Evaluation of alternative sustainable ingredients for use in companion animal diets

by

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Abstract

The pet food industry has a unique position in the food system as it largely relies on by-products or co-products from the human food systems for ingredient sourcing. Considering the substantial size of the pet food market, identifying alternative ingredients for use in the pet food industry that are sustainability sourced will be essential. The first objective of this work was to evaluate fermentation characteristics of alternative fiber ingredients using an *in vitro* canine fecal inoculum model. Organic matter disappearance (OMD) and postbiotic production were determined for apple pomace (AP), blueberry pomace (BP), cranberry pomace (CP), tomato pomace (TP), and pea fiber (PF) incubated with inoculum for 1, 3, 6, and 12 h at 39ºC. The OMD was similar ($P>0.05$; average of 18.5%) between treatments with no effect of time ($P>0.05$). Total VFA concentration was highest for AP ($P<0.05$), followed by TP, BP and PF, and lowest for CP (1.17, 0.75, average of 0.48, and 0.21 mmol*g^{-1} of substrate, respectively). AP and TP had greater butyrate concentrations (average of 0.0476mmol*g^{-1} of substrate) than all other treatments (0.0093 to 0.0344mmol*g^{-1} of substrate). Overall, the fiber substrates evaluated were marginally to moderately fermentable when incubated for up to 12 h with canine fecal inoculum. The second objective was to evaluate the use of a yeast biomass as a novel protein source in feline diets. An extruded feline diet containing Torula yeast (TY) was evaluated for diet processing, palatability, and apparent total tract digestibility (ATTD) compared to diets containing pea protein (PP), soybean meal (SM), and chicken meal (CM). During diet production, specific mechanical energy of TY and SM (average of 187 kJ/kg) was greater ($P<0.05$) than for PP (138 kJ/kg) but not different ($P>0.05$) from CM (167 kJ/kg). Kibble sectional expansion and piece volume were greatest for TY ($P<0.05$). Cats fed TY had greater intake ratios ($P<0.05$) than CM and PP (0.88 and 0.73,

respectively), but were not different ($P>0.05$) from SM. ATTD of dry matter (DM), organic matter (OM), and gross energy (GE) was greater ($P<0.05$) for CM (87.43, 91.34, 90.97%, respectively) than TY. The ATTD of DM, OM, and GE was similar ($P<0.05$) for TY to PP and SM (averages of 86.20, 89.76, and 90.22%, respectively). Crude protein ATTD of TY was similar to other treatments (average of 89.97%), but fat ATTD was lower ($P<0.05$; 92.52%) than other treatments (93.76 to 94.82%). Total dietary fiber ATTD was similar between TY and CM (average of 66.20%) and greater ($P<0.05$) than PP and SM (average of 58.70%). In summary, the Torula yeast facilitated kibble formation, increased diet preference, and was highly digestible when fed to cats. The results here provide valuable data on the use of high-fiber food processing by-products and yeast derived proteins in companion animal diets. Alternative ingredients, such as these, have great potential to provide valuable, sustainably sourced ingredients for pet diets.

Table of Contents

List of Tables

List of Figures

List of Equations

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Chapter 1 - Literature review

Introduction

According to the 2021-2022 American Pet Products Association (APPA) National Pet Owners Survey, about 70% of U.S. households owned a pet in 2021 (APPA, 2022) representing around 90.5 million homes. This is a 25% increase in the proportion of American households owning a pet from the first year this study was conducted in 1988. Among households that own a pet, 45.3 million own at least one cat whereas 69 million own at least one dog. Additionally, pet ownership is not aggregated to a particular age group and spans generations (Baby boomers, Gen X, Millennials, etc.). In 2021, over $123 billion was spent on pets in the United States alone with pet food and treat sales accounting for $50 billion of this total expenditure (APPA, 2022). This was a substantial increase from the previous year of 2020 in which total pet expenditures were just under $104 billion. This large single-year increase was largely driven by the Covid-19 pandemic that boosted market sales with large increases in pet adoption and bulking purchasing of food (Mintel, 2021). Prior to this short-term market boost, single year increases in spending saw more modest, yet significant, increases from $90.5 billion in 2018 to $97.1 billion in 2019 to $103.6 billion in 2020 (APPA, 2022). Pet food sales specifically rose by about 20% from 2016 to 2021 and, while 2021 saw an unprecedented increase related to the pandemic, Mintel (2021) predicts this market will continue along a steady growth trajectory of about 16% between 2021 and 2026.

The pet food industry has steadily grown over the last several decades and has continuously segmented and splintered into an almost endless amount of options catering to different price points, product form, packaging, ingredient selection, lifestyles, life-stage, breed specific diets, and well as prescription diets (Aldrich, 2006). Pet owners are motivated by a

number of different factors when it comes to choosing a product for their animal, including the popular trends of the moment. For a number of years, there has been an increasing migration towards “humanization” in pet foods (Aldrich, 2006). Additionally, trends in the human food sector tend to appear in the pet food space in a short time period. One such topic has been the growing concern over sustainability. Within the human food market, consumer demand has pressured retailers and manufacturers to minimize waste streams and reduce or eliminate negative impacts on the environment (Mintel, 2021). Within the pet food market, about 3 out of 10 pet owners express the desire for increased sustainably in pet foods (Mintel, 2021). However, this interest is most highly concentrated in younger pet owners who inevitably will determine the direction of the pet food market in decades to come. With this in mind, sourcing of raw materials that satisfy name appeal, nutritional needs of the animal, functionality, availability, and economic considerations will continue to be a challenge for the pet food industry for the foreseeable future.

Extrusion Food Processing

Extrusion has long been utilized for industry applications, particularly for the manufacturing of rubber and plastics since the later part of the 19th century (Alavi et al., 2011). “Extrusion is commonly defined as the operation of forming and shaping a molten or dough-like material by forcing it through a restriction, or die” (Bouvier and Campanella, 2014). More recently, extrusion has been applied towards food production for humans, pets, and even livestock in some cases. The general extrusion process for food applications is the same as that of synthetic polymers. Raw material is fed into a barrel containing a screw that conveys, mixes, and cooks the material into a molten dough, often referred to as a melt, that is then formed under pressure by being forced through a small space called a die that then expands upon exit from the

extruder due to pressure differential with the atmosphere. Extrusion food processing is unique in its reliance on intense mechanical shearing to cook the food material. Depending upon the particular application, the raw material may be subjected to preconditioning prior to extrusion in which water, steam, or both are applied to the material and mixed for a given period of time before being discharged into the extruder barrel (Riaz, 2000). Additionally, there is often a subsequent drying process as well. Extrusion cooking is a high temperature, high pressure, and high mechanical shear process that can produce a wide variety of products ranging in shape, size, and density. Generally, starch gelatinization is necessary to generate melt formation, wherein with sufficient moisture and energy the starch granule swells, loses its crystalline structure, and forms a molten-like phase that binds all the raw materials into a homogeneous mass that will expand upon exit while also maintaining its structure (Guy, 2001; Riaz, 2000). Extrusion is a continuous, high throughput, and economical method of food production that utilizes low-cost starchy plant sources such as grains, legumes, and plant-based byproducts to produce value-added ready-to-eat dry expanded foods.

Extruded Pet Foods

In the human food industry, extrusion is commonly used for the for the production of ready-to-eat snack foods and breakfast cereals that are generally high in starch but low in protein. Depending upon the intended purpose, these products may or may not require a preconditioning step. Pet foods, on the other hand, are complete and balanced diets which must supply the entire nutritional requirement of the animal in a single product. Just like with human food, starch is hydrated under high shear, temperature, and pressure and acts as a thermoplastic polymer that binds the raw material into a plasticized uniform dough (Kallu et al., 2017; Monti et al., 2016). However, high protein pet foods require much greater water input (~25 to 30%) to properly

hydrate and cook both the starch and protein polymers within the food matrix (Baller et al., 2018). This requires a thorough preconditioning step where the raw material is softened with water and the thermal energy of steam begins the cooking process. Thus, the total energy input into the system is a combination of both specific mechanical energy (SME) and specific thermal energy (STE). While water is necessary to promote starch gelatinization and protein denaturation within the matrix, too much water will decrease dough viscosity and reduce mechanical energy between the screw and material in the barrel (Ding et al., 2005). Similarly, fat acts as a lubricant during the extrusion process which also reduces the mechanical energy input; therefore, fat is usually surface applied after drying. Lower mechanical energy input will in turn result in a less expanded, denser finished product.

Sensory characteristics and textural properties of the finished product must be carefully considered when developing a product. Quality characteristics of kibbles have been defined by a number of metrics including expansion ratio, bulk/piece density, texture analysis, and scanning electron micrograph to name a few (Alvarenga et al., 2018; Kallu et al., 2017; Monti et al., 2016). Diminished textural properties, particularly increased hardness, tend to have negative effects on sensory evaluation in human panels (Kallu et al., 2017; Liu et al., 2011; Monti et al., 2016). It is not currently clear whether these changes in textural properties have a negative impact on pets as well (Alvarenga et al., 2018; Monti et al., 2016). While processing conditions such as the feed rate of raw material into the extruder barrel, in-barrel moisture, screw design, and shaft speed all influence the quality of the finished product, the raw materials have a major impact as well.

Extruded Pet Foods Containing Fiber

Fiber is commonly included in pet food formulations due to the associated health benefits (Monti et al., 2016). Despite the nutritional benefit of fiber in the diet, the incorporation of these ingredients can present challenges to the formation of a final product (Alvarenga et al., 2018; Kallu et al., 2017; Karkle et al., 2012; Monti et al., 2016). When fiber levels are increased within the formula, textural properties can be negatively impacted by a decrease in the radial expansion which forms a denser, harder product (Alvarenga et al., 2018; Karkle et al., 2012; Monti et al., 2016). Decreases in radial expansion can be accompanied by increases in longitudinal expansion depending upon the chemical structure, particularly long unbranched filamentous fibers (Monti et al., 2016). Yanniotis et al. (2007) also reported that cellulose had a negative impact on expansion, but treatments containing pectin had in an increased extensibility of the kibble cell walls; thus, the type of fiber has substantial influence. The addition of fiber also requires greater energy to extrude and tends to decrease starch gelatinization (Monti et al., 2016). Some of these issues can be mitigated through the manipulation of ingredient particle size, starch type, and the amount of SME applied. Smaller fiber particle sizes exhibit greater expansion (Kallu et al., 2017; Monti et al., 2016) and greater starch gelatinization (Monti et al., 2016), compared to larger particle sizes. Increased screw speed, and subsequent increased SME, tend to result in greater expansion due to increased breakdown of the fiber structure. The ratio of amylose:amylopectin also has a big impact on expansion. Without the inclusion of fiber, a higher proportion of amylopectin leads to greater expansion. This is likely due the highly branched structure of amylopectin, whereas amylose is an unbranched linear polymer. Interestingly, Kallu et al. (2017) reported that when fiber is included in the formula, the greatest expansion was obtained with a 50:50 ratio of amylose to amylopectin. The authors postulated that a complex relationship exists

between fiber and starch structures during processing. More detailed information regarding this subject appears to be lacking.

Food Processing By-Products

For the last few decades, the use of fruits and vegetables and their by-products in extruded foods has become a growing area of research (Alavi et al., 2014; Karkle et al., 2012). In human foods, inclusions of these ingredients have been used to increase vitamins, minerals, antioxidants, and fiber in ready-to-eat extruded snack products (Alavi et al., 2011). For pet food, fruit and vegetable by-products represent potential alternatives to more costly traditional fiber ingredients such as crystalline cellulose and beet pulp. Thus, the use of fruit and vegetable by-products from the food processing industry is both an economical and sustainable option as this can add great value to by-products that would otherwise be discarded. Some fruit and vegetable fibers have pre-biotic functionality, whereas others are largely resistant to degradation (Swanson et al., 2001). Research regarding the biological functionality of many of these ingredients is still scarce, but potential remains for them to be effective ingredients in the formulation of both weight management diets and gut health diets for companion animals.

The residual biomass, termed pomace, remaining from the processing of fruit and vegetables into various consumer goods is a major by-product of the food industry (Ross et al., 2017). Fruit and vegetable pomaces are recognized as being good sources of dietary fiber and healthy bioactive substances including antioxidants and phenolic compounds (Cao et al., 2009; Kruczeketal., 2017; Van der Sluis et al., 2002; da Silva et al., 2020). In 2017, world apple production was approximately 81 million tons (Antonic et al., 2020). While the largest consumption of apples is in the form of fresh fruit, estimates range between 20 to 30% are processed into consumer goods such as juice, cider, wine/vermouth, jams, and various dried

products (Antonic et al., 2020; Shalini and Gupta, 2010). During the manufacturing of these products, 25 to 30% of total fresh apple mass is discarded as apple pomace (Bhushan et al., 2008), generating several million tons annually. Apple pomace is primarily composed of the skin and flesh from the apple, but also contains minor amounts of seeds and stems; however, the specific composition is dependent on the manufacturing process, the apple cultivar, and the year of harvest (Antonic et al., 2020). Apple pomace contains a good ratio of soluble to insoluble dietary fiber and is a major source of chemically isolated pectin (Kruczek et al., 2017; Morales-Contreras et al., 2020). For the berry processing industry, 20 to 30% of fresh mass remains as pomace after juicing. Juice production generally begins with fresh or frozen berries crushed, treated with pectinases, and heated prior to pressing and juice extraction (Struck et al., 2016). In blueberries, the residual mass primarily consists of the skin with minor amounts of seeds and stems. Cranberry pomace is the main by-product from cranberry juice and canning production (White et al., 2010). It is also composed primarily of skin, seeds, and stems left over after processing. Compositional differences exist depending upon the specific process.

In 2016, global tomato production was about 180 million metric tons (Lu et al., 2019). A large proportion of total tomato consumption comes in the form of various processed consumer goods including canned tomatoes, juices, sauces and tomato paste, among others (Lu et al., 2019). In the tomato processing industry, 5 to 30% of initial mass remains after processing as pomace by-product or is discarded as waste (Saini et al., 2018); thus, millions of tons of tomato pomace are generated annually. Tomato pomace can contain large portions of seeds and pulp as well as the skin (Poojary & Passamonti, 2015), but, similar to previous examples, the composition is largely dependent on the type of product being manufactured and the method of production. Due to the large range of consumer goods produced from tomato, processing

methods are diverse. As an example, peeling method and the use or absence of enzymatic treatment will greatly affect yield of a final product as well as the composition of residual pomace. Regardless of precise method of production, tomato pomace has large concentrations of dietary fiber characterized by the presence of both cellulose and pectin (Alancay et al., 2017; Lu et al., 2019). Additionally, tomato pomace can also have significant portions of protein depending on the abundance of seeds present (Lu et al., 2019).

Pea fiber is a primary by-product from the processing of whole peas into protein isolates and concentrates, pea starch, and pea flour. Pea fiber can be produced by both dry milling and wet extraction processes and the total fiber content can vary between 50 to 90% (Tulbek et al., 2017). Regardless of the intended finished product, pulse processing typically begins with the removal of the hulls (seed coat). The cotyledon is then milled and used as flour or for extraction of protein and/or starch. During pulse milling, hulls represent the main waste stream in production and are commonly used as fiber ingredients in animal feed. Pea hulls, in particular, are composed of up to 90% dietary fiber, of which most is insoluble (Dalgetty and Baik, 2003). The dehulling process usually results in product loss in the form of powdered and broken cotyledons, leading to lost starch and protein in the by-product stream (Patras et al., 2011). These by-products of pulse milling can account for 20 to 30% of the total unprocessed mass (Narasimha et al., 2004). Pea fiber can also be extracted from the cotyledon using wet fractionation. These contain larger proportions of soluble fiber (Tulbek et al., 2017). However, wet extraction techniques are more costly and have mainly been explored as functional ingredients in human food applications (Patras et al., 2011).

Utilizing the large quantity of by-products produced annually from fruit and vegetable processing is necessary to prevent their bioaccumulation in landfills. For fruit by-products in

particular, the residual mass after processing is high in moisture. This makes them highly susceptible to microbial degradation and acidic pH, which present environmental concerns when deposited in the soil or landfills (Vattem and Shetty, 2002). To reclaim these by-products necessitates an initial drying step, after which the material is usually subjected to milling, and the powder is then sieved to achieve the required particle size distribution. Apple pomace has been explored as fuel source in steam generation, enrichment of food products, pectin extraction, as well as biogas production (Shalini and Gupta, 2010). Similarly, berry pomace has been used food enrichment and biogas production (Tagliani et al., 2019; Struck et al., 2016). Tomato pomace has also been explored as a value-added food ingredient and for the extraction of carotenoids (Bhat et al., 2017; Saini et al., 2018).

Nutritional Benefits of Fiber

Over the history of human-canine co-evolution, the dog has changed from a carnivorous lifestyle with a diet high in protein and high in fat, to an urban lifestyle with a carbohydrate rich diet (Sabbioni et al., 2016). The increased intake of highly digestible carbohydrates may play a role in the prevalence of companion animal obesity seen today. In weight management diets, nutritionists often increase fiber content in the diet which can help promote satiety and dilute caloric value. When a substantial portion of a diet is supplemented with high-fiber foods, both total calories digested and glycemic index of the diet tend to be significantly reduced (Wolever, 2013). The American Association of Cereal Chemists has defined dietary fiber as the following: ‘‘Dietary fibre is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fibre includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fibres promote beneficial physiological effects including

laxation, and/ or blood cholesterol attenuation, and/or blood glucose attenuation'' (AACC, 2001). Similar to that of human digestion, dietary fiber in pet diets represent carbohydrate sources that escapes digestion and arrive intact in the large bowel. Fiber is intrinsic in many whole foods (fruits, vegetables, legumes, whole grains) and is widely recognized to have a diverse set of health benefits when consumed at appreciable levels. Dietary fiber sources are derived from the plant cell wall, where the main components are comprised of cellulose, hemicellulose, lignin and pectin among others. The physiological benefits associated with dietary fiber can differ greatly from one source to another and is determined by specific physico-chemical properties of the source (Wahlqvist, 2016).

Fiber Categorizations

Fiber is usually classified as either being insoluble or soluble (Williams et al., 2019). However, this is an over-simplification and does not address the different biological impacts of fiber sources within the same category of solubility. Today, it is generally recognized that fiber can fall into four broad categories: 1) Insoluble, non-fermented; 2) Soluble, non-viscous, readily fermented; 3) Soluble, viscous, readily fermented; and 4) Soluble, viscous, non-fermented (McRorie, 2015a). These classifications are based around three characteristics: solubility, viscosity, and fermentablity. Solubility describes whether a fiber source will dissolve in water (soluble) or remain as discrete solid particles (insoluble) (Dikeman and Fahey, 2006). Viscosity is the impediment to flow and is caused by a soluble fibers ability to absorb water, forming a gel-like substance. Fermentablity refers to the ability of a fiber to be fermented in the large bowel by colonic bacteria into various metabolic by-products, some of which have important implications to mammalian health (Morrison and Preston, 2016).

These different chemical properties give rise to the diverse set of health impacts including glycemic control, cholesterol lowering, depressed appetite, improvements in gastro-intestinal health, and have even been associated with lowering the risk for certain cancers (Alexander et al., 2019; Velázquez et al., 1996). Notable for the prevalence of obesity, gel-forming fibers greatly increase the viscosity of chyme in the small intestine, slowing the digestion of complex nutrients and absorption of glucose, leading to a reduced glycemic index (Wolever, 2013). More relevant to human health, viscous gel-forming soluble fibers have noticeable ability for lowering cholesterol by trapping bile acids from being reabsorbed in the small intestine (McRorie, 2015a). The trapping of bile acids and prevention from reentering the enterohepatic recirculation forces the body to replenish lost bile acids from serum stores of cholesterol, lowering overall blood cholesterol. Fibers that are fermented by the gut microbiota primarily result in the production of short chain fatty acids (SCFA); these metabolic by-products have a complex interaction between host, diet, and gut microbiome that seem to play an important role in long-term colonic health, metabolic control, and inflammatory status (Velazquez et al., 1996; Morrison and Preston, 2016).

