Evaluation of corn fermented protein (CFP) in pet food applications

by

Logan Rylee Kilburn-Kappeler

B.S., Iowa State University, 2016
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
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Abstract

Co-products from the ethanol industry, such as distillers dried grains with solubles (DDGS) and corn fermented protein (CFP), can provide alternative protein sources for pet food. Corn fermented protein is produced using post-fermentation technology to split the protein and yeast from fiber prior to drying. This results in a higher protein ingredient compared to DDGS, increasing its appeal for pet food while still providing a sustainable and cost-effective ingredient. Corn fermented protein also contains a substantial yeast component which may provide health benefits. Therefore, the objectives of this work were to determine: 1) the optimal inclusion level of CFP for use in pet food and 2) the contribution from the yeast component of CFP on overall nutrition and animal health. To achieve these objectives, the effects of CFP on extrusion processing, kibble quality, palatability, nutrient digestibility, stool quality, and the fecal microbiome were assessed when fed to both dogs and cats. The first experiment evaluated diets with increasing levels of CFP in exchange for soybean meal at 0, 5, 10, and 15% (0C, 5C, 10C, 15C). The second experiment compared a diet containing 17.5% CFP to diets containing either 15% soybean meal (CON), 3.5% brewer's dried yeast (BDY), or 2.5% brewer's dried yeast plus 17.5% DDGS (BDY+DDGS). Titanium dioxide was added to all diets as a marker to estimate digestibility. Diets were fed to 12 dogs or 11 cats in a 4x4 replicated Latin square design. Animals were fed each dietary treatment for 9-d adaptation followed by 5-d total fecal collection. Fresh fecal samples from the second experiment were analyzed by 16S Metagenomic Sequencing. All data were analyzed using a mixed model in SAS (version 9.4, SAS Institute, Inc., Cary, NC) with treatment as a fixed effect and animal and period as random effects. For the first experiment, dry bulk density of kibble decreased while kibble toughness increased with CFP inclusion ($P < 0.05$). Dry fecal output was greater for dogs and cats fed increased levels of CFP

($P < 0.05$). Overall, nutrient digestibility decreased with increased levels of CFP. For palatability assessment, cats preferred 5C over 0C whereas dogs preferred 0C over 10C and 15C ($P < 0.05$). For the second experiment, preconditioner discharge temperature was greater for CON and BDY compared to BDY+DDGS and CFP ($P < 0.05$). Extruder screw speed, die temperature, kibble toughness, and kibble hardness were greatest for CFP ($P < 0.05$). The bulk density of BDY+DDGS at 392 g/L was greater than BDY and CFP at an average of 342 g/L ($P < 0.05$). The sectional expansion index of kibble for CFP was greater than BDY+DDGS and smaller than CON ($P < 0.05$) but similar to BDY. Fecal output was greatest for cats and dogs fed BDY+DDGS ($P < 0.05$). Overall, nutrient digestibility was lowest for BDY+DDGS when fed to both dogs and cats. There were no differences in total short chain or branched chain fatty acid concentrations in fresh fecal samples of dogs or cats fed dietary treatments ($P > 0.05$). For palatability assessment, dogs and cats had no preference when CON was compared to BDY or BDY+DDGS ($P > 0.05$). However, they appeared to prefer CON over CFP ($P < 0.05$). For the microbiome analysis, alpha-diversity indices (Observed, Chao1, Shannon, Simpson) and beta-diversity metric (principal coordinate analysis) were similar among all treatments in fecal samples from dogs and cats. There were no quantifiable shifts in predominant phyla among treatments in dogs ($P > 0.05$). However, in cats, the relative abundance of Firmicutes and Actinobacteria was lower for BDY+DDGS compared to CFP and BDY, respectively ($P < 0.05$). There were multiple significant differences in the relative abundance of genera among dietary treatments in both dogs and cats. Overall, the variation in dietary fiber content among treatments likely contributed to the difference in results. In order to maintain stool quality, nutrient digestibility, and palatability when fed to dogs or cats, a 10% inclusion of CFP would be

recommended if exchanged for soybean meal in pet food. Further research is warranted to determine the ideal inclusion level of CFP in pet food to promote animal health.

