0.2421	0.2421	0.2516	0.111	0.230
leucylalanine	-0.048	0.046	0.240	0.3119	0.3119	0.3254	0.376	0.417
leucylglutamine*	0.059	0.302	0.376	0.2949	0.2949	0.3088	0.281	0.374
leucylglycine	0.025	-0.098	0.073	0.2296	0.2296	0.2390	0.500	0.477
lysylleucine	0.068	0.154	0.188	0.2676	0.2676	0.2792	0.786	0.582
phenylalanylalanine	-0.045^{b}	0.359^{ab}	0.565^{a}	0.3102	0.3102	0.3254	0.026	0.110
phenylalanylglycine	-0.078	0.054	0.134	0.2536	0.2536	0.2662	0.501	0.477
prolylglycine	-0.322	0.291	0.115	0.3687	0.3687	0.3903	0.101	0.218
threonylphenylalanine	-0.241	0.070	0.115	0.4171	0.4171	0.4317	0.306	0.393
tryptophylglycine	0.012^{b}	0.158^{ab}	0.364^{a}	0.2070	0.2070	0.2155	0.041	0.135
tyrosylglycine	-0.049	0.138	0.120	0.2617	0.2617	0.2751	0.559	0.495
val-val-ala	-0.064	0.011	0.029	0.2724	0.2724	0.2850	0.870	0.611
valylglutamine	-0.036	0.117	0.074	0.3136	0.3120	0.3237	0.716	0.560
valylglycine	0.136	-0.019	0.020	0.1857	0.1846	0.1926	0.432	0.441
valylleucine	-0.010	-0.045	0.004	0.2952	0.2952	0.3093	0.970	0.640

Table C.8.

Fecal Putrefactive Compounds Metabolomics of Dogs (N=24) fed Diets produced at High, Medium and Low Shear (HS, MS and LS, respectively).

Analyte	Estimate HS	Estimate MS	Estimate LS	Std Error HS	Std Error MS	Std Error LS	*P*	q-value
3-indoleglyoxylic acid	-0.223[b]	-0.201[b]	0.148[a]	0.2357	0.2357	0.2403	0.001	0.042
3-hydroxyindolin-2-one	-0.560[a]	-1.357[b]	-1.279[b]	0.3994	0.3994	0.4122	0.002	0.042
indolepropionate	-0.283[b]	0.436[a]	0.223[ab]	0.6058	0.6055	0.6145	0.008	0.068
indole	0.233[a]	-0.601[b]	-0.434[b]	0.4204	0.4204	0.4379	0.010	0.073
phenol sulfate	0.413[a]	-0.440[b]	-0.264[ab]	0.6018	0.6018	0.6158	0.014	0.087
indolin-2-one	0.450[a]	-0.917[b]	-0.375[ab]	0.8927	0.8927	0.9153	0.015	0.090
2-hydroxy-3-methylvalerate	-0.980[b]	0.201[a]	0.025[ab]	0.6032	0.6032	0.6337	0.020	0.095
4-methyl-2-oxopentanoate	-0.397[ab]	-0.382[a]	-0.975[b]	0.4071	0.4071	0.4208	0.025	0.109
indoleacetylglycine	-1.012[b]	-0.323[a]	-0.588	0.4675	0.4675	0.4826	0.044	0.138
alpha-hydroxyisocaproate	-1.173	-0.124	-0.198	0.7001	0.7001	0.7325	0.063	0.164
indolelactate	-0.278	1.068	0.636	0.6927	0.6927	0.7386	0.068	0.170
imidazole lactate	-0.039	0.857	0.454	0.5375	0.5375	0.5643	0.077	0.185
alpha-hydroxyisovalerate	-1.244	-0.414	-0.415	0.6060	0.6060	0.6343	0.085	0.197
5-hydroxyindoleacetate	-0.195	-0.470	-0.528	0.3977	0.3977	0.4045	0.111	0.230
phenyllactate (PLA)	0.051	0.825	0.602	0.5430	0.5430	0.5684	0.118	0.240
4-hydroxyphenylpyruvate	0.022	0.072	-0.590	0.4193	0.4193	0.4507	0.149	0.273
3-methyl-2-oxovalerate	-0.523	-0.473	-0.937	0.4792	0.4792	0.4949	0.196	0.316
indoleacetate	-0.027	-0.047	-0.219	0.1846	0.1845	0.1923	0.272	0.369
indoleacrylate	-0.175	-0.347	-0.502	0.3578	0.3578	0.3694	0.292	0.380
3-methyl-2-oxobutyrate	-0.486	-0.494	-0.764	0.3857	0.3857	0.3975	0.346	0.409
phenylpyruvate	-0.131	-0.117	-0.431	0.3602	0.3602	0.3771	0.372	0.417
3-formylindole	-0.266	-0.235	-0.391	0.1975	0.1978	0.2088	0.443	0.449
3-(4-hydroxyphenyl) propionate	-0.078	0.127	0.007	0.4893	0.4896	0.4989	0.614	0.519
indole-3-carboxylate	-0.251	-0.413	-0.396	0.3334	0.3334	0.3499	0.758	0.578
2-oxindole-3-acetate	0.065	-0.028	0.082	0.2847	0.2847	0.2937	0.768	0.580
4-hydroxyphenylacetate	0.116	0.229	0.133	0.2998	0.2998	0.3101	0.791	0.583
phenylacetate	0.255	0.278	0.347	0.5457	0.5457	0.5647	0.958	0.637

Table C.9.

Fecal advanced glycation end-products (AGEs) Metabolomics of Dogs (N=24) fed Diets produced at High, Medium and Low Shear (HS, MS and LS, respectively).

Analyte	Estimate HS	Estimate MS	Estimate LS	Std Error HS	Std Error MS	Std Error LS	*P*	q-value
carboxy-methyl-arginine	-0.810	-0.811	-0.559	0.4230	0.4230	0.4309	0.298	0.384
N6-carboxyethyllysine	-0.245	-0.219	-0.060	0.2650	0.2650	0.2713	0.310	0.394
N6-carboxymethyllysine	-0.267	-0.524	-0.488	0.2084	0.2087	0.2193	0.137	0.261
pyrraline	-0.109[b]	0.380[a]	0.478[a]	0.2966	0.2966	0.3050	0.001	0.042