Insoluble and non-fermentable soluble fibers are either mostly or completely resistant to microbial degradation as they pass through the digestive tract. Insoluble and non-fermentable soluble fibers can aid in stool forming/hardening in the case of diarrhea, act as a laxation agent in constipation, as well as a calorie diluent since there is no harvesting of caloric energy from the substrate (McRorie, 2015a; McRorie, 2015b).

Colonic Fermentation

Dietary factors, such as increased fiber intake, can drive changes in the composition of gut microbiota. The gastro-intestinal tract (GIT) of the dog represents a rich ecosystem,

composed of a wide range of metabolically active microorganisms (Swanson et al., 2011; Suchodolski et al., 2008; Kerr et al., 2013). The microbiota of the GIT acts as a metabolic organ that can break down food components that escape mammalian digestion. This can result in the synthesis of certain vitamins, and amino acids, as well as postbiotic SCFAs (den Besten et al., 2013). The diet is a major determinant of the colonic microbiome, however other factors including genetic makeup of the host and colonic environment also influence the composition of the microbiota in the large bowel (den Besten et al., 2013). In turn, the microbiota itself influences the colonic environment. Taken together, these complex interactions between host, microbial population, and diet result in a high degree of variation in microbiota composition among individuals of the same species. Even though there is great diversity in microbiome makeup, many of the biochemical pathways are conserved throughout the bacterial kingdom (Abubucker et al., 2012; den Besten et al., 2013).

Prebiotics are food ingredients that are selected to promote the growth and activity of specific species of bacteria in the GIT, usually of the genera Bifidobacterium and Lactobacillus (Cummings et al., 2001). The consumption of prebiotics, such as dietary fiber, promote a network of metabolic interactions among the bacterial species of the microbiota. This results in indirect growth of certain microbes via the utilization of metabolic end-products produced by other species in a process termed “cross-feeding” (Holscher et al., 2017). Thus, dietary modulation will result in metabolic consequences that may not be predicted by the interaction of a single isolated species with a substrate.

The SCFA acetate, propionate, and butyrate are the major post-biotics produced from microbial fermentation of otherwise indigestible plant polysaccharides in the colon (Cummings et al., 2001; Miller and Wolin, 1996). The production of SCFA can provide significant

nutritional and health benefits to the animal. Acetate is absorbed into many body tissues including heart, adipose tissue, kidney, and muscle where it is metabolized as an energy source (Den Besten et al., 2013). The majority of acetate is taken up by the liver (Bloemen et al., 2009), where it can be used as an energy source as well as a substrate for cholesterol, long-chain fatty acids, glutamine, and glutamate synthesis. Propionate acts as a precursor to gluconeogenesis in the liver (Miller and Wolin, 1996). Butyrate is the most biologically significant of all the SCFA. Butyrate is preferentially utilized by enterocytes as a source of energy, serves as a regulator of cellular proliferation and differentiation, and has been shown to have beneficial effects on colonic disease (Alexander et al., 2019; Velazquez et al., 1996). Increases in *bifidobacteria* and *lactobacilli* populations are associated with increased butyrate concentrations. However, *bifidobacteria* and *lactobacilli* are lactic acid bacteria, which produce lactate and acetate as their major fermentation end products (De Vuyst et al., 2011; Holscher et al., 2017; Moens et al., 2017). Lactate and acetate produced by these bacteria are utilized by a variety of other species of bacteria in the colon as a substrate for the production of butyrate, emphasizing the role cross-feeding has on the overall colonic environment.

The major fermentation pathways of SCFA production are the Embden-Meyerhof-Parnas pathway (glycolysis, for six-carbon sugars) and the pentose-phosphate pathway (for five-carbon sugars), which convert monosaccharides into pyruvate and phosphoenolpyruvate (PEP), respectively (den Besten et al., 2013; Miller and Wolin, 1996). Subsequently, PEP is converted into pyruvate or other organic acids and further converted into SCFA. A major part of pyruvate is converted to acetyl-CoA with the parallel formation of H2 and CO2. Acetate is either formed by hydrolysis of acetyl-CoA or from CO2 via the Wood-Ljungdahl pathway (den Besten et al., 2013). Propionate can be formed from PEP through the succinate de-carboxylation pathway or

through the acrylate pathway in which lactate is reduced to propionate (den Besten et al., 2013; Miller and Wolin, 1996). Lactate-utilizing bacteria start the formation of butyrate by first producing acetyl-CoA from lactate. Butyrate is formed by the condensation of two molecules of acetyl-CoA with the subsequent reduction to butyryl-CoA followed by the conversion to butyrate by the enzyme butyrate kinase (den Besten et al., 2013; Duncan et al., 2004).

In vitro modeling

In the late 19th century, researchers began to recognize that microorganisms of the rumen and large intestine of herbivorous animals fermented plant polysaccharides into various volatile compounds (Bergman, 1990). Several decades later the metabolic role of fermentation started to be understood, specifically in regard to ruminant nutrition (Barcroft et al., 1944; Elsden et al., 1946). Since then, extensive research has been devoted to the metabolism of forages and the production of volatile fatty acids in the rumen as these represent the major source of energy in forestomach fermenters. Accordingly, early models and techniques of fermentation were primarily focused on ruminant and other herbivore species. Tilley and Terry (1963) developed one of the first *in vitro* techniques to estimate the fermentation of forages in ruminants, which began to be used and modified by several subsequent researchers (Goering and Van Soest, 1970; Grant et al., 1974). It would be a few decades later that these methods would be modified and adapted to the human model (Ehle et al., 1982; McBurney et al., 1985). In a series of publications, Sunvold et al. (1995a, 1995b, 1995c, 1995d) would later be the first to pioneer the use of *in vitro* fermentation models with companion animals.

Since the conception of the *in vitro* fermentation model, many researchers have applied these techniques across a range of species including ruminants (Garleb et al., 1988; Sunvold et al., 1995d), swine (Awati et al., 2006; Sappok et al., 2013; Sunvold et al., 1995d), human

(Casterline et al., 1997; Flickinger et al., 2002; Kaur et al., 2011), as well as companion animals (Bednar et al., 2001; de Godoy et al., 2015; Faber et al., 2011). These methods primarily emphasize the degree of substrate degradation and the production of volatile organic acids, particularly regarding the concentration and relative proportions of SCFA. Substrate degradation is typically quantified as dry or organic matter disappearance during incubation, calculated by substrate recovery after fermentation (Bourquin et al., 1992, 1993, 1996; Donadelli et al., 2019; Sunvold et al., 1995a, 1995b, 1995c, 1995d; Swanson et al., 2001). However, substrate degradation has also been expressed by a variety of different metrics, including water-holding capacity (Adiotomre et al., 1990; Bourquin et al., 1996; McBurney et al., 1985), gas production (Spears et al., 2007; Bosch et al., 2008; Swanson et al., 2001), as well as the disappearance of non-starch polysaccharides (Barry et al., 1995), soluble and insoluble substrate components (Titgemeyer et al., 1991), and individual monosaccharides (Bourquin et al., 1993; Lebet et al., 1998; Titgemeyer et al., 1991). Additionally, Donadelli et al. (2019) calculated estimated organic matter disappearance as an attempt to predict substrate degradation based upon the total concentration of SCFA present after fermentation.

Protein ingredients in pet foods

Animal by-products have long been an essential source of protein ingredients for the pet food industry. These have mostly come in the form of animal protein meals including meat and bone meal, chicken meal, lamb meal, poultry meal, poultry by-product meal, and fish meal among others (Aldrich, 2006). These animal-derived protein meals are a function of the rendering process which reclaims the remaining tissues not utilized in human foods (Meeker and Hamilton, 2006). Meat and bone meal has traditionally been a major source of animal protein in pet foods (Aldrich, 2006). However, within the last few decades the use of this ingredient has

fallen out of favor compared to other rendered animal proteins. Improved efficiency during the slaughtering of animal meat has led to higher ash concentrations which limit its use as a primary protein source in pet food formulations. Today, pet food producers have shifted towards the use of poultry-derived protein meals, such as chicken meal, as they contain large amounts of essential nutrients for dogs and cats, are well digested, and have good acceptance (Aldrich, 2006). Utilization of rendered protein meals is a necessary role that the pet food industry plays in the global sustainability effort. Without the use of value-added rendered ingredients, these food processing by-products that are unfit for human consumption would otherwise be discarded in landfills and would pose serious risk to the environmental as well human and animal health (Meeker and Hamilton, 2006). However, issues still remain regarding this use of these products. Not the least of which being the wide compositional variation seen within the same types of rendered animal proteins (Yamka et al., 2003).

Pea protein ingredients generally come in two major forms, isolates or concentrates (>80% and 50-75% protein, respectively (Boukid et al., 2021). The protein extraction from raw pea seeds for the preparation of these ingredients are traditionally achieved through wet extraction and dry fractionation methods, respectively. Prior to either method, the hulls are removed from whole pea seeds and milled for protein extraction from ground cotyledons. For isolate production, peas are subjected to alkaline extraction, isoelectric precipitation, and final drying; whereas concentrate production relies merely on air classification for the fractionation of protein and starch material (Boukid et al. 2021). While the air classification process is a chemical free and cost-effective method, it results in a lower protein content and contains larger proportions of starch, fiber, and lipids. Defatted soybean meal is a primary by-product of soybean oil processing that is widely used as a cost-effective protein source in animal diets

(Luján-Rhenals et al., 2014). The process involves dehulling of raw soybeans, solvent extraction of oil, and heat treatment. Generally, there are two kinds of soybean meal that are produced: a high protein variety (~49% protein) that is complete after heat treatment and a lower protein variety (~44% protein) that has the hulls added back, effectively diluting protein content with larger portions of non-starch polysaccharides (Karr-Lilienthal et al., 2005).

Single cell proteins

Microbial derived proteins are alternative ingredients which are gaining attention in both the human and animal food sectors. Microorganisms including bacteria, algae, fungi, and yeasts have been successfully utilized for protein production and are generally termed single cell protein (SCP) (Anupama and Ravindra, 2000; Boze et al., 1992; Kuhad et al., 1997). Microorganism have been utilized for a millennia in the preparation of fermented foods, bread, and alcoholic beverages. These processes take advantage of the unique metabolic capabilities of microbes and usually rely on the fermentation of a starting material, such as sugars, into post-biotic compounds including lactic acid, CO2, and ethanol for use in food products. The idea of SCP is different in that the cellular biomass of a particular species is inactivated (often thermally or enzymatically lysed) and dried prior to being directly fed to humans or animals (Bekatorou et al., 2006; Øverland and Skrede, 2016). Microbial SCP can result as a co-product from other primary cultivation purposes such as the production of alcohol, biofuels, bioactive food ingredients, lipids, as well as microbial enzymes and metabolites (Belloch et al., 2011; Kurtzman and Robnett, 2003; Øverland and Skrede, 2016). However, many SCP are purposefully cultivated solely to increase total microbial biomass to serve as a food product. Purpose grown SCP is a more recent concept and has not been as extensively researched as other food grade products derived from microorganisms; however, this is not a new concept. In the modern era, it

was reported that during the first world war the German people cultured and harvested SCP as other protein sources became scarce (Buerth et al., 2016; Sousa-Silva et al., 2021)

Nutritional yeasts

Nutritional yeasts are those directly fed as an inactive, dry biomass for the primary purpose of nutritional value. Yeasts are the only group of unicellular microorganisms within the fungi kingdom with characteristic growth patterns of budding or fission (Buerth et al., 2016; Kutty and Philip, 2008). Unlike bacteria, Yeasts are eukaryotic microorganisms meaning they have membrane-bound organelles similar to plant and animal cells. However, unlike plants, yeast do not contain chloroplasts and are considered heterotrophic species that must obtain their energy and nutrients from other sources of organic material (Shurson, 2018). The use of microorganisms for the production of protein offers some unique advantages over that of plant and animal origin. Single cell proteins have high protein content that is produced in a short amount of time and is not limited by environmental conditions (Bekatorou et al., 2006). Yeasts, in particular, have wide flexibility for growth substrates and can be cultivated using inexpensive materials. Additionally, yeast derived SCP contain lower amounts of nucleic acids compared to those produced from bacteria (Anupama and Ravindra, 2000; Bekatorou et al., 2006).

Saccharomyces cerevisiae is the most commonly used food grade yeast and perhaps one of the most well studied microorganisms overall. *S. cerevisiae* is also known as baker's yeast or brewer's yeast and has long been used for the production of bread and baking products as well as alcoholic beverages. Many other species are also utilized for similar roles such as those used in sourdough breads belonging to the *Wickerhamomyces*, *Candida*, and *Torulaspora* genus (Rández-Gil et al., 2014) as well as the more closely related species from the same *Saccharomyces* genus (*S. bayanus, S. cariocanus, S. kudriavzevii, S. mikatae, S. paradoxus*, etc.)

utilized for the production of various alcoholic products (Kurtzman and Robnett, 2003). While many species are used through the food and brewing industry, *S. cerevisiae* is a model organism for these types of roles. S. cerevisiae is highly efficient in the fermentation of hexose sugar into ethanol and is commonly grown on cane and beet molasses substrates; however, wild-type strains are unable to assimilate pentose sugars (Øverland and Skrede, 2016). During the brewing process, *S. cerevisiae* can increase in biomass which is seen as a co-product (similar to spent brewer's grain) to ethanol fermentation (Ferreira et al., 2010). Spent brewer's yeast is the inactive dry biomass remaining after the brewing process which has no leavening ability and has found little use outside of an inexpensive supplementation to animal feed. It should be emphasized that spent brewer's yeast is a co-product of the brewing industry and is distinct from purpose grown nutritional yeasts that are cultivated specifically as a source of protein (Ferreira et al., 2010). Additionally, biomass yields during ethanol fermentation are usually minor in comparison or may even be completely absent. *S. cerevisiae* can also be purpose grown as a nutritional yeast, wherein the primary goal is to increase total biomass. However, for metabolic reasons described below this can be an inefficient species for this purpose.

Kluyveromyces marxianus is a closely related species to S. cerevisiae and is a sister-species to the better studied *Kluyveromyces lactis* (Lane and Morrissey, 2010). *K. marxianus* and *K. lactis* are the only lactose-fermenting species found in dairy products and are sometimes referred to as dairy yeast or whey yeasts (Belloch et al., 2011). The ability to efficiently metabolize lactose as a sole source of carbon under anaerobic conditions is a unique feature of these *Kluyveromyces* species and is critical to the development of cheeses and other fermented dairy products. While *K. lactis* has been more widely researched, *K. marxianus* has been more widely favored in industry for biotechnical applications due to rapid growth rates and

thermostability (Bekatorou et al., 2006; Lane and Morrissey, 2010). *K. marxianus* can also be used for SCP production. Unlike *S. cerevisiae, K. marxianus* has the ability to metabolize both hexose and pentose sugars and has a wide spectrum of substrates that it can be cultivated on (Øverland and Skrede, 2016); additionally, it is the only commercially produced nutritional yeast grown on whey (Bekatorou et al., 2006). For reasons discussed in the next section, *K. marxianus* also has its own set of drawbacks related to biomass production.

The species *Candida utilis*, commonly referred to as Torula yeast, has been utilized commercially as a food and fodder yeast for over 75 years. The common name "Torula" is derived from the original species designation of *Torulopsis utilis* in 1934. The nomenclature of this particular species has changed many times since its discovery and has been summarized by Barnet (2004) in a section titled "The tortuous history of *Candida utilis*." *Cyberlindnera jadinii* is another name used today which represents the teleomorph (or sexual) state of the same species (Sousa-Silva et al., 2021). *C. utilis* is the name of the anamorphic (asexual) form and is still the most popular in common parlance. Based on the most current system of nomenclature, the teleomorph state is the primary name of a species (*C. jadinii* in this case), but the anamorphic name is also valid (Sousa-Silva et al., 2021). This is just one example of the confusing and complex nature of yeast nomenclature and is indicative of the expansive number and genetic diversity of yeasts. *S. cerevisiae, K. marxianus,* and *C. utilis* have all obtained generally recognized as safe (GRAS) status by the US Food and Drug Administration (Boze et al., 1992; Øverland and Skrede, 2016; Sousa-Silva et al., 2021), which means they are approved from food grade use and are not known to be hazardous to health. It should be noted that *C. utilis* (or *C. jadinii*) is phylogenetically distant from the species *Candida albicans* which is a significant

fungal pathogen in human health that causes dangerous infections (Buerth et al. 2016; Sousa-Silva et al., 2021).

Torula yeast has been favored due to its flexible utilization of carbon sources and robust growth patterns (Bekatorou et al., 2006, Buerth et al., 2016). Similar to *K. marxianus, C. utilis* also has the ability to metabolize both hexose and pentose sugars (Øverland and Skrede, 2016). Torula yeasts have been cultivated on a wide range of substrates including beet pulp (Athar et al., 2009), distillery waste (Hosken et al., 2015; García et al., 2014), pineapple effluent (Nigam, 1998), and wheat bran (Yunus et al., 2015) among others. Torula yeast has the ability to metabolize xylose and xylose oligomers (Yanai and Sato, 2001), allowing for growth on lignocellulosic waste materials from the forestry and agriculture industries (Øverland and Skrede, 2016). Thus, representing sustainable production of a high-value protein ingredient from low-value, cost-effective raw materials. Furthermore, protein production from yeasts grown on woody by-products has a lower carbon footprint in its life-cycle compared to soybean meal production and does not compete for resources with the human food system (Øverland and Skrede, 2016; Spiller et al, 2020).

Yeast protein production

Carbon metabolism is perhaps the main consideration for biomass growth of yeasts. All yeasts can metabolize glucose as an energy source, but the ability to utilize other carbon sources differs greatly between species and directly influences what substrates can be used as a growth medium (Boze et al., 1992). Additionally, the mode of metabolism varies between species which has critical implications to the efficacy of biomass production. Many yeast species are facultative anaerobes, meaning they can survive under both aerobic (presence of oxygen) and anaerobic (absence of oxygen) conditions (Bekatorou et al., 2006). Generally, in the presence of oxygen

these species will metabolize sugar molecules via aerobic respiration. During respiration, sugar molecules are fully oxidized leading to production of CO2 and an abundance of the high energy molecule adenosine triphosphate (ATP) which allows for the accumulation of cell biomass (Bekatorou et al., 2006; Hagman et al., 2014). In the absence of oxygen, these species can only survive via fermentation. Compared to respiration, fermentation is an inefficient mode of energy generation which produces a small amount of ATP, CO2, and ethanol as a metabolic end product (Bekatorou et al., 2006; Steensels et al., 2014). Because of the severe reduction in energy generated, fermentation results in little to no increase to cell biomass. Both respiration and fermentation begin with glycolysis which oxidizes a sugar molecule into to pyruvate (Compagno et al., 2014). This generates a small amount of ATP which is only possible through the reduction of an electron carrying molecule. During respiration, pyruvate enters the mitochondria where it feeds into the krebs (or citric acid) cycle where a series of enzymatically catalyzed reduction and oxidation reactions take place resulting in more high-energy phosphate compounds, via substrate-level phosphorylation, as well as the reduction of more electron carrier molecules. The reduced electron carriers will feed these electrons into the electron transport chain, which requires oxygen as the final electron acceptor, to further produce a substantial amount of ATP via oxidative phosphorylation (Compagno et al., 2014). When oxygen is not present, these mechanisms are not able to function, thus fermentation occurs. During fermentation, pyruvate formed during glycolysis is reduced to ethanol (or lactate depending on the organism) through an intermediate step (Compagno et al., 2014). This is necessary in order to oxidize the electron carrying molecule needed to continue glycolysis for further energy production needed for survival.