Keywords: cats, corn fermented protein, dogs, extrusion, fecal microbiome, nutrient digestibility, palatability, stool quality

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Major Professor
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recommended if exchanged for soybean meal in pet food. Further research is warranted to determine the ideal inclusion level of CFP in pet food to promote animal health.

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Table of Contents

List of Figures xv
List of Tables xvi
Acknowledgements xix
Chapter 1 - Literature Review: The utilization of corn as a protein source in pet food 1
Introduction 1
Overview of Corn 1
Corn Protein 2
Corn in the Pet Food Industry 2
Corn Co-products 3
Corn Gluten Meal (CGM) 5
Corn Gluten Feed (CGF) 7
Distillers Dried Grains with Solubles (DDGS) 8
Enhanced Dried Distillers Grains 14
High-Protein Dried Distillers Grains (HPDDG) 14
Corn Fermented Protein (CFP) 16
Conclusion 19
References 20
Chapter 2 - Evaluation of graded levels of corn fermented protein (CFP) on stool quality, apparent nutrient digestibility, and palatability in healthy adult cats 28
Abstract 28
Introduction 29
Materials and Methods 31
Diet Formulation and Production 31
Feeding Trial 32
Digestibility Calculations 33
Nutrient Analysis 33
Palatability Trial 33
Statistics 34
Results and Discussion 34

Diet Chemical Analyses 34
Feed Intake and Fecal Characteristics 35
Apparent Total Tract Digestibility 36
Palatability 37
Summary 38
Conclusion 39
References 40
Chapter 2 Tables 43
Chapter 3 - Evaluation of graded levels of corn fermented protein (CFP) on extrusion processing and diet utilization in healthy adult dogs 49
Abstract 49
Introduction 50
Materials and Methods 51
Diet Formulation 52
Diet Production 52
Physical Characteristics of Kibble 54
Feeding Trial 55
Digestibility Calculations and Nutrient Analysis 56
Palatability Trial 56
Statistics 57
Results 57
Extrusion Processing and Kibble Characteristics 57
Diet Chemical Analyses 59
Feed Intake and Fecal Characteristics 59
Apparent Total Tract Digestibility 59
Palatability 60
Discussion 60
Extrusion Processing and Kibble Characteristics 60
Diet Chemical Analyses 63
Feed Intake and Fecal Characteristics 64
Apparent Total Tract Digestibility 65

Palatability 66
Application of CFP in the Pet Food Industry 66
Conclusion 67
References 68
Chapter 3 Tables 72
Chapter 4 - Comparison of corn fermented protein (CFP) to distillers dried grains with solubles (DDGS) fed to healthy adult dogs 79
Abstract 79
Introduction 80
Materials and Methods 81
Diet Formulation and Production 81
Feeding Trial 83
Digestibility Calculations 84
Nutrient Analysis 84
Fecal Chemical Analysis 85
Palatability Trial 86
Statistics 86
Results 87
Nutrient Analysis 87
Feed Intake and Fecal Characteristics 88
Apparent Total Tract Digestibility 89
Fecal Chemical Analysis 89
Palatability 90
Discussion 90
Nutrient Analysis 90
Feed Intake and Fecal Characteristics 93
Apparent Total Tract Digestibility 95
Fecal Chemical Analysis 96
Palatability 97
Conclusion 98
References 99