As stated previously, *S. cerevisiae* is widely used in the production of baked good and alcoholic beverages. While these two examples are very different consumer goods, they both utilize the same metabolic pathway for their production. As already alluded to, yeast generate ethanol for alcoholic products via fermentation of sugars during the brewing process. In baking products, yeast acts as a leavening agent in the dough, where CO2 produced during fermentation causes the bread to rise and ethanol is evaporated off (Rández-Gil et al., 2014). *S. cerevisiae* rapidly ferments hexose sugars into ethanol and CO2 under anaerobic conditions. While this species is capable of aerobic respiration, *S. cerevisiae* will produce ethanol aerobically when glucose is present; a phenomenon known as the Crabtree effect (Hagman et al., 2014; Rández-Gil et al., 2014). Crabtree-positive yeasts will favor a fermentative metabolism when glucose is accessible even in the presence of oxygen, thus biomass production is severely hampered by this tendency (Rández-Gil et al., 2014; Steensels et al., 2014).

K. marxianus is a facultative anaerobe and has a mixed model of fermentation and respiratory metabolisms. Under strictly anaerobic conditions, *K. marxianus* can ferment a variety of substrates, including lactose, into ethanol (Belloch et al., 2011). The fermentation of lactose into ethanol is relatively rare among yeast species - a useful application in the dairy industry (Fonseca et al., 2008). As would be expected, *K. marxianus* is unable to increase biomass under strictly anaerobic conditions. *K. marxianus* is a Crabtree-negative species, meaning that the presence of glucose does not favor fermentation during aerobic conditions, thus the formation of ethanol is exclusively linked to the presence of oxygen (Fonseca et al., 2008). However, this species is capable of concurrent fermentation and respiratory metabolism and can produce both ethanol and biomass simultaneously (Lane and Morrissey, 2010; Øverland and Skrede, 2016).

There is an obvious trade-off between two competing modes of metabolism and when the production of biomass is the goal, this can be a limitation.

Unlike the previous two species, *C. utilis* is a strictly aerobic species and is incapable of producing ethanol (Øverland and Skrede, 2016). Therefore, *C. utilis* exclusively uses a respiratory metabolism to efficiently produce energy from its growth substrates which is used to increase biomass. When selecting a yeast species for the production of biomass, it is imperative to select a species with strong respiratory ability. Thus, a fermentatively dominate species, such as one that is Crabtree-positive, would not be the most suitable for this purpose. Additionally, Crabtree-negative species including *K. marxianus* may not be the most ideal. While Crabtree-negative yeasts do not have a glucose inhibiting effect on aerobic respiration, they can display mixed metabolisms that may compete for substrates.

Research objectives

Considering the substantial size of the pet food market and the strong year-over-year growth, increased efforts to improve the sustainability of the pet food industry will be critical. Sustainability can involve many aspects including the optimization of energy, water, and land usage. The pet food industry, in particular, has a unique position in the food system as it largely relies on by-products or co-products from the human food systems for ingredient sourcing. Additionally, novel ingredients that are produced using waste products from other industries can be valuable ingredients with lower environmental impacts. Thus, ingredient selection in pet foods represents a key area for improving overall sustainability.

The first objective of this work was to evaluate the fermentation characteristics of several alternative fiber ingredients using an *in vitro* canine fecal inoculum model. These fibers are by-products derived from the fruit and vegetable processing industry and could potentially be

sustainably sourced ingredients with important health benefits to companion animals. The second objective was to evaluate the use of a yeast biomass as a protein source in feline diets. In this study, this yeast protein was evaluated for diet processing behavior, palatability, and apparent digestibility. This is a novel protein source cultivated using woody by-products from the timber industry and has not been previously fed to felines. Thus, they could potentially represent a sustainably produced high-quality protein ingredient for companion animal diets.

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Chapter 2 - Substrate degradation and post-biotic analysis of alternative fiber ingredients fermented using an *in vitro* canine fecal inoculum model

Abstract

Many fiber ingredients are used within the pet food industry; however, little data is available regarding the fermentation characteristics of alternative fibers currently being used. The objectives of this study were to determine organic matter disappearance (OMD), estimated organic matter disappearance (EOMD), and postbiotic production of various fruit and vegetable fiber sources using an *in vitro* dog fecal inoculum model. Apple pomace (AP), blueberry pomace (BP), cranberry pomace (CP), tomato pomace (TP), and pea fiber (PF) were used as experimental treatments. Inoculum was prepared using newly voided feces under anaerobic conditions. Predigested fibers were inoculated and incubated for 1, 3, 6, and 12 h at 39ºC. Short-chain fatty acids (SCFA), branched-chain fatty acids (BCFA), total volatile fatty acids (VFA), OMD, and EOMD were determined for each fiber source and time point in triplicate. The OMD was similar ($P>0.05$; average of 18.5%) between treatments and no effect of time ($P>0.05$) was observed. Proportionally, acetate was greater for BP and AP ($P<0.05$; average of 80.1%) than the other treatments (68.3 to 71.2%). Propionate proportions was greatest ($P<0.05$) for CP (26.8%) compared to the remaining treatments (13.6 to 20.7%). Butyrate was proportionally greater for PF (7.7%; $P<0.05$) than BP and CP (average of 4.8%) and was lowest for AP (3.8%); however, TP was not different from PF ($P>0.05$; average of 7.25%). Total VFA concentration was highest for AP ($P<0.05$) followed by TP (1.17 and 0.75 mmol*g^{-1} of substrate, respectively). Both BP and PF were similar (average of 0.48 mmol*g^{-1} of substrate) and lower than TP, with CP having the lowest

VFA concentration (0.21 mmol*g^{-1} of substrate) among all treatments. Additionally, when comparing absolute concentrations, AP and TP (average of 0.0476mmol*g^{-1} of substrate) had greater butyrate concentrations than PF (0.0344mmol*g^{-1} of substrate). The treatments AP, BP, and TP had linear relationships with time ($P<0.05$) for total VFA and butyrate concentrations, whereas CP and PF only showed quadratic relationships ($P<0.05$). Both total (VFA) concentrations and EOMD increased with time ($P<0.05$) and were highly correlated with one another ($r^2=0.9998$). Overall, the fiber substrates evaluated were marginally to moderately fermentable when incubated for up to 12 h with canine fecal inoculum.

Keywords: companion animals, fermentation, fruit, pomace, short-chain fatty acids, volatile fatty acids

Introduction

Dietary fiber is a broad term that encompasses any carbohydrate substrate that escapes digestion in the small intestine and arrives intact within the large bowel. Fiber is commonly included in pet food formulations due to the associated health benefits (Monti et al., 2016). Dietary fibers are usually classified as either being insoluble or soluble (Williams et al., 2019). However, this binomial classification is an oversimplification and is not necessarily predictive of impact in the colon. Solubility describes whether a fiber source will be dissolved by the digestive system secretions (soluble) or remain as discrete solid particles in the chyme (insoluble) (Dikeman and Fahey, 2006). Fibers that are fermented by the gut microbiota primarily result in the production of short chain fatty acids (SCFA); these postbiotic compounds have a complex interaction between host, diet, and gut microbiome that seem to play an important role in long-term colonic health, metabolic control, and inflammatory status (Velázquez, 1996; Morrison and Preston, 2016). Fibers that are not degraded by the microbiota and make it through the digestive tract intact aid in stool formation and act as a calorie diluent.

For the last few decades, the use of fruit and vegetable by-products in extruded foods has become a growing area of research (Alavi et al., 2014; Karkle et al., 2012). The residual biomass resulting from the processing of fruit and vegetables into consumer goods, termed pomace, is a major by-product of the food industry (Ross et al., 2017). Further, the large quantity of these by-products produced across the globe annually present challenges to prevent bioaccumulation in landfills. Pet food companies are constantly searching for novel ingredients to use in place of traditional fiber sources. Fruit and vegetable by-products represent cost-effective, sustainable ingredient alternatives to purified wood cellulose and beet pulp. With the exception of Swanson et al. (2001), data regarding fruit and vegetable fibers fermentation using a canine model is extremely

limited. Thus, there is need to characterize the fermentation patterns of a wider array fiber ingredients. It was hypothesized here that fruit pomaces derived from apple, blueberry, and cranberry would contain the most fermentable fiber, tomato fiber would be intermediate, and pea fiber would be the least fermentable. The objective of this study was to investigate substrate degradation and postbiotic production of several fibers that are currently used in the pet diets, but have limited published data, using an *in vitro* canine inoculum model.

Materials and Methods

Fiber sources

Apple pomace (AP), blueberry pomace (BP), cranberry pomace (CP), tomato pomace (TP), and pea fiber (PF) were sourced from a local mill (Lortscher Animal Nutrition, Inc., Bern, Ks 66408) and used as experimental treatments. These fiber sources were selected based on their prevalence in diet formulations within the pet food industry but limited reported data on potential benefit to gut health for companion animals. Prior to the *in vitro* protocols, fiber treatments were analyzed for dry matter (DM; AOAC 930.15), organic matter (AOAC 942.05), crude protein (AOAC 990.03), insoluble dietary fiber and total dietary fiber (TDF; AOAC 991.43; TDF kit, K-TDFR-200A, Megazyme Ltd., Bray, Ireland) according to AOAC international approved analytical methodologies. Additionally, soluble fiber concentrations were calculated by the difference between TDF and insoluble fiber content. Crude protein of the residual samples remaining after the digestion procedure was also analyzed for the estimation of protein concentration entering fermentation.

Media and dilution solution preparation

The medium composition and the anaerobic dilution solution were prepared according to Donadelli et al. (2019) and are detailed in Table 1. All components were autoclaved with the

exception of heat-liable vitamins which were filtered-sterilized and added to medium solution afterwards.

Substrate preparation

To prepare treatments for inoculation, fiber sources underwent an *in vitro* digestion procedure to simulate small intestinal digestion adapted from the enzymatic total dietary fiber procedure described by Donadelli et al. (2019). Residual samples remaining after enzymatic digestion were ground through a 1mm screen in a fixed blade laboratory mill (Retsch, type ZM200, Haan, Germany). For each treatment, 300±0.1 mg of predigested and ground substrate was placed into a 50 mL polypropylene centrifuge tube (Falcon brand conical centrifuge tubes; Corning, Inc., Corning, NY) in triplicate. Additionally, 3 blank tubes were used during each time point for correction of background postbiotic concentrations. All tubes were added with 26 mL of media solution while purged with CO_2 to maintain anaerobic conditions. Tubes were sealed with rubber stoppers equipped with one-way values and allowed to hydrated overnight at 4 ºC.

Dog donors and inoculum preparation

Beagle dogs were used as donors for the preparation of fecal inoculum in this study. Dogs (n=12, neutered/spayed, average age 5.4±0.3 years old, average weight 12.7±1.5 kg) were group-housed in indoor kennels located at the Large Animal Research Center of Kansas State University in Manhattan, KS. Dogs were fed a commercial maintenance diet twice a day (Table 2) with constant access to fresh water. Fecal inoculum was prepared as described by Donadelli et al. (2019). Briefly, newly voided fecal samples were collected from two dogs (one male and one female) within 5 min of defecation, sealed in a sterile polyethylene bag (Whirl-Pak; Nasco sampling, Madison, WI), and stabilized at 39 ºC until inoculum preparation. Fecal inoculum was produced by combining 50 g of sample from each donor and diluting 1:10 (w/v) with anaerobic

dilution solution (100 g of feces: 900 mL of dilution solution). Inoculum was blended and filtered through 4 layers of cheese cloth under constant CO_2 purge. The filtrate was then retained in 1 L screw cap Pyrex bottle (Corning, Inc., Corning, NY) at 39 °C until incubation.

Fiber Incubation and OMD determination

Prior to fecal collection, tubes containing treatments and media were placed in a water bath at 39 ºC for at least 1 h to equilibrate temperature to 39 ºC prior to inoculation. All treatments were incubated at four timepoints: 1, 3, 6, and 12 h. Tubes were inoculated with 4 mL of fecal inoculum starting from the longest time point (12 h) and ending with the earliest time point (1 h). During inoculation, tubes remained under CO_2 purge until resealed with one-way valves. After each incubation time, duplicate 1 mL aliquots of clear supernatant were collected, acidified with 0.25 mL of 25% m-phosphoric acid, centrifuged at 25,000 x G for 20 min, and frozen at -20 °C. The remaining sample contents of each tube were transferred to a beaker and precipitated by diluting with 112 mL of 95% ethanol overnight. The precipitated samples were later filtered under vacuum using ashless paper filter (Whatman 541 ashless paper filter; catalog no. 1541-110) and 78% ethanol. When all sample volume had been filtered, residue was rinsed with two 10 mL volumes of 95% ethanol followed by two 10 mL volumes of acetone. After filtration, residues and filter paper were dried in a convection oven overnight at 105 °C. Dry residue weight was recorded the following day and placed in muffle furnace at 450 °C overnight for determination of inorganic material. Organic matter was calculated by difference and organic matter disappearance (OMD) was calculated using the following equation:

Equation 2.1. Calculation of organic matter disappearance.

$$\text{OMD (\%)} = \left[1 - \left(\frac{\text{OM residue - OM blank}}{\text{Initial OM}}\right)\right] \times 100$$

wherein, OM residue is the organic matter remaining in sample after fermentation and filtration in g, OM blank is the organic matter remaining in blank after fermentation and filtration in g, and initial OM is the organic matter of the raw sample in g. Additionally, EOMD was calculated using short-chain fatty acid (SCFA) concentration according to Donadelli et al. (2019). Briefly, each molecule of acetate, propionate, and butyrate were assumed to be produced exclusively from anhydrous glucose (molecular weight of 162 g/mol). A single glucose molecule was assumed to yield either 2 acetates, 2 propionates, or a single butyrate molecule. The mass of glucose needed to produce the concentrations of SCFA was calculated as follows:

Equation 2.2. Estimated mass of glucose.

$$\text{Glucose} = \left(\frac{\text{acetate}}{2} + \frac{\text{propionate}}{2} + \text{butyrate}\right) \times 162$$

wherein, glucose is the mass of glucose in g, and acetate, propionate, and butyrate are the molar concentrations of each respective SCFA in each tube after incubation. Estimated organic matter disappearance was calculated using the following equation:

Equation 2.3. Calculation of estimated organic matter disappearance.

$$\text{EOMD (\%)} = \left[1 - \left(\frac{\text{Glucose}}{\text{Initial OM}}\right)\right] \times 100$$

wherein, glucose is the mass of glucose in g and initial OM is the organic matter of the raw sample in g.

Postbiotics determination

The duplicate supernatant subsamples removed from each tube at the end of each time point were used for postbiotic analysis. Supernatants were extracted from GC vials and placed into a gas chromatography (GC) system (7890A GC System, Agilent Technologies, Santa Clara, CA) via direct liquid injection using a 10:1 split ratio with injection volume of 1 µL. The GC system used helium as a carrier gas with a flow rate of 3.5 mL/min and the volatile organic compounds

were separated using a capillary GC column (15 m x 0.35 mm internal diameter, 0.5 µm film thickness; Nukol™ column, Sulpeco, Bellefonte, PA). A flame ionization detector was used for the determination of volatile compounds and was configured with nitrogen as the makeup gas with a flow rate of 25 mL/min to clarify peak resolution. Peak area of chromatograms were determined using integrative software (Agilent OpenLAB CDS version A.01.04, Agilent Technologies, Santa Clara, CA). Short chain fatty acids (acetate, propionate, butyrate, and valerate) and branched-chain fatty acids (isobutyrate and isovalerate) were quantified by comparing the sample peak area to a known standard of 10 mM concentration (Volatile Free Acid Mix, Sigma-Aldrich, St. Louis, MO). The mean value of the duplicate subsamples was used as concentration for each tube and concentrations were reported as mM^{-1} g of substrate on a dry matter basis. For the purpose of this work, acetate, proportionate, butyrate, and valerate were collectively referred to as SCFA. The volatile iso-acids, isobutyrate and isovalerate, were referred to as branch chain fatty acids (BCFA). Total VFA refers to the sum of them all.

Statistical Analysis

Data were analyzed as a complete randomized design with triplicate centrifuge tubes as experimental units (4 time points * 5 fiber sources * 3 replicates = 60 experimental units). Treatment least square means within each timepoint for OMD, EOMD, and volatile fatty acids were compared using the general linear model (GLM) procedure from statistical analysis software (SAS; V. 9.4, Cary, NC, USA). Pairwise treatment comparisons were conducted using Fisher's Least Significance Difference to minimize type I error. Linear and quadratic trends across time within treatments were evaluated for butyrate and total volatile fatty acid (VFA) concentrations using single degree of freedom orthogonal polynomial contrasts. Additionally, overall trends for all treatments combined of OMD, EOMD, and total VFA concentrations over time were evaluated

using linear and quadratic contrasts. Orthogonal polynomial contrasts were used due to unequally spaced timepoints, and contrast coefficients were estimated using the interactive matrix language (IML) procedure in SAS. Pearson correlation coefficients between total VFA concentrations and OMD and EOMD were estimated using the CORR procedure in SAS (v 9.4). For all statistical analyses, differences were considered significant at $P < 0.05$.

Results

The nutrient composition of experimental fiber treatments is presented in Table 3. Treatment fibers had a large range in crude protein content (8.17 to 24.20%) with TP having the largest concentration, doubling that of the other treatments (average 11.91%). When evaluating TDF, TP was comparably lower (52.67%) than the other treatments (average 63.52%). Cranberry pomace had the largest TDF content (68.31%) but was almost completely insoluble (98.68% of TDF). As a proportion of total fiber content, AP had a much greater amount of soluble fiber (17.25% of TDF) compared to the other treatments (1.32 to 7.61% of TDF).

Prior to the *in vitro* fermentation procedure, fiber treatments were defatted using hexane as an organic solvent followed by an enzymatic digestion and filtration. Expectations here would be that fat, starch, and soluble sugars present in the initial samples would be removed and not contribute to fermentation. Most protein would be removed as well, however some portion remains bound to fiber and is quantified for the residual substrate. Similar to initial samples, TP had the highest concentration of protein (21.37%) in residue samples, again roughly double that of the other treatments (average 10.83%).

After 12 h of incubation, there was no treatment effect ($P>0.05$) on OMD (Table 4; average 18.53%), but EOMD was greatest for AP ($P<0.05$). On a proportional basis, acetate comprised the highest percentage of total VFA concentration of each treatment across all time points (Table 4;

68.34 to 83.58%). After 1 h of incubation, all treatments had similar proportions of acetate ($P>0.05$; average 78.37%); however, by the end of incubation (12 h), AP and BP had larger proportions of acetate ($P<0.05$; average 80.07%) than the other treatments. Acetate proportions of AP and BP remained relatively constant as total VFA pool increased across time, whereas CP (78.15 to 68.34%), PF (75.79 to 70.39%) and TP (81.18 to 71.22%) tended to decrease throughout incubation. Propionate was the second most abundant VFA at all treatment and time point combinations (13.64 to 26.78%) and differed ($P<0.05$) between all treatments after 12 h of incubation. At the end of incubation, CP had the largest proportion of propionate ($P<0.05$) and tended to increase across time (16.86 to 26.78% from 1 to 12 h, respectively). Proportionally, butyrate made small (-0.31 to 7.69%) contributions to the total VFA pool for each treatment and time point. After 12 h of incubation, PF had greater butyrate proportions ($P<0.05$; 7.69%) than AP, BP, and CP but was similar ($P>0.05$) to that of TP (average 7.23%). With the exception of TP, BCFA contributed marginally (-0.87 to 1.25%) to the total VFA pool.

Results for absolute concentrations presented here will be exclusively focused on the 12 h time point. Total VFA concentrations were greatest (Table 5; $P<0.05$) for AP (1.1647 $mmol*g^{-1}$ of substrate) followed by TP (0.7530 $mmol*g^{-1}$ of substrate), BP and CP (average 0.4804 $mmol*g^{-1}$ of substrate), and lowest for CP (0.2049 $mmol*g^{-1}$ of substrate). Butyrate concentrations were greater ($P<0.05$) for AP and TP (average of 0.0476 $mmol*g^{-1}$ of substrate), followed by PF (0.0344 $mmol*g^{-1}$ of substrate), BP (0.0249 $mmol*g^{-1}$ of substrate), and lowest for CP (0.0093 $mmol*g^{-1}$ of substrate). Branch chain fatty acid concentrations were much greater for TP ($P<0.05$; 0.0240 $mmol*g^{-1}$) compared with the remaining treatments (ranging from -0.0101 to 0.0057 $mmol*g^{-1}$).

The AP, BP, and TP treatments had linear and quadratic relationships (Table 6; $P<0.05$) for both butyrate and VFA concentrations across time, whereas CP and PF only had quadratic

relationships for these two variables (P<0.05). No relationships were observed for OMD across incubation times (Table 7; P>0.05); however, EOMD and VFA both had linear and quadratic relationships with time (P<0.05). Correlations were found between VFA vs OMD (P<0.05) and VFA vs EOMD (P<0.05). However, only VFA vs EOMD showed a high correlation coefficient (0.9998), whereas VFA vs OMD showed a weak correlation (0.5289).

Discussion

The nutrient composition of treatment fibers was evaluated to provide context to fermentation patterns. Apple pomace has been well studied and its nutrient composition was within values reported throughout the literature (Fekete et al., 2001; Grigelmo-Miguel and Martin-Belloso, 1999; Swanson et al., 2001). Less work has been published on berry processing by-products such as blueberry and cranberry pomace. The protein content of BP was greater than previously reported (6.64 to 9.00%; Hotchkiss et al, 2021; Ross et al, 2017; Crizel et al., 2016; Tagliani et al., 2019) and fiber composition ranges widely within the reported literature (26.2 to 60.8%; Crizel et al., 2016; Hotchkiss et al., 2021; Šarić et al., 2018; Tagliani et al., 2019). The protein content of CP was also greater than previous reports (2.2 to 8.2%; Park and Zhao, 2006; Spadoni Andreani and Karboune, 2020; Ross et al., 2017; White et al., 2010) but TDF was between that previously reported by Spadoni Andreani et al. (2020) and White et al. (2010) evaluating cranberry pomace by-products (63.0 and 71.2%, respectively). Protein was greater for PF than that reported by Bosch et al., (2008) and Titgemeyer et al. (1991) evaluating pea fiber ingredients (5.5 and 4.9%, respectively) but was similar to that reported by Swanson et al. (2001) for pea hull (16.2%). The fiber content of PF was lower than previous reports (69.7 to 89.7%; Donadelli et al., 2019; Swanson et al., 2001; Titgemeyer et al., 1991). The protein content of TP was on the upper limits of previous reports (16 to 25%; Fuentes et al., 2013; Savadkoohi and Farahnaky, 2012; Shao

et al., 2013; Del Valle et al., 2006) but fiber was lower than other work (Shao et al., 2013; Swanson et al., 2001; Fahey et al., 1990).

Colonic transit times of beagle dogs has been reported between 12.2 and 14.8 h (De Cuyper et al., 2017). It is possible that the addition of various fiber sources could impact retention in the large bowel; however, previous results have been conflicting (Burros et al., 1982; Fahey et al., 1990) and it is reasonable to assume that a transit time of at least 12 h to be appropriate for modeling. In the present study, substrate degradation was quantified by the disappearance of organic matter after each incubation time point; however, after 12 h of incubation, there was no differences between treatments due to large variation within treatment replicates. Throughout incubation, the OMD values of TP remained relatively constant, similar to that reported previously by Swanson et al. (2001) for tomato pomace incubated between 0 and 12 h (24.4 to 26.5%, respectively); however, the values here were slightly lower. Due to the relative consistency in OMD values, Swanson et al. (2001) considered TP to be poorly fermented at 12 h and only after 24 h did these authors report an increase in OMD up to 35.0%. For the remaining treatment substrates evaluated here, OMD values across time didn't show a reliable consistency or increase. Treatment substrates exhibited modest fermentation during 12 h of incubation in terms of organic acid concentrations and likely only experienced a minor degree of substrate degradation during inoculation. Small fluctuations across time were likely error associated with fecal inoculum. Estimated organic matter disappearance was much lower than that of OMD values for each treatment and time point combination. Unlike OMD, EOMD was greater for substrates with larger concentrations of volatile organic acids and was observed to have both linear and quadratic effects with time. This is not surprising, considering EOMD is derived from SCFA concentrations.

Considering the variability OMD values obtained here, EOMD may offer an alternative for estimating fiber degradation during fermentation.

Among the postbiotic compounds produced from colonic fermentation, acetate, propionate, and butyrate usually comprise greater than 95% of molar concentrations (Bergman, 1990; den Besten et al., 2013). In most mammals, acetate is the most predominate SCFA and usually present in greater concentrations than all other organic acids combined; however, butyrate is commonly recognized as the most important organic acid produced during fermentation, particularity for the health of the colonic epithelium. Acetate is transported into circulation where it is taken up and metabolized as an energy source by many peripheral organs (den Besten et al., 2013), whereas propionate is primarily absorbed by the liver and serves as a precursor for gluconeogenesis (Miller and Wolin, 1996). Almost all of the butyrate formed in the colon is absorbed by the colonocytes where it is preferentially utilized as an energy source, serves as a regulator of cellular proliferation and differentiation, and has been shown to have beneficial effects on colonic disease (Alexander et al., 2019; Velázquez et al., 1996).

Apple fibers are known to contain significant portions of pectin (Guillon et al., 1995; Lebet et al., 1998) and several authors have previously reported that the fermentation of pectin structures greatly increases the production of acetate (Adiotomre et al., 1990; Barry et al., 1995; Titgemeyer et al., 1991). The relatively large portion (~17% of total fiber concentration) of soluble fiber of AP likely consisted mainly of pectic substances and would generally be expected to be susceptible to fermentation. Indeed, this was evident by the greatest concentration of VFA during incubation. The blueberry cell wall also consists of small proportions of soluble pectin as well as insoluble cellulose and hemicellulose materials (Hotchkiss et al., 2021; Lin et al., 2019). While BP exhibited fermentation patterns characteristic of pectin substrates, such as an extremely high

acetate:propionate ratio (Adiotomre et al., 1990; Bourquin et al., 1996; Sunvold et al., 1995a; Titgemeyer et al., 1991), the total concentration of VFA produced during incubation was very modest, which is consistent with the small fraction of soluble fiber. For the remaining treatment substrates, while still comprising the greatest contribution to the VFA pool, acetate proportionally declined as incubation time progressed.

While the total VFA concentrations of CP appeared to plateau after only 3 h of incubation, propionate concentrations continued to increase. Majeed et al. (2018) fermented cranberry seed fiber *in vitro* using isolated probiotic species *Bacillus coagulans* and found that after 6, 12, and 24 h of incubation, propionate concentrations far exceeded that of acetate and butyrate. The greater proportions of propionate are likely a function of the unique polysaccharide structure of the cranberry cell wall. Previous reports have identified relatively abundant amounts of arabinose and galactose within its structure (Fan et al., 2010; Spadoni Andreani et al., 2021; Spadoni Andreani and Karboune, 2020). Human fermentation studies have shown that the degradation of pentose sugars including arabinose and xylose have led to greater concentrations of propionate *in vitro* (Mortensen et al., 1988; Salvador et al., 1993). In the current experiment, the greater proportion of propionate coupled with the lower proportion of acetate across incubation times may indicate a lower degradation of pectin.

Generally, PF had the largest proportion of butyrate during incubation; however, at 12 h PF was not different from TP. Butyrate proportions were greater that than that previously reported for pea fiber (4.2%; Donadelli et al., 2019) and pea hulls (2.9%; Swanson et al., 2001); however, the molar concentration of butyrate was relatively similar to these groups (0.02824 and 0.0300 $mmol*g^{-1}$ of substrate, respectively). While the molar proportion of butyrate from PF was large compared to other sources, AP and TP produced greater absolute concentrations of butyrate. Pea

fibers can contain large amounts of insoluble cellulose (Guillon et al., 1995; Lebet el al., 1998), which is largely resistant to degradation in the colon of canines (Donadelli et al., 2019; Sunvold et al., 1995b; Swanson et al., 2001) and may explain the lower overall VFA production.

The total VFA concentration of TP was about 65% that of AP with most of the difference represented by acetate. Tomato pomace had similar concentrations of butyrate to that of AP, but proportionally butyrate made up a larger contribution to the total VFA pool. Therefore, TP contributed both greater concentrations and relative proportions of butyrate compared to other treatment fibers. Perhaps the most striking feature of TP fermentation is the relatively large concentration of BCFA. The presence of BFCA is a direct indicator of protein fermentation, whereby isobutyrate and isovalerate are formed by the metabolism of the branched-chain amino acids valine and leucine, respectively (Macfarlane et al., 1992). Protein fermentation results in a complex mixture of metabolites including SCFA, BCFA, ammonia, biogenic amines, phenolic, and indolic compounds to name a few. While only SCFA and BCFA were quantified, many of these other compounds are toxic to the colonocytes and have been associated with gut health impairment such as inflammation, reduced epithelial barrier function, and even tumor-promotion (Gilbet et al., 2018; Hughes et al., 2008; Trefflich et al., 2021).

While the postbiotic analysis showed good consistency between treatment replicates, the OMD values had large variation. Incomplete homogenization of fecal material during inoculum preparation was likely a source of error. Differences in fermentation patterns were likely a function of the monosaccharides present in the cell walls and the unique physical arrangement of polysaccharide structure in treatments. Unfortunately, monosaccharide composition was not measured in the current study. Future work should further characterize the composition and disappearance of monosaccharides in these and other fiber sources to help elucidate the

mechanisms driving differences in postbiotic compounds which could have beneficial impacts in the diets of companion animals.

Conclusion

In the current study, there were no effects of treatment or time on OMD. Unlike OMD, EOMD was greater for substrates with larger concentrations of volatile organic acids and increased over time. Pea fiber generally had greater proportions of butyrate but was not different from TP after 12 h. Total VFA concentrations were greatest for AP, followed by TP, BP and PF, and was lowest for CP. Tomato pomace had similar concentrations of butyrate to that of AP, but proportionally butyrate made up a larger contribution to the total VFA pool.

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Tables

Table 2.1. Composition of the inoculum medium and anaerobic dilution solution.

Component	Medium	Anaerobic dilution solution
Autoclaved		
Solution A[1], mL	330.0	-
Solution B[2], mL	330.0	-
Trace mineral solution[3], mL	10.0	-
Resazurin solution[4], mL	1.0	1.0
Yeast extract, g	0.5	-
Trypticase, g	0.5	-
Sodium carbonate monohydrate, g	4.0	-
Distilled water, mL	296.0	854.0
Cysteine hydrochloride monohydrate, g	0.5	0.5
Mineral solution #1[a], mL	-	37.5
Mineral solution #2[b], mL	-	37.5
Sodium bicarbonate solution[c], mL	-	70.0
Added after autoclave		
Vitamin premix solution[5], mL	20.0	-
Folate: Biotin solution[6], mL	5.0	-
Riboflavin solution[7], mL	5.0	-
Hemin solution[8], mL	2.5	-

[1]Solution A. 5.4 g sodium chloride, 5.4 g ammonium sulfate, 2.7 g potassium phosphate monobasic anhydrous, 0.18 g calcium chloride dihydrate, 0.12 g magnesium chloride hexahydrate, 0.06 g manganese chloride tetrahydrate, 0.06 g cobalt chloride hexahydrate. (Dilute to 1 L of distilled water).
[2]Solution B. 2.7 g potassium phosphate dibasic anhydrous. (Dilute to 1 L of distilled water).
[3]Trace mineral solution. 500 mg of EDTA, 200 mg iron (II) sulfate heptahydrate, 30 mg m-phosphoric acid, 20 mg cobalt chloride hexahydrate, 10 mg zinc sulfate heptahydrate, 3 mg manganese chloride tetrahydrate, 3 mg sodium molybdate dihydrate, 2 mg nickel (II) chloride hexahydrate, 1 mg copper (II) chloride dihydrate. (Dilute to 1 L of distilled water).
[4]Resazurin solution. 100 mg resazurin. (Dilute to 100 mL of distilled water).
[5]Vitamin premix solution. 100 mg thiamin hydrochloride, 100 mg pantothenic acid, 100 mg niacin, 100 mg pyridoxine hydrochloride, 10 mg ammonium carbonate, 5 mg ρ-aminobenzoic acid, 0.25 mg vitamin B-12. (Dilute to 1 L of distilled water).
[6]Folate: Biotin solution - 100 mg ammonium carbonate, 10 mg folic acid, 2 mg biotin. (Dilute to 1 L of distilled water).
[7]Riboflavin solution - 130 mg HEPES, 1 mg riboflavin. (Dilute to 1 L of distilled water).
[8]Hemin Solution - 50 mg hemin, 40 mg sodium hydroxide. (Dilute to 100 mL of distilled water).
[a]Mineral solution #1. 3 g potassium phosphate dibasic anhydrous, 1 g sodium citrate dehydrate. (Dilute to 500 mL of distilled water).
[b]Mineral solution #2. 10 g sodium citrate dehydrate, 6 g sodium chloride, 6 g ammonium sulfate, 3 g potassium dihydrogen phosphate anhydrous, 1.23 g magnesium sulfate heptahydrate, 0.6 g calcium chloride dehydrate. (Dilute to 500 mL of distilled water).
[c]Sodium bicarbonate solution. 91 g sodium bicarbonate. (Dilute to 1 L of distilled water)

Table 2.2. Nutrient composition (DM-basis) of diet fed to inoculum donors.

Nutrients	Diet composition
Crude protein, %	27.5
Fat (acid-hydrolyzed), %	17.8
Fiber (crude), %	2.5
Neutral detergent fiber, %	8.3
Acid detergent fiber, %	3.0
Nitrogen free extract (difference), %	37.4
Gross energy, kcal/kg	4710
Digestible energy, kcal/kg	3900
Ash, %	6.6

Ingredients: poultry by-product meal, ground yellow corn, ground rice, corn gluten meal, poultry fat persevered with ethoxyquin, meat meal, ground wheat, beet pulp, animal digest, brewers dried yeast, animal fat preserved with BHA, dried whole egg, dried whey, soybean oil, calcium carbonate, L-lysine, potassium chloride, salt, pyridoxine hydrochloride, choline chloride, DL-methionine, menadione dimethylpyrimidinol bisulfate (source of vitamin K), cholecalciferol, biotin, lecithin, vitamin A acetate, DL-alpha tocopheryl acetate, inositol, ethoxyquin (a preservative), sodium selenite, calcium pantothenate, thiamin mononitrate, riboflavin, nicotinic acid, cyanocobalamin, L-tryptophan, folic acid, ferrous sulfate, manganous oxide, zinc oxide, copper sulfate, calcium iodate, cobalt carbonate

Table 2.3. Nutrient composition of treatment fiber sources.

Composition, %	Fiber sources				
	Apple pomace	Blueberry pomace	Cranberry pomace	Pea fiber	Tomato pomace
Dry matter	91.31	91.44	94.78	90.88	91.89
			Dry matter basis		
Organic matter	97.89	98.69	98.01	97.21	95.87
Crude protein[1]	8.17 (9.40)	14.97 (13.84)	10.15 (10.96)	14.35 (9.11)	24.20 (21.37)
Total dietary fiber	63.43	62.83	68.31	59.50	52.67
Insoluble fiber[2]	52.49 (82.75)	58.91 (93.76)	67.41 (98.68)	54.97 (92.39)	50.02 (94.97)
Soluble fiber[3]	10.94 (17.25)	3.92 (6.24)	0.90 (1.32)	4.53 (7.61)	2.65 (5.03)

[1]Numbers in parenthesis represent crude protein concentration of residue after enzymatic digestion

[2,3]Numbers in parenthesis represent percentage of total fiber concentration

Table 2.4. OMD, EOMD, SCFA, and BCFA expressed as a percent of their total for treatment fibers incubated for 1, 3, 6, and 12h.

Fermentation time, h	Fiber sources					SEM	P-value
	Apple	Blueberry	Cranberry	Pea	Tomato		
OMD, %							
1	23.84^{a}	15.70^{abc}	14.85^{bc}	11.28^{c}	20.63^{ab}	1.776	0.0040
3	22.62^{a}	11.98^{b}	11.83^{b}	8.20^{b}	21.05^{a}	2.052	0.0007
6	31.92^{a}	17.30^{b}	18.33^{b}	14.69^{b}	20.79^{b}	2.519	0.0051
12	24.66	16.06	18.35	13.64	19.95	2.902	0.1605
EOMD, %							
1	1.11^{b}	0.87^{cd}	0.84^{d}	1.10^{cb}	1.36^{a}	0.0506	0.0002
3	6.07^{a}	1.97^{c}	1.81^{c}	1.94^{c}	3.50^{b}	0.1847	<0.0001
6	8.66^{a}	3.01^{c}	1.46^{d}	2.44^{cd}	5.01^{b}	0.2509	<0.0001
12	10.09^{a}	4.33^{c}	1.76^{d}	4.00^{c}	6.59^{b}	0.2261	<0.0001
Acetate, %							
1	78.88	79.51	78.15	75.79	79.54	0.933	0.0873
3	83.58^{a}	81.68^{b}	72.64^{c}	73.15^{c}	81.18^{b}	0.344	<0.0001
6	80.91^{a}	77.02^{b}	69.11^{d}	68.17^{d}	73.81^{c}	0.385	<0.0001
12	79.86^{a}	80.27^{a}	68.34^{c}	70.37^{bc}	71.22^{b}	0.607	<0.0001
Propionate, %							
1	18.40^{a}	13.91^{c}	16.86^{b}	19.40^{a}	16.51^{b}	0.310	<0.0001
3	15.42^{c}	14.21^{cd}	22.90^{a}	19.58^{b}	13.90^{d}	0.321	<0.0001
6	16.10^{cd}	15.45^{d}	25.15^{a}	21.27^{b}	16.49^{c}	0.166	<0.0001
12	17.22^{d}	13.64^{e}	26.78^{a}	20.68^{b}	18.82^{c}	0.284	<0.0001
Butyrate, %							
1	-0.31^{b}	2.30^{a}	3.66^{a}	3.76^{a}	2.81^{a}	0.457	0.0005
3	2.22^{c}	4.50^{b}	4.19^{b}	7.42^{a}	4.46^{b}	0.165	<0.0001
6	3.56^{c}	5.60^{b}	4.12^{c}	7.50^{a}	6.03^{b}	0.210	<0.0001
12	3.79^{c}	4.89^{bc}	4.68^{bc}	7.69^{a}	6.77^{ab}	0.507	0.0015
Valerate, %							
1	2.79^{a}	3.055^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.420	0.0004
3	0	0	0	0	0	0	-
6	0	0	0	0	0	0	-
12	0	0	0	0	0	0	-
SCFA, %							
1	99.76^{a}	98.77^{bc}	98.66^{c}	98.95^{b}	98.86^{bc}	0.0567	<0.0001
3	101.22^{a}	100.39^{ab}	99.72^{b}	100.14^{b}	99.54^{b}	0.1898	0.0007
6	100.57^{a}	98.06^{bc}	98.38^{b}	96.93^{cd}	96.33^{d}	0.2728	<0.0001
12	100.87^{a}	98.80^{ab}	99.80^{a}	98.75^{ab}	96.81^{b}	0.5981	0.0084
Isobutyrate, %							
1	0	0	0	0	0	0	-
3	-0.26^{b}	-0.01^{ab}	0.15^{a}	0.05^{ab}	0.29^{a}	0.072	0.0034
6	0.05^{c}	0.61^{b}	0.66^{b}	1.25^{a}	1.41^{a}	0.119	<0.0001
12	-0.07^{a}	0.78^{ab}	0.40^{ab}	0.66^{ab}	1.37^{a}	0.269	0.0386
Isovalerate, %							
1	0.24^{c}	1.23^{ab}	1.34^{a}	1.05^{b}	1.14^{ab}	0.0567	<0.0001
3	-0.97^{b}	-0.38^{a}	0.13^{a}	-0.20^{a}	0.17^{a}	0.122	0.0004
6	-0.62^{d}	1.32^{bc}	0.96^{c}	1.82^{ab}	2.26^{a}	0.164	<0.0001
12	-0.80^{b}	0.42^{ab}	-0.20^{b}	0.59^{ab}	1.82^{a}	0.332	0.0027
BCFA, %							
1	0.24^{c}	1.23^{ab}	1.34^{a}	1.05^{b}	1.14^{ab}	0.0567	<0.0001
3	-1.22^{e}	-0.39^{d}	0.28^{bc}	-0.14^{cd}	0.46^{ab}	0.1898	0.0007
6	-0.57^{d}	1.94^{bc}	1.62^{c}	3.07^{ab}	3.67^{a}	0.2728	<0.0001

12	-0.87^{b}	1.20^{ab}	0.20^{b}	1.25^{ab}	3.18^{a}	0.5981	0.0084

Table 2.5. Short-chain, branched-chain, and total fatty acid concentrations of treatment fibers incubated for 1, 3, 6, and 12h.