Chapter 4 Tables 103
Chapter 5 - Diet production and utilization of corn fermented protein (CFP) compared to traditional yeast in healthy adult cats 111
Abstract 111
Introduction 112
Materials and Methods 113
Diet Formulation 113
Diet Production 114
Physical Characteristics of Kibble 116
Feeding Trial 117
Digestibility Calculations 118
Nutrient Analysis 119
Fecal Chemical Analysis 119
Palatability Trial 119
Statistics 120
Results and Discussion 120
Extrusion Processing and Kibble Characteristics 120
Diet Chemical Analyses 125
Feed Intake and Fecal Characteristics 126
Apparent Total Tract Digestibility 127
Fecal Chemical Analysis 129
Palatability 130
Conclusion 131
References 132
Chapter 5 Tables 135
Chapter 6 - Evaluation of corn fermented protein (CFP) on the fecal microbiome of dogs 142
Abstract 142
Introduction 142
Materials and Methods 144
Diet Formulation, Production, and Nutrient Composition 144
Feeding Trial 145

Sample Collection 146
Fecal DNA Extraction and Sequencing 146
Data Analysis 146
Results 147
Beta and Alpha-Diversity 147
Phyla Relative Abundance 147
Genera Relative Abundance 148
Discussion 148
Beta and Alpha-Diversity 148
Phyla Relative Abundance 149
Genera Relative Abundance 150
Conclusion 153
References 154
Chapter 6 Tables and Figures 159
Chapter 7 - Evaluation of corn fermented protein (CFP) on the fecal microbiome of cats 166
Abstract 166
Introduction 167
Materials and Methods 168
Diet Formulation, Production, and Nutrient Composition 168
Feeding Trial 170
Fecal DNA Extraction and Sequencing 170
Data Analysis 171
Results and Discussion 171
Beta and Alpha Diversity 171
Phyla Relative Abundance 172
Genera Relative Abundance 174
Conclusion 178
References 179
Chapter 7 Tables and Figures 184
Chapter 8 - Conclusion and future steps 191

List of Figures

Figure 6.1. Principal coordinate analysis (PCoA) explaining 9.3% and 5.4% of the variability in operational taxonomic units (OTU) of Bray-Curtis UniFrac distances for fecal samples from dogs fed dietary treatments. Treatments: T1 = control; T2 = brewer's dried yeast; T3 = brewer's dried yeast and distillers dried grains with solubles; T4 = corn fermented protein. 161

Figure 6.2. Alpha diversity measures for fecal samples from dogs fed dietary treatments. Treatments: T1 = control; T2 = brewer's dried yeast; T3 = brewer's dried yeast and distillers dried grains with solubles; T4 = corn fermented protein. 162

Figure 7.1 Principal coordinate analysis (PCoA) explaining 7.1% and 4.3% of the variability in operational taxonomic units (OTU) of Bray-Curtis UniFrac distances for fecal samples from cats fed dietary treatments. Treatments: T1 = control; T2 = brewer's dried yeast; T3 = brewer's dried yeast and distillers dried grains with solubles; T4 = corn fermented protein. 186

Figure 7.2 Alpha diversity measures for fecal samples from cats fed dietary treatments. Treatments: T1 = control; T2 = brewer's dried yeast; T3 = brewer's dried yeast and distillers dried grains with solubles; T4 = corn fermented protein. 187