Fermentation time, h	Fiber sources					SEM	P-value
	Apple	Blueberry	Cranberry	Pea	Tomato		
Acetate, mM^{-1}g							
1	0.1100^{ab}	0.0864^{c}	0.0780^{c}	0.0970^{bc}	0.1256^{a}	0.0047	0.0002
3	0.5927^{a}	0.1865^{c}	0.1530^{c}	0.1581^{c}	0.3236^{b}	0.0161	<0.0001
6	0.8130^{a}	0.2723^{c}	0.1194^{d}	0.1907^{cd}	0.4278^{b}	0.0227	<0.0001
12	0.9302^{a}	0.4086^{c}	0.1399^{d}	0.3182^{c}	0.5363^{b}	0.0217	<0.0001
Propionate, mM^{-1}g							
1	0.0256^{a}	0.0152^{b}	0.0168^{b}	0.0248^{a}	0.0261^{a}	0.00110	<0.0001
3	0.1094^{a}	0.0325^{c}	0.0478^{bc}	0.0423^{bc}	0.0554^{b}	0.00360	<0.0001
6	0.1617^{a}	0.0546^{c}	0.0434^{c}	0.0595^{c}	0.0955^{b}	0.00456	<0.0001
12	0.2005^{a}	0.0695^{d}	0.0546^{d}	0.0933^{c}	0.1417^{b}	0.00476	<0.0001
Butyrate, mM^{-1}g							
1	-0.0005^{b}	0.0025^{ab}	0.0037^{a}	0.0048^{a}	0.0045^{a}	0.00070	0.0019
3	0.0158^{ab}	0.0103^{bc}	0.0088^{c}	0.0161^{ab}	0.0178^{a}	0.00141	0.0040
6	0.0358^{a}	0.0198^{b}	0.0072^{c}	0.0212^{b}	0.0349^{a}	0.00180	<0.0001
12	0.0441^{a}	0.0249^{c}	0.0093^{d}	0.0344^{b}	0.0510^{a}	0.00174	<0.0001
Valerate, mM^{-1}g							
1	0.0038^{a}	0.0033^{a}	0^{b}	0^{b}	0^{b}	0.00051	0.0003
3	0	0	0	0	0	0	-
6	0	0	0	0	0	0	-
12	0	0	0	0	0	0	-
SCFA, mM^{-1}g							
1	0.1390^{ab}	0.1074^{cd}	0.0984^{d}	0.1266^{bc}	0.1562^{a}	0.0057	0.0002
3	0.7178^{a}	0.2292^{c}	0.2096^{c}	0.2165^{c}	0.3968^{b}	0.0210	<0.0001
6	1.0104^{a}	0.3467^{c}	0.1700^{d}	0.2713^{cd}	0.5582^{b}	0.0287	<0.0001
12	1.1749^{a}	0.5030^{c}	0.2038^{d}	0.4458^{c}	0.7290^{b}	0.0268	<0.0001
Isobutyrate, mM^{-1}g							
1	0	0	0	0	0	0	-
3	-0.0018^{c}	0.0000^{b}	0.0003^{b}	0.0002^{b}	0.0012^{a}	0.00017	<0.0001
6	0.0005^{c}	0.0022^{bc}	0.0012^{c}	0.0035^{b}	0.0082^{a}	0.00038	<0.0001
12	-0.0008^{d}	0.0040^{b}	0.0012^{cd}	0.0030^{bc}	0.0103^{a}	0.00059	<0.0001
Isovalerate, mM^{-1}g							
1	0.0003^{b}	0.0013^{a}	0.0013^{a}	0.0013^{a}	0.0018^{a}	0.00013	0.0001
3	-0.0068^{c}	-0.0008^{b}	0.00032^{ab}	-0.0003^{ab}	0.0007^{a}	0.00028	<0.0001
6	-0.0062^{d}	0.0047^{b}	0.0017^{c}	0.0052^{b}	0.0131^{a}	0.00062	<0.0001
12	-0.0093^{c}	0.0022^{b}	0.0000^{b}	0.0027^{b}	0.0137^{a}	0.00070	<0.0001
BCFA, mM^{-1}g							
1	0.0003^{b}	0.0013^{a}	0.0013^{a}	0.0013^{a}	0.0018^{a}	0.00013	0.0001
3	-0.0087^{c}	-0.0008^{b}	0.0007^{ab}	-0.0002^{b}	0.0018^{a}	0.00043	<0.0001
6	-0.0057^{d}	0.0068^{bc}	0.0028^{c}	0.0087^{b}	0.0213^{a}	0.00098	<0.0001
12	-0.0101^{c}	0.0062^{b}	0.0012^{b}	0.0057^{b}	0.0240^{a}	0.00126	<0.0001
Total, mM^{-1}g							
1	0.1393^{ab}	0.1087^{cd}	0.0998^{d}	0.1279^{bc}	0.1581^{a}	0.00581	0.0002
3	0.7092^{a}	0.2284^{c}	0.2103^{c}	0.2164^{c}	0.3986^{b}	0.02125	<0.0001
6	1.0048^{a}	0.3535^{c}	0.1728^{d}	0.2800^{cd}	0.5795^{b}	0.02921	<0.0001
12	1.1647^{a}	0.5092^{c}	0.2049^{d}	0.4515^{c}	0.7530^{b}	0.02748	<0.0001

Table 2.6. Linear and quadratic relationships of butyrate and total VFA within treatments.

Treatment	Variable	Time points				Contrasts	
		1	3	6	12	Linear	Quadratic
Apple pomace							
	Butyrate	-0.0005	0.0158	0.0358	0.0441	<0.0001	0.0018
	Total VFA	0.1393	0.7092	1.0048	1.1647	<0.0001	<0.0001
Blueberry pomace							
	Butyrate	0.0025	0.0103	0.0198	0.0249	0.0002	0.0107
	Total VFA	0.1087	0.2284	0.3535	0.5092	0.0024	0.0030
Cranberry pomace							
	Butyrate	0.0037	0.0088	0.0072	0.0093	0.1589	0.0033
	Total VFA	0.0998	0.2103	0.1728	0.2049	0.1525	0.0067
Pea fiber							
	Butyrate	0.0048	0.0161	0.0212	0.0344	0.0920	0.0099
	Total VFA	0.1279	0.2164	0.2800	0.4515	0.2306	0.0087
Tomato pomace							
	Butyrate	0.0045	0.0178	0.0349	0.0510	<0.0001	<0.0001
	Total VFA	0.1581	0.3986	0.5795	0.7530	<0.0001	<0.0001

Table 2.7. Linear and quadratic relationships of VFA, OMD, and EOMD and Pearson correlations between them.

Variables	Time points				Contrasts		Pearson Correlation vs VFA	
	1	3	6	12	Linear	Quadratic	P-value	R^2
VFA, $mM^{-1}g$	0.1268	0.3526	0.4781	0.6167	0.0332	0.0477	-	-
OMD, %	17.26	15.37	19.80	18.53	0.3463	0.1995	<0.0001	0.5289
EOMD, %	1.06	3.06	4.12	5.35	0.0329	0.0405	<0.0001	0.9998

Chapter 3 - Evaluation of Torula yeast as a protein source in extruded feline diets

Abstract

The objective of this work was to evaluate the use of a Torula yeast on diet processing, palatability, and total tract nutrient digestibility in extruded feline diets. Four dietary treatments were compared, differing by protein source: Torula yeast (TY), pea protein concentrate (PP), soybean meal (SM), and chicken meal (CM). Diets were produced using a single-screw extruder under similar processing conditions. Palatability assessment was conducted as a split plate design where both first choice and intake ratio (IR) were determined. Apparent total tract digestibility (ATTD) of nutrients was estimated using Titanium dioxide as an indigestible marker. During diet production, specific mechanical energy (SME) of TY and SM (average of 187 kJ/kg) was greater ($P<0.05$) than for PP (138 kJ/kg); however, CM was similar to all treatments (167 kJ/kg). Kibble diameter, piece volume, and sectional expansion ratio were greatest for TY ($P<0.05$). Additionally, both bulk and piece density were lowest ($P<0.05$) for TY. Kibble hardness was lower for TY and SM ($P<0.05$; average of 2.10 Newtons) compared to CM and PP (average of 2.90 Newtons). During the palatability trial, TY was chosen first a greater number of times than CM ($P<0.05$; 36 vs 4, respectively), but differences were not found between TY and PP (25 vs 15, respectively) or TY and SM (24 vs 16, respectively). TY was preferred to CM and PP, having an IR greater ($P<0.05$) than 0.67 (0.88 and 0.73, respectively). However, TY was not found to be preferred over SM. ATTD of dry matter (DM) and organic matter (OM) was greater ($P<0.05$) for CM (87.43 and 91.34%, respectively) than other treatments. Both DM and OM ATTD of TY were similar ($P<0.05$) to PP and SM (average of 86.20 and average of 89.76%, respectively). Ash ATTD was greater ($P<0.05$) for cats fed TY and SM (average of 37.42%), intermediate for PP (32.79%), and

lowest for CM (23.97%). Crude protein (CP) ATTD of TY was similar to all other treatments (average of 89.97%), but fat ATTD was lower ($P<0.05$; 92.52%) than other treatments (93.76 to 94.82%). Gross energy (GE) ATTD was greater ($P<0.05$) for CM than TY (90.97 vs 90.18%, respectively); however, TY was similar to PP and SM (average of 90.22%). Total dietary fiber (TDF) ATTD was similar between TY and CM (average of 66.20%) and greater ($P<0.05$) than PP and SM (average of 58.70%). In summary, the Torula yeast used in this study facilitated diet processing, increased diet preference, and was highly digestible when fed to cats.

Keywords: *Candida utilis*, cat, companion animal, extrusion, palatability, novel protein

Introduction

In 2021, over $123 billion was spent on pets in the United States alone, with pet food and treat sales accounting for $50 billion of this total expenditure (APPA, 2022). Consumers often demand new "high-quality" ingredients and generally prefer high protein formulated diets (Swanson et al., 2013). Traditionally these diets are sourced from animal proteins. However, the sustainability of these diets has been challenged as some of these formulations use ingredients that directly compete with human food systems or potentially contribute to a greater environmental footprint (Acuff et al., 2021; Swanson et al., 2013). The use of plant-based proteins including pulses, legumes, and tubers have been proposed as a cost effective, sustainable alternative to animal proteins (Reilly et al., 2020). However, plant-based ingredients come with their own liabilities, including limitations in essential amino acids, lower palatability, and negative perceptions by consumers to name a few. Alternatively, microbial proteins produced from heterotopic microorganisms, such as yeasts, have been proposed. These microorganisms utilize the elementary components of waste materials, that would otherwise be inaccessible to higher organisms such as humans and pets and convert them into bioavailable high-quality proteins with minimal environmental impact (Matassa et al., 2016; Spiller et al, 2020).

Yeast and yeast derived products have been fed to animals for over a century (Stone, 2006). Brewer's yeast, whey yeast, and Torula yeast have been categorized as nutritional yeasts when fed as inactive microbial biomass principally for nutritional value (Shurson, 2018). Among these traditionally used in livestock nutrition, Torula yeast has been favored due to its flexible utilization of carbon sources and robust growth capabilities (Bekatorou et al., 2006, Buerth et al., 2016). Torula yeast has the ability to metabolize xylose and xylose oligomers (Yanai and Sato, 2001), allowing for growth on low-value cellulosic waste materials. Thus, representing an opportunity to

produce large amounts of microbial protein from a sustainable and cost-effective growth medium. Furthermore, yeast production from cellulosic material has a lower carbon footprint compared to soybean meal and does not compete for resources with the human food system (Øverland and Skrede, 2016; Spiller et al, 2020).

In companion animal research, most work involving yeast products have primarily been focused on immune response and gastrointestinal microflora modulation in dogs (Gouveia et al., 2006; Grieshop et al., 2004; Kroll et al., 2020; Pawar et al 2017; Swanson et al., 2002; Van den Abbeele et al. 2020) and cats (Santos et al., 2018; Calabrò et al., 2020). Two previous reports found brewer's yeast and sugarcane yeast to be an adequate protein source in dog diets (Martins et al., 2013; Reilly et al., 2021). A recently developed proprietary Torula yeast product has been introduced into the animal food marketplace (Arbiom, 2021) and previously evaluated in weaning pig diets (Espinosa et al., 2020; Lagos and Stein, 2020). In these studies, the researchers concluded that Torula yeast could be included into weaning pig diets in exchange for fish meal and plasma protein. Additionally, a previous press release detailed the use of this Torula yeast in exchange for chicken meal in dog diets (Arbiom, 2019). Currently little has been published on the application of yeast products in feline diets and, to our knowledge, no data are available regarding the nutritional utilization of this type of yeast biomass as a protein source for felines. It is assumed here that this Torula yeast ingredient can provide an alternative high-quality protein source for commercial feline diets. Therefore, the objective of this work was to evaluate the use of a wood-effluent grown Torula yeast (SylPro; Arbiom Inc., Durham, NC) and its influence on diet processing and kibble formation, palatability, and nutrient utilization in extruded feline diets.

Material and Methods

Diet formulation and production

Four dietary treatments were designed using concept 5 formulation software (Creative Formulation Concepts (CFC) Tech Services Inc., Pierz, Minnesota) to meet AAFCO (AAFCO, 2019) minimum recommendations for "Growth and Reproduction" for cats (Table 1).). Diets differed primarily by protein source and were formulated to be similar in terms of crude protein (CP), crude fat, and gross energy (GE). Test protein ingredients included a Torula dried yeast (SylPro; Arbiom Inc., Durham, NC), pea protein concentrate (VITESSENCE® Pulse 1550; Ingredion Inc., Westchester, IL), high-protein soybean meal (SBM) and low-ash chicken meal sourced from a local mill (Lortscher's Animal Nutrition Inc. Bern, KS). Torula yeast, pea protein, and SBM were included at 20% of their respective treatment formulas (TY, PP, and SM, respectively) to offset a portion of chicken meal, whereas the last treatment contained only chicken meal (CM) as its sole protein source. The remainder of the treatment formulas included brewers rice, beet pulp, fish oil, taurine, DL-methionine, vitamin and mineral premixes. Each dietary treatment also included titanium dioxide (0.40%) as an indirect marker to estimate digestibility. Dry ingredients were mixed, extruded, and kibbles were dried prior to the addition of topical chicken fat and dry flavor digest.

Diets were produced using a pilot-scale single screw extruder (Wenger single screw X-20, Wenger Manufacturing, Sabetha, KS), with a screw diameter of 82.55 mm and a length to diameter ratio of 10. The extruder screw profile is presented in Figure 1. The die was a 4 mm single opening diameter (resulting in die open area of 12.6mm) and was fitted with six short, hard blades. Raw material was fed into the preconditioner (PC) at a rate of 88.5 kg/h. Material in PC was hydrated to form a dough by water injection that fluctuated between 9.2 to 9.3 kg/h. Thermal energy was

applied to dough in the PC via steam injection that fluctuated between 8.0 to 8.2 kg/h, resulting in discharge temperatures between 88 to 92ºC. Extruder screw speed was set at 398 rpm for all treatments. Water was injected into the extruder at a rate of 7.0 to 7.7 kg/h; slight adjustments were made during processing to achieve a target bulk density of 300 to 350 g/L. No additional thermal energy was applied to the extruder.

All diets were produced on a single day in sequential order. Once processing stability was achieved, treatment order began with CM, followed by SM, then PP, and last for TY. Treatments were switched once target product amounts were reached, and collection of the following treatment began after allowing extruder to clear out for 30min. After extrusion, kibbles were dried in a double pass forced-air oven (Series 4800, Wenger Manufacturing, Sabetha, KS) at 121°C for ~5 minutes each pass, to achieve a target moisture content of ~7%, followed by a subsequent ~5 minutes cooling prior to bagging. Chicken fat and dry flavor digest were surface applied to the kibble in a rotating barrel mixer at a later date. Processing parameters and samples were collected in triplicate at three equally spaced time intervals (~45min apart) during the production of each treatment. Recorded processing parameters included feed rate (kg/h), preconditioner water flow (kg/h), preconditioner steam flow (kg/h), preconditioner discharge temperature (°C), extruder screw speed (rpm), extruder water flow (kg/h), die pressure (psi), and die temperature (°C). Additionally, extruder mass flow rate was measured at the end of each experimental treatment by collecting material out of the extruder into a bucket for one minute, then weighed for mass per unit time (kg/min). At each observation time, samples were collected from the preconditioner, extruder and dryer and stored at −20°C for further analysis. Specific mechanical energy (SME) was calculated using the following equation:

Equation 3.1. Calculation of specific mechanical energy.

$$\mathrm{SME}\left(\frac{\mathrm{kJ}}{\mathrm{kg}}\right) = \frac{\frac{\tau - \tau_0}{100} * \left(\frac{\mathrm{N}}{\mathrm{N_r}}\right) * \mathrm{P_r}}{\mathrm{m}}$$

where τ is the % torque, or motor load, τ_o is the no-load torque (34%), N is the screw speed in rpm, N_r is the rated screw speed (508 rpm), P_r is the rated motor power (37.3 kW), and m is the total mass flow in kg/s. In-barrel moisture (IBM) was calculate as described below:

Equation 3.2. Calculation of in-barrel moisture.

$$\mathrm{IBM}\ (\%) = \frac{\mathrm{mf} * \mathrm{Xf} + \mathrm{mps} + \mathrm{mpw} + \mathrm{mes} + \mathrm{mew}}{\mathrm{mf} + \mathrm{mps} + \mathrm{mpw} + \mathrm{mes} + \mathrm{mew}} * 100$$

where mf is the dry feed rate, Xf is moisture content of the feed material, mps is the steam injection rate in the preconditioner (kg/h), mpw is water injection rate in the preconditioner (kg/h), mes is the steam injection rate in the extruder (kg/h), and mew is the rate of water injected in the extruder (kg/h).

Kibble characteristics

Kibble samples were collected out of the dryer during each replicate to evaluate final product macrostructure characteristics. From each time point, length, diameter, and weight were measured from 15 kibbles for calculation of piece volume, piece density, sectional expansion index (SEI), and specific length (l_{sp}) as follows:

Equation 3.3. Calculation of piece volume.

$$\mathrm{V} = \frac{\pi * \mathrm{l_e} * \mathrm{d_e}^2}{4}$$

Equation 3.4. Calculation of piece density.

$$\rho = \frac{\mathrm{m_e}}{\mathrm{V}}$$

Equation 3.5. Calculation of sectional expansion index.

$$\mathrm{SEI} = \frac{\mathrm{d_e}^2}{\mathrm{d_d}^2}$$

Equation 3.6. Calculation of specific length.

$$l_{sp} = \frac{l_e}{m_e}$$

where V is the volume in cm^3, l_e is the kibble length in mm, d_e is the average of two measurements of the kibble diameter in mm, ρ is the piece density in $g*cm^{-3}$, m_e is the kibble mass in g, SEI is the sectional expansion index, and d_d is the die hole diameter in mm. Additionally, bulk density and true density (gas displacement) were measured for each treatment at each collection time point. Bulk density was measured during production both off the extruder and out of the dryer in duplicate, collected using a 1L steel cup.

Texture analysis

Texture analysis was performed using a texture analyzer (model TA-XT2, Texture Technology Corp., Scarsdake, NJ) equipped with a 30 kg load cell. A cylindrical probe (25 mm diameter) was used to compress 30 kibbles in triplicate from each collection time point for each diet (30 kibbles x triplicate x 3 time points). Prior to texture analysis, kibbles were dried in a convection oven at 55ºC for 48h to equilibrate samples; after drying, samples were removed and placed in a desiccator (airtight with SiO_2 desiccant) at room temperature for an additional 48 hours to stabilize dry samples. The pre-test speed was 2 $mm*s^{-1}$, test speed was 1 $mm*s^{-1}$, and a post-test speed was 10 $mm*s^{-1}$ (adapted from Dogan and Kokini, 2007). Strain level was set at 50%. Kibble hardness (Newtons) was considered to be the peak force of the first major kibble breakage. The average values of 30 kibbles for hardness was used as the experimental unit for statistical analysis to help account for variation among individual kibbles.

Palatability testing

Experimental diets were evaluated for palatability at a commercial kennel (Summit Ridge Farms, Susquehanna, PA). The cattery facility is registered with the USDA No. 23-R-0126 under

the Animal Welfare Act. Palatability tests were conducted as a split plate design (Griffin, 2003). A total of 20 healthy adult cats (6 neutered males and 14 spayed females) with average age of 9.57±3.25 years (mean±SD) were used in this experiment. Three split plate tests were conducted: TY vs CM, TY vs PP, and TY vs SM. Since the purpose of this palatability testing regimen was to compare the novel Torula yeast protein to currently utilized protein ingredients, the other treatments (CM, PP, and SM) were not directly compared to one another. During a two-bowl test, two stainless steel bowls each containing ~100 g of a single test diet were presented once daily for up to 4 hours. Each test was evaluated over a 2-day period with bowl placement being switched (left-right) between days; thus, the three tests were completed over a total of six days. If one experimental diet was completely consumed prior to the end of the 4 h feeding window both bowls were removed. Both first choice (first diet consumed) and intake ratio (IR) were reported for the present study. Intake ratio was determined using to the formula below:

Equation 3.7. Calculation of intake ratio.

$$IR = \frac{consumption\ of\ diet\ A}{(consumption\ of\ diet\ A\ +\ consumption\ of\ diet\ B)}$$

Digestibility assessment

Experimental diets were evaluated for apparent total tract nutrient digestibility (ATTD) at the Kansas State University College of Veterinary Medicine. All experimental procedures were reviewed and approved by the Kansas State University Institutional Animal Care and Use Committee (IACUC) at under protocol # 4348 prior to beginning of study. A total of 12 healthy adult American shorthair cats (10 neutered males and 2 spayed females) with average age of 2.72±1.52 years (mean±SD) and weight of 5.60±1.27 kg (mean±SD) were used in this experiment. Cats were fed over four 14-day periods which included 9 days of diet adaption followed by 5 days of fecal collection. Stainless steel liter pans (12in x 8in x 4in) fitted with rubber turf and elevated

drainage mats were used for the collection of fecal and urine samples. All cats exclusively utilized liter pans for elimination during collection. Cats were randomized to treatment and period in a 4 x 4 replicated Latin square design according to Kim and Stein (2009). This design allows each cat to serve as its own control.

Initial food amounts offered were estimated based on the chemical composition of the diets and individual energy requirements of each cat to maintain body weight according to the National Research Council (NRC, 2006; ME, kcal*day^{-1} = 62*BW in kg). Daily food allowance was adjusted weekly, if necessary, to maintain body weight. Cats were fed twice daily (0900 and 1600) and excess food was collected. Water was provided *ad libitum.* Cats were kept in a temperature-controlled room (22°C ± 1°C) with a 12h light cycle (lights automatically shut off from 1945 to 0745 daily). Each day was considered to start at 0900, coinciding with the first feeding. Cats were group housed with 4 cats to a room during adaptation (3 rooms of 4 cats each) but were individually fed for 1h. After the 1h feeding, refused food was collected and weighed for intake calculation. To acclimate cats to metabolic cages, two phases were used during adaptation. Phase 1 of adaptation (days 1 to 4), the cats were only kept in the cages during the two 1 h feeding periods (0900 to 1000 and 1600 to 1700). Phase 2 of adaptation (days 5 to 9), cats were kept in individual cages from the beginning of the first feeding to the end of the last feeding (0900 to 1700). During fecal collection (days 10 to 14), cats were housed individually for the entire 120 h. During fecal collection, excess food was collected 1 h prior to the start of the next feeding day (0800).

Feces were collected prior to each meal and whenever observed throughout the day. Fecal samples were used to calculate ATTD of nutrients but also to characterize fecal scores, defecation frequency, dry and wet fecal output, and fecal pH. Upon collection, feces were scored subjectively according to a 5-point scale (1 runny to 5 hard, in 0.5 point increments; Carciofi et al., 2008) then

stored in sterile polyethylene bags (Whirl-Pak; Nasco sampling, Madison, WI) and frozen at -20°C for later analysis. Due to their qualitative measurements, fecal scores were evaluated based on frequency of occurrence rather than on average of aggregate scores. One fresh fecal sample (within 15 minutes of defecation) was also collected from each cat during each period and stored at -80°C.

Digestibility calculations

At the culmination of the feeding assay, fecal samples were placed in an aluminum pan, weighed, and dried in an oven (Cat 52755-20, Matheson Scientific, Morris Plains, NJ) at 55°C for 72h. Dried feces were later ground through a 1-mm screen in a fixed blade laboratory mill (Retsch, type ZM200, Haan, Germany). Both food and feces were analyzed for titanium concentration using an adaptation of the procedure described by Leone (1973). Briefly, 0.3 g of fecal sample or 0.6 g of food sample were incinerated overnight in muffle furnace at 450°C and allowed to cool to room temperature. Next, 1.0 g of sodium sulfate and 5 mL of sulfuric acid were added to the incinerated samples and were digested on a hot plate at 280°C for 25 min. After cooling to room temperature, samples were transferred to 50mL centrifuge tubes and brought to 50 g with distilled water. The tubes were centrifuged at 1,000 x G for 10 min and allowed to rest for 24 h. The following day, 0.25 mL of each sample was pipetted, in duplicate, into a 96-well plate. Then 30µL of 30% hydrogen peroxide solution was added to each well and the plate was allowed to rest for at least 15 min. Absorbance values were measured at 410 nm using a microplate reader (Synergy H1, Biotek, Winooski, VT, USA). Apparent total tract nutrient digestibility (ATTD) was calculated using Titanium Dioxide (TiO_2) as an indigestible marker, using the following equations:

Equation 3.8. Calculation of apparent total tract nutrient digestibility.

$$\mathrm{TiO_2} = \frac{[1 - (\%\mathrm{TD} * \%\mathrm{NF})]}{(\%\mathrm{TF} * \%\mathrm{ND})} * 100$$

wherein %ND is the percent nutrient in the diet, %NF is the percent nutrient in the feces, %TD is the percent Titanium in the diet, and %TF is the percent Titanium in feces.

Nutrient analysis

Test ingredients, experimental diets, and dried fecal samples were analyzed for dry matter (DM; AOAC 930.15), organic matter (OM; AOAC 942.05), ash (inorganic matter calculated by difference), crude protein (CP; AOAC 990.03), crude fat by acid hydrolysis (AOAC modified 954.02), and total dietary fiber (TDF; AOAC 991.43; TDF kit, K-TDFR-200A, Megazyme Ltd., Bray, Ireland), according to AOAC international approved analytical methodologies. All nutrients were reported on a DM-basis. Gross energy (GE) was determined by bomb calorimetry (model 6200, Parr Instrument Company, Moline, IL). Additionally, diets were also analyzed for crude fiber (AOAC Ba 6a-05), insoluble dietary fiber (AOAC 991.43; TDF kit, K-TDFR-200A, Megazyme Ltd., Bray, Ireland), and soluble dietary fiber by difference between total dietary fiber and insoluble dietary fiber.

Statistical analysis

For kibble measurements and texture analysis, diet was considered the fixed effect and replication nested within diet was considered as the random effect. Kibble length, diameter, weight, volume, piece density, true density, SEI, bulk densities out of the extruder and dryer, and hardness were all dependent variables. The digestibility experiment was performed as a replicated 4x4 Latin square design, where cat and period served as blocking factors. Diet was considered the fixed effect, whereas square, period, and cat nested within square were considered as random effects. Nutrient digestibility, daily food intake, defecation rate, wet and dry fecal output, percent fecal dry matter, fecal pH, and urine pH were dependent variables. Data was analyzed using statistical software via the general linear mixed models procedure (GLIMMIX in SAS; v. 9.4).

Least square means were considered significant at $P<0.05$ and multiple comparisons were adjusted using tukey post-hoc method. Fecal scores of each treatment were also separately analyzed using the GLIMMIX procedure with cat and period as random effects. Fecal score frequencies were then determined using the frequency procedure (PROC FREQ in SAS; v. 9.4). For palatability testing, first choice and IR were evaluated using a chi-square test and 2-way ANOVA, respectively. Differences were also considered significant at a $P<0.05$ for both tests.

Results

Diet formulation and production

The nutrient composition of experiment protein ingredients are presented in Table 2 to provide context to dietary differences. Chicken meal had a much higher CP and fat content (70.66 and 15.02%, respectively) compared to the other experimental ingredients (average of 50.92 and 4.45%, respectively). Total dietary fiber was much greater for pea protein, SBM, and Torula yeast ingredients (average of 17.14) compared to that of chicken meal (6.49%,). Dietary treatments had similar concentrations of most nutrients but varied in fiber measurements (Table 3). Total dietary fiber and soluble fiber content were greatest for TY (11.32 and 4.77%) compared to other treatments. Additionally, TY had the greater relative proportion of soluble fiber content (42% of TDF) among treatments. Both PP and SM had intermediate TDF values (9.59 and 9.63%, respectively) which both primarily consisted of insoluble fiber (>99 and 94% of TDF, respectively). The TDF of CM was lower than the other treatments (7.01%), but CM had a larger amount (1.89%) and relative proportion (27% of TDF) of soluble fiber compared PP and SM. Crude fiber content was slightly greater for SM (1.71%) compared to other treatments (average of 1.07%). Gross energy was also found to be slightly lower for CM (5236 kcal/kg) than the other treatments (average of 5399 kcal/kg).

During diet production, dry feed rate, water and stream injection into PC, and extruder screw speed were held constant across treatments with minor fluctuations (Table 4). Water injection was adjusted between treatments and ranged from 7.0 to 7.7 kg/h. Die pressure was greater ($P<0.05$) for TY than for other treatments (358 vs average of 294 psi; Table 5). In-barrel moisture differed among all treatments ($P<0.05$) and was greatest for SM, followed by CM, PP, and TY (29.3, 29.2, 28.3, and 27.8%, respectively). Specific mechanical energy was greater for TY and SM ($P<0.05$; average of 187 kJ/kg) than for PP (138 kJ/kg), but CM was similar to all treatments (167 kJ/kg).

Kibble characteristics

Bulk density out of the extruder was different between all treatments ($P<0.05$; Table 5), being greatest for CM, decreasing in order by PP, SM, and lowest for TY (450, 431, 410, and 365 $g*L^{-1}$, respectively). Bulk density was initially targeted between 300-350 $g*L^{-1}$; however due to constraints of diet processing, bulk densities were higher than expected. Extruder startup and steady state were achieved using the raw material of the CM diet prior to diet collection. The steady state achieved here was used to set processing parameters for the subsequent treatments but was not able to meet the target bulk density. After drying, the bulk density of CM and PP were similar (average of 385 $g*L^{-1}$) and greater ($P<0.05$) than SM (350 $g*L^{-1}$), with TY again having the lowest density (324 $g*L^{-1}$). Kibble length was greatest for SM ($P<0.05$), whereas kibble diameter was greatest for TY ($P<0.05$). Piece volume was greatest ($P<0.05$) for TY, followed SM, and lowest for CM and PP which were similar to one another (0.268, 0.238, and average of 0.206 cm^3). As expected, piece density showed the opposite trend to piece volume and mirrored that of bulk density. Piece density was lowest for TY, intermediate for SM, and greatest for CM and PP (0.382,

0.431, and average of 0.497 g*cm^{-3}, respectively). Accordingly, hardness was greater for CM and PP ($P<0.05$; average of 2.90 Newtons) than for SM and TY (average of 2.10 Newtons).

Palatability and dietary utilization assessment

Out of the 40 observations (20 cats x 2 days) during the palatability trial, TY was chosen first ($P<0.05$; Table 6) over CM (36 vs 4, respectively), but differences were not found between TY and PP (25 vs 15, respectively) or TY and SM (24 vs 16, respectively). When comparing preference, TY had an IR greater than 0.67 ($P<0.05$) when compared to CM and PP (0.88 and 0.73, respectively). However, the IR of TY was below that of 0.67 when comparing to SM (0.59).

During the digestibility experiment, daily food intake was greater for CM than SM ($P<0.05$ Table 7). Defecation frequency was greater ($P<0.05$) for cats fed SM compared to those fed TY (0.73 vs 0.53 defecations*day^{-1}). Cats fed SM and PP had greater total fecal output ($P<0.05$; average of 31.85 g*day^{-1}) than those fed TY and CM (average of 23.65 g*day^{-1}). However, on a DM-basis, fecal output was only different between SM and CM ($P<0.05$), wherein SM was greater than CM (10.47 vs 8.64 g*day^{-1}, respectively). Fresh fecal pH of TY was similar (5.54) to that of other treatments, but PP and SM had lower fecal pH ($P<0.05$; average of 5.34) than those fed CM (5.75). Fecal scores were evaluated based on frequency of occurrence rather than on average of aggregate scores. Fecal score frequencies were different ($P<0.05$; Figure 2) between SM, CM, and TY; wherein SM had the greatest frequency of larger fecal scores, followed by CM with greater frequency of intermediate scores, and last for TY which had the greatest tendency of lower fecal scores. The fecal score frequency of PP did not differ from those of SM and CM. Urine pH was greatest for cats fed SM ($P<0.05$; 7.41), followed CM and PP (average of 6.96), and was lowest for TY (6.55).

Apparent total tract digestibility of DM and OM was greater (P<0.05; Table 8) for cats fed CM (87.43 and 91.34%, respectively) compared to all other treatments. Both DM and OM digestibility of cats fed TY was similar to PP and SM (average of 86.20 and average of 89.76%, respectively). Ash digestibility was greater (P<0.05) for TY and SM (average of 37.42%), intermediate for PP (32.79%), and lowest for CM (23.97%). For cats fed TY, CP digestibility was not different from other treatments (average of 89.97%), but fat digestibility was lower (P<0.05; 92.52%) than all other treatments (93.76 to 94.82%). Gross energy digestibility was greater (P<0.05) for CM than TY (90.97 vs 90.18%, respectively); however, TY was similar to PP and SM (average of 90.22%). Total dietary fiber digestibility was similar between TY and CM (average of 66.20%) and greater (P<0.05) than PP and SM (average of 58.70%).

Discussion

The purpose of the present work was to investigate the utility of a Torula dried yeast product (SylPro; Arbiom Inc., Durham, NC) as a novel source of protein in extruded cat diets. The Torula yeast used here had a DM content similar to that of several previous reports (Lagos and Stein, 2020; Olvera-Novoa et al, 2002; Ringrose, 1948; Øverland et al., 2013). Figueroa et al. (1990) and Ringrose (1948) reported similar ash but lower CP values, whereas Olvera-Novoa et al. (2001) reported greater ash and lower CP content. Øverland et al. (2013) reported lower ash, similar CP, and greater GE concentrations. On dry matter basis, CP, GE, and TDF were all similar to that reported by Lagos and Stein (2020); however, ash content reported here was much lower. Interestingly, fat content of the Torula yeast used in the current work was higher than all other reports (0.9 to 3.22%; Lagos and Stein, 2020; Olvera-Novoa et al, 2002; Ringrose, 1948; Øverland et al., 2013), and almost double the next leading amount (Lagos and Stein, 2020).

Differences in nutrient composition within and between yeast species are not uncommon. Torula yeasts have been grown under a wide range of conditions and substrates (Buerth et al., 2016). They are specifically known for their flexible utilization of carbon substrates, which have included beet pulp (Athar et al., 2009), distillery waste (Hosken et al., 2015; Garcia et al., 2014), pineapple effluent (Nigam, 1998), and wheat bran (Yunus et al., 2015) among others, whereas the particular yeast used in the present experiment was grown on forestry by-products from the timber industry. Additionally, nitrogen source is a critical variable influencing protein composition. Many different sources of nitrogen have been utilized in growth medium of Torula yeast including ammonia, urea, amino acids, peptides, or nucleic acids (Boze et al., 1992). Differences in growing conditions likely play a large part in the nutrient variation reported throughout the literature. Thus, emphasizing the need to individually qualify these products when being included in animal diets.

The Torula yeast used in this experiment was evaluated in comparison to pea protein concentrate, SBM, and chicken meal. Torula yeast, pea protein, and SBM were supplemented in their respective diets with chicken meal to complement amino acid deficiencies in order to fulfill minimum requirements. During diet production, all processing variables were kept constant with the exception of water injection into the extruder. Although PC settings were held constant, discharge temperature was slightly lower for CM. It may be possible that this is due to exclusively using chicken meal as protein, which would have a reduced endothermic capacity due to the denaturing of proteins during the rendering process. Additionally, the raw dry ration of CM had a larger amount of native fat owing to the higher fat in chicken meal compared to the other proteins, which may have also resulted in a lower absorption of steam into the dough.