List of Tables

Table 2.1 Analyzed chemical composition of experimental ingredients, soybean meal (SBM) and corn fermented protein (CFP), reported on a dry matter basis 43
Table 2.2 Ingredient composition of feline diets with increasing levels of corn fermented protein 44
Table 2.3 Analyzed chemical composition of feline diets with increasing levels of corn fermented protein reported on a dry matter basis 45
Table 2.4 Food intake and stool quality parameters of cats fed diets with increasing levels of corn fermented protein 46
Table 2.5 Apparent total tract digestibility of cats fed diets with increasing levels of corn fermented protein estimated by TiO_2 as a dietary marker 47
Table 2.6 First choice (FC) and intake ratio (IR) of cats fed diets with increasing levels of corn fermented protein 48
Table 3.1 Ingredient composition of canine diets with increasing levels of corn fermented protein 72
Table 3.2 Least squares means and contrasts (0C vs 5C-15C (T), linear (L); quadratic (Q); cubic (C) level of corn fermented protein) for processing parameters and physical characteristics of diets with increasing levels of corn fermented protein 73
Table 3.3 Analyzed chemical composition of canine diets with increasing levels of corn fermented protein on a dry matter basis 75
Table 3.4 Least squares means and contrasts (0C vs 5C-15C (T), linear (L); quadratic (Q); cubic (C) level of corn fermented protein) for food intake and stool quality parameters of dogs fed diets with increasing levels of corn fermented protein 76
Table 3.5 Least squares means and contrasts (0C vs 5C-15C (T), linear (L); quadratic (Q); cubic (C) level of corn fermented protein) for apparent total tract digestibility, estimated by titanium dioxide as a dietary marker, of diets with increasing levels of corn fermented protein 77
Table 3.6 First choice (FC) and intake ratio (IR) of dogs fed diets with increasing levels of corn fermented protein 78

Table 4.1 Ingredient composition of canine diets containing yeast and ethanol co-products on an as-is basis 103
Table 4.2 Nutrient composition of experimental ingredients on a dry matter basis 104
Table 4.3 Amino acid composition of experimental ingredients on a dry matter basis..... 105
Table 4.4 Analyzed chemical composition of canine diets containing yeast and ethanol co-products on a dry matter basis 106
Table 4.5 Food intake and stool quality parameters of dogs fed diets containing yeast and ethanol co-products[1] 107
Table 4.6 Apparent total tract digestibility of dogs fed diets containing yeast and ethanol co-products estimated by titanium dioxide as a dietary marker on a dry matter basis[1] 108
Table 4.7 Fecal chemical analysis of dogs fed diets containing yeast and ethanol co-products[1] 109
Table 4.8 First choice (FC) and intake ratio (IR) of dogs fed diets containing yeast and ethanol co-products[1] 110
Table 5.1 Ingredient composition of feline diets containing yeast and ethanol co-products on an as-is basis 135
Table 5.2 Processing parameters and physical characteristics of feline diets containing yeast and ethanol co-products 136
Table 5.3 Analyzed chemical composition of feline diets containing yeast and ethanol co-products on a dry matter basis 137
Table 5.4 Food intake and stool quality parameters of cats fed diets containing yeast and ethanol co-products[1] 138
Table 5.5 Apparent total tract digestibility of diets containing yeast and ethanol co-products estimated by titanium dioxide as a dietary marker on a dry matter basis[1] 139
Table 5.6 Chemical analysis of feces from cats fed diets containing yeast and ethanol co-products[1] 140
Table 5.7 First choice (FC) and intake ratio (IR) of cats fed diets containing yeast and ethanol co-products[1] 141
Table 6.1 Ingredient composition of canine diets containing yeast and ethanol co-products on an as-is basis 159
Table 6.2 Analyzed chemical composition of canine diets containing yeast and ethanol co-products on a dry matter basis 160

Table 6.3 Relative abundance of bacterial phyla among the 50 most abundant operational taxonomic units (OTU) in fecal samples from dogs fed dietary treatments 163
Table 6.4 Relative abundance of bacterial genera among the 50 most abundant operational taxonomic units (OTU) in fecal samples from dogs fed dietary treatments 164
Table 7.1 Ingredient composition of feline diets containing yeast and ethanol co-products on an as-is basis 184
Table 7.2 Analyzed chemical composition of feline diets containing yeast and ethanol co-products on a dry matter basis 185
Table 7.3 Relative abundance of bacterial phyla among the 50 most abundant operational taxonomic units (OTU) in fecal samples from cats fed dietary treatments 188
Table 7.4 Relative abundance of bacterial genera among the 50 most abundant operational taxonomic units (OTU) in fecal samples from cats fed dietary treatments 189

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Chapter 1 - Literature Review: The utilization of corn as a protein source in pet food

Introduction

Overview of Corn

Cereals are the world's most important food crop. Cereal grains are the seeds that come from grasses such as wheat, millet, rice, barley, oats, rye, triticale, sorghum, and corn. Corn is one of the largest cereal crops worldwide. In 2022, 13.7 billion bushels of corn were produced (USDA ERS 2022), which at 56 lb/bushel is equivalent to approximately 384 million US tons. Over a third, 5.3 billion bushels of corn were used for animal feed and residual use.