After initial hydration and heating in the PC, the dough enters the extruder where the material becomes a continuous molten-like state, referred to as a melt, through the high shear

extrusion process. Processing conditions must be tailored to each unique food recipe and the adjustment of water injection into the extruder between treatments was necessary to facilitate melt movement through extruder. Traditional food extrusion of high starch, low protein materials rely solely on starch gelatinization to generate melt formation and expansion upon exit of extruder. This requires adequate energy input but low moisture to produce a dry expanded product. Inside the extruder barrel water acts as a plasticizing agent (Guy, 2001), which lowers the viscosity of the melt and the frictional, or mechanical, energy with the screw. Thus, increasing plasticizers such as water consequently decreasing expansion of the product. However, high protein pet foods require much greater water input to properly hydrate and cook both the starch and protein polymers into a matrix that will move though the extruder and expand upon exit (Baller et al., 2018). In this case, there is a balancing act between achieving enough water to cook the macromolecules in the ration (specifically starch) and not too much in which the mechanical energy decreases and hinders expansion. The in-barrel moisture of TY was lower than all other diets but the SME was only greater than that of PP. Whereas, SM had the greatest in-barrel moisture but had a similar SME to that of TY. It would be expected from previous work that IBM would be inversely correlated with SME (Ding et al., 2005), wherein as IBM decreases, SME would increase. This would undoubtably be true if treatment rations were identical in terms of raw ingredients. It is evident that test ingredients had different hydration and rheological properties inherent to those materials. Thus, it is likely that ingredient differences had a greater impact on SME than IBM.

All four diets were formulated to be nutritionally similar and primarily differed by protein source; thus, differences were largely attributed to protein ingredients. The DM of CM was slightly higher than desired (target moisture content ~7%) due to a longer drying time after extrusion. Processing parameters are estimated prior to start of diet production and occasionally require

adjustment during the manufacturing process. The CM treatment was the first to be produced, and drying time was reduced for subsequent treatments. Ash content was slightly lower for PP than for other treatments, owing to the lower ash content observed for the pea protein ingredient used here. Fat content was greater for TY compared to other diets, due to greater amount of native fat in the Torula yeast ingredient than was expected. Crude fiber was reported for diets because it is a common method which is required as part of the guaranteed analysis for pet food by the American Association of Feed Control Officials (AAFCO, 2019). Crude fiber was greater for SM than other treatments; however, this measurement mainly includes cellulose, only small portions of hemicellulose and lignin, and no soluble components (Fahey et al., 2019). Despite labeling requirements, this measurement accounts for a small portion of the true fiber content and holds little nutritional relevance for monogastric animals. Total fiber and soluble fiber were greater for TY than for other dietary treatments. Generally, dietary fiber is not equalized across treatments when formulating diets to be similar in protein, fat, and energy; however, it is still an important constituent of many ingredients, especially for yeast and legume sources. The Torula yeast used here had a TDF concentration over 18%, which was similar to that reported by Lagos and Stein (2020). Those authors also identified about 80% of the total fraction as being soluble. Considering that 10 to 30% of yeast biomass consists of cell wall (Lipke and Ovalle, 1998; Nguyen et al, 1998), which is composed largely of β(1,3)/(1,6)-glucans, a large proportion of soluble fiber is not surprising. The diets containing vegetable-based proteins had moderate fiber content that were almost completely insoluble. The total fiber content of SBM observed here was similar to that reported by Grieshop et al. (2003), who analyzed the nutrient composition of soybean meals produced at many different processing facilities across the United States. Additionally, total fiber of pea protein analyzed here matched that of manufacturer product specifications (Ingredion,

2020). As would be expected, CM had the lowest concentration of total fiber among dietary treatments. Interestingly, CM was found to have a higher concentration of soluble fiber than PP and SM. It would not be expected that a rendered poultry meal would contribute notable levels of soluble fiber. Thus, this may have been an artifact of the fiber assay potentially capturing structural proteins and connective tissue partially solubilized during diet processing. Lastly, GE was lower for CM compared to other treatments, likely due to greater concentration of cartilage and other connective tissues present in rendered meals (Aldrich, 2006). Additionally, the lower fat and fiber values of CM likely also contributed to slightly lower energy value.

Final product characteristics are a result of the interaction between processing conditions and ingredient composition of the formula. While SME for TY was not found to be greater than that of CM or SM, it did result in the greatest die pressure which is typically accompanied with greater expansion (Pasqualone et al, 2020). Consequently, bulk density out of the extruder and dryer were lightest for TY due to this greater expansion. For individual kibble measurements, TY also had the greatest diameter, SEI, and piece volume as well as the lightest piece density among all treatments. Further reinforcing the influence of TY on expansion. The SM treatment had the second lightest piece and bulk densities, accompanied with the greatest kibble length and l_{sp}. This would indicate that TY had a better radial expansion, whereas SM had greater longitudinal expansion. As would be expected, the greater expansion properties associated with TY and SM also led to reduced kibble hardness compared to the other two diets. It would be expected that CM had less expansion with harder, denser kibbles (Fournier, 2013). It is commonly accepted that rendered animal-based proteins tend to have less functionality compared to plant proteins. Rendered meals are subjected to excessive heating during the rendering process, causing protein denaturation. When these proteins are denatured, the native three-dimensional structure is

compromised and unfolds, essentially eliminating any existing functionality. It is surprising that PP had similar expansion characteristics to that of CM in terms of piece volume, density, and hardness. However, PP had a lower SME compared to other dietary treatments when produced at similar processing parameters. Thus, it is possible that PP had a lower water-binding capacity and that the elevated moisture content had a stronger plasticizing effect, reducing the amount of energy imparted into the melt.

Yeast in brewing and bread making has been widely utilized for millennia, but their evaluation in extrusion cooking is extremely limited. Recently, yeast and yeast derived products have been explored for the role as emulsifying and thickening agents in food production. Vélez-Erazo et al. (2021) found that with increasing concentration of yeast cell protein, emulsion properties improved and resulted in highly viscous systems. The emulsification process involves the joining of hydrophobic and hydrophilic material with an emulsifying agent when adequate energy is applied to the system (Quek, Chen, & Shi, 2016). In the current experiment, the high energy extrusion process could have provided the external energy required to join these elements together and generate a highly viscous melt. The use of pea protein in extrusion cooking is still a relatively new area of research with most new work investigating its role as a texturized vegetable protein for use as meat analogues or extenders. Webb et al. (2020) evaluated ratios of pea protein and chickpea flour on texturized characteristics of extrudates. These authors demonstrated that at high protein levels (~80%) texturization was able to occur in the creation of an expanded low-density product. However, these authors also reported that when a small portion of protein was offset with starch, expansion diminished, and density increased as starch gelatinization began to compete with protein texturization. In the current study it is possible that there was not enough pea protein to generate adequate viscosity within the melt, diminishing mechanical energy and

expansion. Pea protein has also been shown to have a lower water binding capacity (Tömösközi et al, 2001; Vose, 1980) and greater solubility in water (Quinn and Paton, 1979) compared to that of soy protein. The high-water binding and low water solubility properties of soy protein have been attributed to greater viscosity development during hydration (Malhotra and Coupland et al., 2004; O'Flynn et al, 2021). Marco and Rosell (2008) reported that protein enriched flours had better water absorption, emulsion properties, and viscosity development when supplemented with soy protein compared with that of pea protein. Lim and Narsimhan (2006) also determined that the addition of soy protein had a synergistic interaction with starch that resulted in increased pasting temperatures and overall viscosity in protein-starch pastes. When included into extruded dog diets, Venturini et al. (2018) found that the inclusion of soybean protein at the expense of poultry meal facilitated phase-transition and better viscosity development of the melt.

The palatability assessment was conducted using the common methodology for preference evaluation by Two-pan (split-plate) method (Griffin, 2003); whereby, the animal is presented with two foods and first choice and intake ratio are recorded. The food that is approached and consumed first is considered to be the "first-choice" and is primarily associated with the animal's response to aromatic characteristics (Aldrich & Koppel, 2015). In this trial, TY was shown to only have a significant first choice preference over CM. Since, the purpose of this palatability testing regimen was to evaluate the efficacy of TY, the other treatments were not compared amongst one another. Thus, making it difficult to ascertain if any differences would be observed among them. It is likely that first choice preference for TY may have actually been an aversion to CM, considering no differences were observed between TY and the other treatments. The CM diet had a much smaller amount of surface applied fat compared with the other three diets, which may have reduced alluring aroma intensity, initial flavor, and texture characteristics of that diet. The remaining diets had

similar amounts of surface applied fat, which would have greatly enhanced their initial appeal and likely diluted any aromatic differences inherent to the uncoated diets. Koppel et al. (2015) evaluated and contrasted the sensory properties of both coated and uncoated extruded kibbles. The authors noted significant changes in appearance, aroma, and flavor after coating; wherein, color darkened, porosity decreased, and masked any off aroma and taste characteristics compared to uncoated kibbles. Although it is known that nutrient composition can also influence preference in cats (Rutherford, 2004), it may have been more appropriate to test diets without surface coatings of fat or flavor in order to determine the true undiluted effect of the experimental ingredients. Preference is demonstrated by a relative food IR of at least 0.67 for the test diet (Aldrich & Koppel, 2015; Griffin, 2003). Based on IR, cats preferred TY over CM and PP. However, while food consumption of TY was greater than that of SM, the IR did not reach the threshold. On average, TY was consumed over six times the amount of CM and over double the amount of PP, suggesting preference along some attributes of the Torula yeast ingredient. However, what those attributes are is unclear. It is possible that the Torula yeast had a more appealing flavor compared to that of chicken meal and pea protein, but it is surprising that it was not favored over SBM as well. Alternatively, TY kibbles were better expanded and lower in hardness compared to CM and PP, whereas kibble hardness was similar between TY and SM. Thus, there may have been textural preferences driving the palatability results. Contrary to this, Baller et al. (2018) found that a dietary formula extruded across a range of SME resulted in range of kibble macrostructures, but these differences did not influence food preferences in cats. Regardless, TY was more or equally preferred to the commonly used proteins.

To our knowledge, palatability of Torula yeasts have not previously been explored in either feline or canine diets. What little research that has been conducted using yeast or yeast products in

the companion animal space has focused on *Saccharomyces cerevisiae* (brewer's yeast, baker's yeast, sugarcane yeast, etc.) and favored canine research. For example, Lin et al. (2019) stated that a 0.2% inclusion of a fermentation product from *S. cerevisiae* resulted in increased palatability relative to a control in a two-bowl dog study. However, those authors only reported a total consumption ratio (1.93:1) in leu of a IR common to the split plate method (Griffin, 2003). A preference is often considered to occur when the animals consume double or more the amount of the other diet, leading to a consumption ratio exceeding 2:1 or intake ratio of 0.67 (Aldrich & Koppel, 2015; Griffin, 2003). Martins et al. (2013) also evaluated the use of *S. cerevisiae* in canine diets, reporting greater average intake ratios (>0.67) for diets containing 7.5% sugarcane yeast relative to a control. In cat diets, the palatability of supplemented yeast extract from *S. cerevisiae* has also been evaluated. de Oliveira et al. (2016) reported a greater intake ratio for a combination of yeast extract and sodium pyrophosphate added to extruded kibbles; however, these ingredients individually did not increase palatability. Lima et al. (2016) evaluated the use of yeast extract in wet cat diets and reported a decrease in palatability.

Contrary to the results of the palatability assessment, in which TY was consumed more than CM and PP, there were no differences in daily food consumption between these three diets. Further, daily food consumption was slightly greater for CM ($P<0.05$) than that of SM (78.1 vs 75.4 g^{-1} day; respectively). However, it is likely that the greater consumption of CM is driven by some other factor than palatability, such as the slightly lower energy content of CM, a satiety promoting effect of SM, or both. It has been reported that felines can regulate their energy intake in order to maintain body weight (Loureiro et al., 2017). Additionally, it is known that high fiber intake in humans can promote satiety (McRorie, 2015), depending upon their specific physical characteristics. The high fiber content of SM possibly had physical qualities capable of promoting

satiety in cats. It is worth noting that the palatability test was summarized as an IR whereas the digestibility trial was recorded using mean total daily consumption. Using an IR during the two-pan test helps to reduce statistical bias that may result from appetite or size effects of the animal, thus equally representing each test animal. Conversely, when reporting only mean consumption animal differences such as body size and appetite can arbitrarily skew the results.

The greater fecal output observed for cats fed SM and PP was largely attributed to a greater moisture content in the feces; whereas on a DM-basis, only SM was observed to have a greater dry fecal output than CM, implicating larger concentrations of indigestible material. This result is unsurprising, considering the lower total fiber content and the greater TDF disappearance of CM. Previous work (Menniti et al., 2014; Clapper et al, 2001; Carciofi et al., 2009) has also shown an increase in fecal production and fecal moisture with the consumption of SBM when substituted for poultry meal or poultry by-product meal. Irrespective of fecal moisture content, TY tended to produce lower fecal scores than all other treatments. This would indicate that the Torula yeast produced slightly softer stools in cats. Martins et al. (2013) found that the inclusion of sugarcane yeast at the expense of poultry by-product meal lead to a decrease in fecal dry matter content and less fecal structure; however, wet and dry fecal outputs were not different on a $g*day^{-1}$ basis. Zentek et al. (2002) also reported a decrease in fecal consistency in dogs supplemented with yeast cell wall components. The high portion of soluble fiber inherent to yeast cell wall is likely responsible for softer fecal structure; wherein, the partially soluble polysaccharides would have a higher affinity for interacting with water, forming a loose gel-like structure (Selvendran et al., 1987). On the contrary, the higher portion of insoluble fiber in legume proteins may have provided a bulking effect that could trap moisture while still maintaining a firmer structure. The differences in fiber structure are also likely responsible for the greater defecation frequency of SM compared

to TY. It has been shown that increasing insoluble fiber content is associated with a laxation effect in cat diets (Loureiro et al., 2017), whereas soluble fiber can slow gastric emptying and the movement of digesta through the GI tract (Schneeman, 1999). Previous work evaluating SBM in comparison to poultry by-product meal in extruded diets indicated that dogs fed SBM produced greater wet fecal output with no differences in fecal scores (Carciofi et al., 2009; Tortola et al., 2013). Bednar et al. (2000) reported that dogs consuming SBM had slightly softer stools compared to several animal-based rendered meals. In the present study, CM tended to produce fecal scores intermediate to that SM and TY. The inconsistencies in reported fecal scores associated with rendered protein meals is, in large part, related to the wide compositional variation seen among these products (Yamka et al., 2003). For example, rendered meals with high ash content lead to greater mineral accumulation in the feces, which tends to decrease fecal moisture and result in more solid fecal formation (Vanelli et al. 2021).

Fecal output, fecal DM, and fecal pH indirectly reflect the colonic environment in response to the structural and nonstructural polysaccharide content in the diets. Carbohydrate fermentation in the colon leads to the production of lactate and short chain fatty acids, which decrease pH and increase luminal osmolarity (Binder, 2010). The presence of the raffinose family of oligosaccharides in SBM and pea protein are completely dispersible in water and particularly susceptible to rapid fermentation (Tosh and Yada, 2010). Clapper et al. (2001) showed that total fecal output decreased with the removal of oligosaccharides and Wiernusz et al. (1995) showed that total fecal output and fecal moisture content linearly decreased with oligosaccharide removal from soy-based diets. The structural ß-glucans of yeast cells are also known to be susceptible to fermentation; however, the ß-glucan structure does not completely dissociate in water. The more complex polysaccharide structure prolongs the fermentation process in the colon, producing

organic acids at a rate in which the colonocytes can readily absorb and blunt the osmolarity and pH changes (Binder, 2010). Urinary pH was elevated among SM, PP, and CM in comparison to TY. Particularly, SM exhibited alkalinity over normal pH (5.5 to 7) values expected of cat urine (Knight and Leitsberger, 2016). Additionally, CM, PP, and SM had pH values above that recommended for the prevention of struvite uroliths (6.0-6.5; Kopecny et al., 2021). Plant-based diets have been suspected to be a potential cause of urinary alkalinization, possibly attributed to lower proportions of acidic amino acids; however, further supporting research is needed (Dodd et al., 2021; Knight and Leitsberger, 2016). Additionally, this would not explain the elevated urine pH observed in CM, which was composed only of animal proteins. One explanation for the lower urinary pH of cats fed TY is the slightly higher methionine inclusion, which is considered an acidifying agent of urine (Queau, 2018; Dodd et al., 2021; Knight and Leitsberger, 2016).

Digestibility coefficients were high across all treatments, but some key differences were observed. Both DM and OM apparent digestibility were slightly greater for CM, whereas the Torula yeast was similar to the legume proteins. The elevated concentrations of fiber in diets containing Torula yeast and plant proteins may have reduced their overall DM and OM digestibility due to greater concentrations of undigested material ending in feces. In a recent review comparing SBM to poultry by-product meal in extruded dog diets, SBM tended to reduce the digestibility coefficients of DM, OM, acid hydrolyzed fat, and GE in the majority of papers examined (Vanelli et al., 2021). Additionally, Bednar et al., (2000) reported that dogs fed poultry meal had a greater digestibility of DM, OM, and CP compared to SBM. This parallels data presented here; however, the digestibility coefficients observed here for cats were greater than reported for dogs (Bednar et al. 2000). The Torula yeast had a similar apparent protein digestibility to all other treatments but had the lowest coefficient of fat digestibility. Martins et al. (2013)

evaluated the use of a brewer's yeast and a sugarcane yeast in dog diets and reported no differences in digestibility coefficients compared to control diet. Theodoro et al. (2019) demonstrated that the inclusion of a soluble yeast cell wall component to dog diets reduced the coefficient of fat digestibility without affecting any other nutrient digestibility. These authors attributed the reduction in apparent fat digestibility to the higher water solubility of yeast cell wall, possibly interfering with fat absorption. The present work utilized a yeast biomass consisting of entire yeast cells. The slight decrease in fat digestibility observed here may be attributed to cellular components such as ß-glucans, mannooligosaccharides, and mannoproteins which may bind water and hinder fat adsorption. The apparent digestibility of TDF was greater for TY and CM than the legume proteins. It would be expected that the Torula yeast would influence colonic fermentation due to the large portion of soluble fiber intrinsic to the Torula yeast and lower fecal scores observed for this diet. As discussed previously, the fiber present in CM was likely a result of non-fiber components being captured as fiber by the assay and these structural proteins and connective tissue were potentially fermentable to certain genus of the microflora. The high portion of non-fermentable structural fibers intrinsic to legumes would result in depressed apparent TDF digestibility. Conversely, most of the fermentable substrates found in legumes are soluble low molecular weight oligosaccharides that do not appear in the TDF analysis (Fahey et al., 2019), thus making their disappearance in the gastrointestinal tract invisible by the methods used here. While many ingredients are used in diet formulation in the pet food industry from both animal and plant origin, most data available have evaluated the use of soy products and rendered animal meals. Further, the majority of data evaluating companion animal diets has favored the dog model. It has been suggested that the cat has an increased ability to digest protein in comparison to the dog

(Golder et al., 2020). Compared with the aggregate of data reported for the dog that was reviewed, the data observed here for cats seems to agree with this assertion.

The increased ash digestibility observed for the Torula yeast and legume proteins may reflect decreased mineral digestibility in CM. It has been reported that phosphorus digestibility of bone tissue is lower than other sources (Sulabo and Stein, 2013). It is arguable that the lower apparent mineral digestibility of rendered animal meals containing large amount of bone material could be more indicative of homeostatic regulation of the animal rather than poor digestion. In the presence of excess minerals such as calcium in bone, it is reasonable to assume that decreases in parathyroid hormone would lead to increased concentration of calcium in the feces (Passlack and Zentek, 2013; Pastoor et al., 1994). Lagos and Stein (2020) demonstrated that a diet containing Torula yeast had improved phosphorus digestibility compared to high ash fish meal when phosphorus intake was similar. However, even though phosphorus intake was not different, the fish meal diet had a much larger calcium concentration compared to the Torula yeast diet, which possibly could interfere with phosphorus digestion (Pastoor et al., 1994). On the contrary, Kim et al. (2014) showed that among diets with similar Ca:P ratios, ethanol and brewers' yeasts had improved phosphorus digestibility compared to SBM and fish meal. It has also been shown in cat diets that increasing dietary phosphorus, accompanied by unchanging adequate calcium concentration, had no effect on calcium absorption or excretion (Pastoor et al., 1995). It is not certain here that phosphorus was the key driver of apparent ash digestion. However, it is important to note that if TY, PP, and SBM lead to increased phosphorus absorption, this would likely increase phosphorus excretion in the urine. In the present work, cats fed diets containing legume proteins were found to produce more alkaline urine pH (>6.5), which in the presence of excess phosphorus increases the risk factor of developing struvite uroliths in felines.

While many interesting observations were made through this work, there were some limitations identified in the present study that should be considered for the interpretation of these data. Diet processing was a point of interest in this work, but the primary goal here was to evaluate nutrient utilization. As mentioned previously, diets were formulated to at least meet minimum requirements of all nutrients as well be similar across diets for CP, fat, and GE concentrations. Therefore, these diets were not necessarily formulated with the intention of evaluating and comparing ingredient functionality during the extrusion process. The greater expansion properties exhibited by this novel Torula ingredient was an interesting observation and could be of great relevance to researchers and industry professionals alike. However, the mechanism of functionality remains unclear. In this study, diets were produced under a single set of processing parameters. Additionally, diets differed in starch and fiber composition, which effect starch gelatinization and nucleation within the melt. Phase transition analysis and water binding capacity of protein ingredients may have provided some insight into the differences in viscosity development between treatments; unfortunately, these measurements were not determined in this work. Future work should investigate the mode of action for expansion in Torula yeast which may involve the presence of ß-glucans, glycoproteins, or both. Future work should also investigate how graded levels of Torula yeast inclusion and processing variables may affect final product characteristics.

Although it is expected that the preference of TY was afforded by the Torula yeast ingredient, it was uncertain if protein ingredients were the only factor driving palatability in these diets. As mentioned previously, all dietary treatments evaluated in palatability trial had different levels of surface applied fat. Additionally, dietary treatments differed in kibble size and hardness. Due to the constraints of this study, it is not possible to determine the magnitude these factors may have influenced palatability results. Further work should be conducted to help discern palatability

differences between Torula yeast-based diets and other protein sources, specifically study designs that are not confounded by texture and surface coating differences.

The greater proportion of soluble fiber and TDF disappearance observed for TY was attributed to greater colonic fermentation of undigested cellular components. Unfortunately, post-biotic analyses were not taken during this study. Additionally, it was noted that diets containing legumes and chicken meal were associated with a more alkaline urine pH. While urinary characteristics were not a major emphasis in this work, urinary health is a significant point of concern in felines and urine pH largely influencing the development of urolithiasis. It is unknown if urinary alkalization was attributed to the lower proportions of acidic amino acids, such as methionine and cystine, alkalizing electrolytes, or another source. Future work should investigate the role protein source may play on urinary parameter in felines and acid-base balance in particular. The last limitation identified here involved the differences in ash digestibility. It was assumed here that the greater ash disappearance may have been attributed to improved phosphorus digestibility. However, evidence to support this increase would require a mineral balance study, which was beyond the present scope.

Conclusions

In summary, the Torula yeast used in this study aided diet processing and kibble formation, increased diet preference, and was highly digestible when fed to cats. Under similar processing conditions, the Torula yeast ingredient resulted in a more highly expanded product, particularly in the radial direction, which resulted in the lowest density and hardness. Preference was observed for TY over PP and CM; however, TY was not found to be preferred over SM. Nutrient digestibility was similar or greater for TY compared with other protein ingredients, with the exception of fat digestibility. Cats fed TY produced feces that were soft and less formed which

may be attributed to fiber composition. It was observed here that Torula yeast could be safely included into feline diets, but inclusion level may be limited by fecal quality considerations. Further work should be conducted to elucidate the mechanisms responsible for greater expansion and physical characteristics of kibbles, palatability characteristics, and implications of protein ingredients on urinary health in cats.

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Tables

Table 3.1. Ingredient composition of dietary treatments.

Ingredient, %	Dietary treatment			
	CM	PP	SM	TY
Rice, brewers	45.96	39.14	38.58	39.97
Chicken meal, low ash	43.09	28.26	28.42	26.60
Pea protein concentrate	-	20.00	-	-
Soybean meal, high protein	-	-	20.00	-
Torula yeast[1]	-	-	-	20.00
Beet pulp	3.00	3.00	3.00	3.00
Fish oil	1.00	1.00	1.00	1.00
Titanium dioxide	0.40	0.40	0.40	0.40
Salt	0.35	0.35	0.35	0.35
Choline chloride, 60% dry	0.20	0.20	0.20	0.20
Taurine	0.20	0.20	0.20	0.20
Vitamin premix[2]	0.15	0.15	0.15	0.15
Trace mineral premix[3]	0.10	0.10	0.10	0.10
Potassium chloride	0.30	0.10	0.10	0.10
DL-Methionine	0.10	0.10	0.10	0.14
Calcium carbonate	-	-	-	0.06
Chicken fat[4]	3.66	5.50	5.90	6.23
Dry flavor digest[4]	1.50	1.50	1.50	1.50

[1]Torula yeast: SylPro
[2]Vitamin E Supplement (79,887 IU*kg-1), Niacin Supplement (64,736 mg*kg-1), Calcium Pantothenate (12,186 mg*kg-1), Vitamin A Supplement (17,162,998 IU*kg-1), Thiamin Mononitrate (14,252 mg*kg-1), Pyridoxine Hydrochloride (5,537 mg*kg-1), Riboflavin Supplement (4,719 mg*kg-1), Vitamin D3 Supplement (920,000 IU*kg-1), Biotin (70 mg*kg-1), Vitamin B12 Supplement (22 mg*kg-1), Folic Acid (720 mg*kg-1).
[3]Zinc Sulfate (88,000 mg*kg-1), Ferrous Sulfate (38,910 mg*kg-1), Copper Sulfate (11,234 mg*kg-1), Manganous Oxide (5,842 mg*kg-1), Sodium Selenite (310 mg*kg-1), Calcium Iodate (1,584 mg*kg-1).
[4]Surface applied to dry kibble

Table 3.2. Nutrient composition of protein sources.

Composition	Experimental Ingredient			
	CM	PP	SM	TY
Dry Matter, %	92.88	92.39	88.02	96.33
		Dry matter basis		
Ash, %	8.09	5.67	8.06	8.41
Crude protein, %	70.66	50.45	47.74	54.58
Acid-hydrolyzed fat, %	15.02	4.71	2.44	6.21
Total dietary fiber, %	6.49	15.30	17.71	18.42
Gross energy, kcal/kg	5852.92	4826.11	4720.30	4846.70

Table 3.3. Nutrient composition of dietary treatments.

Composition	Dietary treatment			
	CM	PP	SM	TY
Dry matter, %	96.43	93.38	93.88	93.92
	Dry matter basis			
Ash, %	5.79	5.32	6.01	6.00
Crude protein, %	38.70	38.13	38.20	38.35
Acid-hydrolyzed fat, %	12.26	12.60	12.97	13.50
Crude fiber, %	1.04	1.05	1.71	1.13
Total dietary fiber, %	7.01	9.39	9.63	11.32
Insoluble, %	5.12	8.83	9.59	6.55
Soluble, %	1.89	0.56	0.04	4.77
Gross energy, kcal/kg	5236	5409	5378	5410

Table 3.4. Extrusion processing parameters during production of dietary treatments.

Processing parameters	Dietary Treatment			
	CM	PP	SM	TY
Raw material				
Feed rate, kg/h	88.5	88.5	88.5	88.5
Preconditioner				
Water injection, kg/h	9.3	9.2	9.2	9.2
Steam injection, kg/h	8.2	8.0	8.1	8.1
Discharge temperature, °C	88.0	90.7	89.7	92.0
Extruder				
Screw speed, rpm	398	398	398	398
Water injection, kg/h	7.7	7.4	7.6	7.0

Table 3.5. Production outputs and kibble characteristics of extruded dietary treatments.

	Dietary Treatment				SEM	P-value
	CM	PP	SM	TY		
Production outputs						
Die pressure, psi	300[b]	300[b]	283[b]	358[a]	9.3	0.0022
Die temperature, °C	110[a]	105[b]	104[b]	107[ab]	0.7	0.0015
Mass flow rate[1], kg/min	1.72	1.74	1.61	1.56	0.038	0.0798
IBM, %	29.2[b]	28.3[c]	29.3[a]	27.8[d]	0.03	<0.0001
SME, kJ/kg	167[ab]	138[b]	182[a]	191[a]	7.1	0.0034
Kibble characteristics						
Bulk density OE[2], $g*L^{-1}$	450[a]	431[b]	410[c]	365[d]	4.0	<0.0001
Bulk density OD[3], $g*L^{-1}$	390[a]	379[a]	350[b]	324[c]	4.6	<0.0001
Length, mm	4.76[c]	4.87[bc]	5.38[a]	5.02[b]	0.056	0.0002
l_{sp}, $cm*g^{-1}$	4.70[c]	4.82[bc]	5.29[a]	4.95[b]	0.051	<0.0001
Diameter, mm	7.47[b]	7.26[c]	7.50[b]	8.24[a]	0.047	<0.0001
SEI	3.49[bc]	3.29[c]	3.52[b]	4.24[a]	0.047	<0.0001
Weight, g	0.101	0.101	0.102	0.102	0.0017	0.9907
Piece volume, cm^3	0.209[c]	0.202[c]	0.238[b]	0.268[a]	0.0052	<0.0001
Piece density, $g*cm^{-3}$	0.488[a]	0.505[a]	0.431[b]	0.382[c]	0.0058	<0.0001
Hardness, N	2.94[a]	2.86[a]	2.21[b]	1.98[b]	0.101	0.0003

[1]Measured directly as weight per unit time
[2]Out of the extruder
[3]Out of dryer
[abc]Means with unlike superscripts differ (P<0.05)

Table 3.6. Palatability comparison of experimental diets assessed by cats.

Diet Comparison (A vs B)	First Choice, n[1]	Intake Ratio[2]
TY vs CM	36[*]	0.88[*]
TY vs PP	25	0.73[*]
TY vs SM	24	0.59

[1]First choice: number of first choices for Diet A (40 observations)
[2]Intake ratio: Diet A/(Diet A+B)
[*]Comparisons differ (P<0.05)

Table 3.7. Food intake and fecal characteristics of experimental diets.

	Dietary Treatment				SEM	P-value
	CM	PP	SM	TY		
Food intake						
g*day^{-1} (DM)	78.1^{a}	76.2^{ab}	75.4^{b}	76.4^{ab}	4.81	0.0329
Fecal characteristics						
Defecations*day^{-1}	0.62^{ab}	0.67^{ab}	0.73^{a}	0.53^{b}	0.072	0.0015
Fecal output (as-is), g*day^{-1}	22.6^{b}	30.3^{a}	33.4^{a}	24.7^{b}	2.52	<0.0001
Fecal dry matter, %	38.8^{a}	33.1^{bc}	31.6^{c}	36.4^{ab}	1.24	<0.0001
Fecal output (DM), g*day^{-1}	8.64^{b}	9.83^{ab}	10.47^{a}	8.94^{ab}	0.776	0.0269
Fecal pH	5.75^{a}	5.30^{b}	5.37^{b}	5.54^{ab}	0.090	<0.0001
Urine pH	6.87^{b}	7.05^{b}	7.41^{a}	6.55^{c}	0.076	<0.0001

^{abc}Means with unlike superscripts differ (P<0.05)

Table 3.8. Apparent total tract digestibility of cats fed dietary treatments estimated by use of indigestible marker TiO_2.

Digestibility, %	Dietary Treatment				SEM	P-value
	CM	PP	SM	TY		
Dry matter	87.43^{a}	86.54^{b}	85.66^{c}	86.39^{bc}	0.422	<0.0001
Organic matter	91.34^{a}	89.71^{b}	89.82^{b}	89.75^{b}	0.414	<0.0001
Ash	23.97^{c}	32.79^{b}	38.25^{a}	36.58^{a}	0.631	<0.0001
Crude protein	90.26^{a}	90.32^{a}	89.35^{b}	89.90^{ab}	0.396	0.0200
Acid-hydrolyzed fat	93.76^{b}	94.82^{a}	93.91^{b}	92.52^{c}	0.264	<0.0001
Gross energy	90.97^{a}	90.30^{ab}	90.18^{b}	90.18^{b}	0.386	0.0154
Total dietary fiber	64.92^{a}	57.52^{b}	59.87^{b}	67.48^{a}	1.710	<0.0001

^{abc}Means with unlike superscripts differ (P<0.05)

Figures

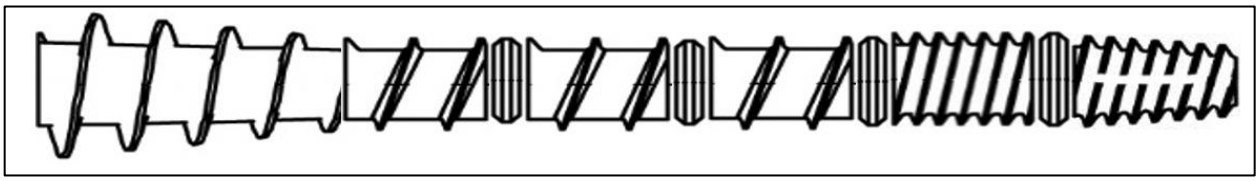

Figure 3.1. Schematic of extruder screw profile.

Inlet starting on the left to discharge ending on the right. Screw element 1: inlet screw, single flight full pitch; 2: single flight, full pitch screw; 3: small steam lock; 4: single flight full pitch screw; 5: small steam lock; 6: Single flight, full pitch screw; 7: medium steam lock; 8: double flight, ½ pitch screw; 9: Large steam lock; 10: double flight, ½ pitch, cut cone screw.

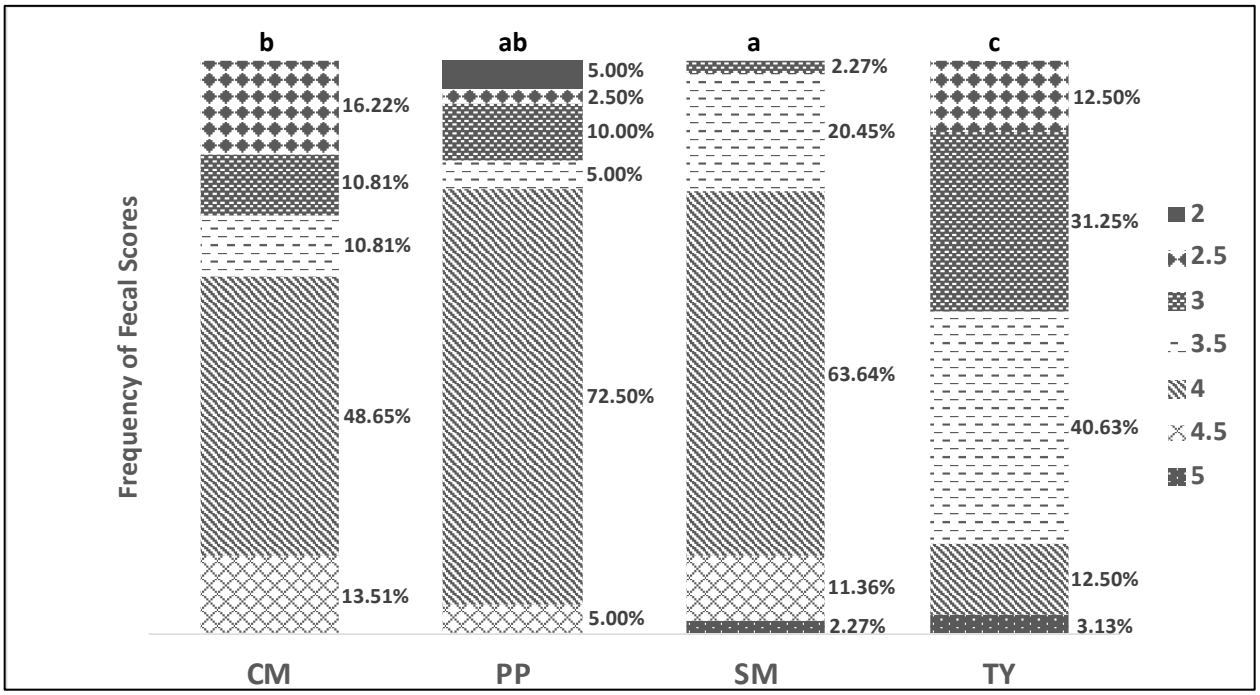

Figure 3.2. Fecal score frequency of cats fed experimental diets.

Fecal scores based on a 1 to 5-point scale in 0.5-point increments (1- liquid stools, 5- hard dry feces)

Appendix A - Supplementary data for Chapter 3

Table A.1. Apparent total tract digestibility of cats fed dietary treatments estimated by the indigestible marker TiO_2 and total fecal collection methods.

Digestibility, %	Dietary Treatment*				SEM‡	P-value
	CM	PP	SM	TY		
TiO2 Marker						
Dry matter	87.43[a]	86.54[b]	85.66[c]	86.39[bc]	0.422	<0.0001
			Dry matter basis			
Organic matter	91.34[a]	89.71[b]	89.82[b]	89.75[b]	0.414	<0.0001
Ash	23.97[c]	32.79[b]	38.25[a]	36.58[a]	0.631	<0.0001
Crude protein	90.26[a]	90.32[a]	89.35[b]	89.90[ab]	0.396	0.0200
Acid-hydrolyzed fat	93.76[b]	94.82[a]	93.91[b]	92.52[c]	0.264	<0.0001
Gross energy	90.97[a]	90.30[ab]	90.18[b]	90.18[b]	0.386	0.0154
Total dietary fiber	64.92[a]	57.52[b]	59.87[b]	67.48[a]	1.710	<0.0001
Total Collection						
Dry matter	89.25[a]	87.61[ab]	86.62[b]	89.05[a]	0.626	0.0136
			Dry matter basis			
Organic matter	92.61[a]	90.44[bc]	89.76[c]	91.68[ab]	0.499	0.0004
Ash	34.48[b]	37.33[ab]	37.55[ab]	47.92[a]	3.296	0.0287
Crude protein	91.71[a]	90.95[ab]	89.93[b]	91.86[a]	0.452	0.0137
Acid-hydrolyzed fat	94.63	95.39	94.62	94.52	0.360	0.2530
Gross energy	92.31[a]	90.98[ab]	90.12[b]	92.04[a]	0.453	0.0043
Total dietary fiber	70.00[a]	60.45[b]	59.66[b]	73.52[a]	1.998	<0.0001

[1]Dietary treatments: Chicken meal (CM), Pea protein (PP), Soybean meal (SM), Torula yeast (TY)

[2]Standard error of the mean

[abc]Means with unlike superscripts differ (P<0.05)