Not all corn kernels are the same. Corn can be classified into the following kernel types: dent, flint, flour, sweet, pop, or pod (Watson and Ramstad, 1991). Dent hybrids are grown in the US corn belt and in certain countries in Europe, with yellow endosperm as the predominant type. Flint corn is grown in South America and Northern Europe. Floury corn is one of the oldest types produced by the Aztecs and Incas. Sweet is the most common corn type consumed by humans as fresh, frozen, or canned (Gyori, 2010).

The corn kernel, or caryopsis, can be divided into three anatomical parts: the pericarp, the endosperm, and the germ, which differ in nutrient composition (Gyori, 2010). The pericarp, or outer layer, is the fibrous fraction which contains primarily cellulose, hemicellulose, lignin, and pectin. The germ, or inside layer, contains lipids such as linoleic acid. The largest portion of the kernel is the endosperm which contains starch granules dispersed in a matrix of storage proteins (Evers and Millar, 2002).

Corn Protein

In the corn kernel, storage proteins are synthesized in the rough endoplasmic reticulum (ER) and deposited in organelles located within the ER or in storage vacuoles (Mainieri et al., 2015). The major function of storage proteins is to accumulate high levels of amino acids within the limited space of the kernel (Shimoni and Galili, 1996). Cereal proteins consist of four main classes of storage proteins which are characterized by their solubility: albumin (water-soluble), globulin (salt-soluble), glutelin (alkaline-soluble), and prolamin (alcohol-soluble; Osborn, 1924). The cereal storage proteins have been given common names specific to the cereal grain: for wheat prolamin, gliadin; for wheat glutelin, glutenin; for corn, zein, for rye, secalin; for barley, hordein; for oats, avenin; for rice, oryzin; and for millet and sorghum; kafirin (Scherf et al., 2016).

Zein, a prolamin, comprises about 45-50% of the protein in corn (Shukla and Cheryan, 2001). Zein is insoluble in water except in the presence of alcohol, high concentrations of urea, high concentrations of alkali (pH $\geq$ 11), or anionic detergents. The insolubility in water is due to the hydrophobic amino acid composition. Zein is rich in glutamic acid (21-26%), leucine (20%), and proline (10%). However, it is deficient in essential amino acids such as lysine and tryptophan (Shukla and Cheryan, 2001).

Corn in the Pet Food Industry

Because both carbohydrates and fats contribute to energy requirements, carbohydrates are often included in pet foods to provide lower cost energy sources and functional properties during processing as compared to more costly fat sources. Although corn supplies protein in addition to carbohydrate, it is primarily used in pet food as an energy source due to its high starch content and relatively lower cost. Little information is available regarding the amount of corn used in US

pet food. However, it can be estimated that 0.85 million US tons of corn are used in pet food. This value was based on the following assumptions: the pet food market consists of 9.4 million US tons, 80% of pet foods have grains, grains compose 45% of the formula, and corn is 25% of these grains (Corsato Alvarenga and Aldrich, 2020; Corsato Alvarenga et al., 2021).

Even with the high utilization of corn in pet food, recent marketing claims have created a negative image for corn as a pet food ingredient. One of these claims is that corn as well as other grains cause allergies in dogs and cats. However, most food allergies are caused by animal proteins (Verlinden et al., 2006; Laflamme et al., 2014). In fact, Laflamme et al. (2014) reported that less than 1.5% of all food allergy cases are caused by grains, which are often caused by wheat not corn. In addition, some consumers believe that corn provides no nutritional value and that it is added as a "filler." However, scientific literature supports the use of corn in pet food. Corn used in pet food contains an average of 10% protein, 6% fat, 77% starch, and 11% total dietary fiber (Corsato Alvarenga et al., 2021). Many studies have reported the dry matter (DM) digestibility of corn in extruded kibble to be high when fed to dogs at an average of 80% (Walker et al., 1994; Murray et al., 1999; Twomey et al., 2002; Gajda et al., 2005; Carciofi et al., 2008; Fortes et al., 2010; Bazolli et al., 2015; Domingues et al., 2019). In addition, dogs fed corn-based diets had firm, high quality feces (Walker et al., 1994; Murray et al., 1999; Twomey et al., 2002). Even with this support, consumers are hesitant to feed corn to their pets. However, changing the narrative of corn to a protein source rather than a carbohydrate source may improve consumer acceptance for inclusion into pet food.

Corn Co-products

Co-products from the processing of corn may provide acceptable protein sources for pet food. Corn as an industrial input can be processed by either wet or dry milling. Starch is the

primary product from the wet milling process and can be converted into fuel ethanol, high-fructose corn syrup, or modified starches. Co-products from corn wet milling include corn gluten meal and corn gluten feed. The primary product of dry milling is ethanol which results in distillers dried grains with or without solubles as co-products. Since the starch in corn is converted to the primary product during both processing methods, the protein content of the co-products is significantly greater than the input corn initially processed. Therefore, the use of corn co-products, from the wet and dry milling of corn, as protein sources for pet food could provide value-added opportunities. Some corn co-products are already being used in pet food today. But most corn co-products are used in ruminant feeds due to their elevated fiber content, incomplete amino acid profile, and high nutrient variability.

The nutrient composition and animal utilization of these corn co-products are heavily influenced by processing methods. Therefore, extensive investigation is warranted to understand the extent of diet utilization for dogs and cats. To evaluate the use of an ingredient in pet food, research is often conducted to support animal health, consumer demands, and market positioning. This research will include such factors as the effects on nutrient digestibility and stool quality (consistency, volume, moisture) when fed to dogs or cats. Furthermore, markers of gut health can also be measured such as end-products of intestinal fermentation and the fecal microbiome. In addition, the palatability or "liking" of the ingredient can be assessed. Although not as common, the impact of ingredients on the physical diet production and final product quality can also be evaluated. These methods have been used in various combinations to evaluate the use of corn gluten meal (CGM), corn gluten feed (CGF), distillers dried grains with solubles (DDGS), and enhanced dried distillers grains in pet food.

Corn Gluten Meal (CGM)

Corn gluten meal (CGM) is produced from corn wet milling in which corn is steeped with water and sulfur dioxide. After steeping, the corn is coarsely ground to separate the germ from the endosperm for oil extraction. The remaining steeping water is condensed into steep liquor. The endosperm undergoes further screenings that separate the fiber from the protein and starch slurry. The fiber free endosperm is then centrifuged in order to separate the starch from protein, with the protein fraction resulting in CGM (CRA, 2023).

Corn gluten meal, which contains 60-75% crude protein (CP), 3% fat, and 1% crude fiber on a DM basis, is the most used corn protein source in pet food. The protein composition of CGM is mainly composed of endosperm proteins, zein and glutelin (Shukla and Cheryan, 2001). Compared to other corn protein sources such as CGF and DDGS, CGM contains less fiber which may support higher nutrient digestibility and better stool quality when fed to both dogs and cats. In addition, CGM may provide health benefits. For example, due to high concentrations of sulfur-containing amino acids, CGM can promote the production of acidic urine when fed to cats and dogs, which is desirable for prevention of struvite uroliths (Lewis et al., 1984).

A body of previous work was conducted which compared CGM to traditional animal meals used in pet food (Funaba et al., 2001, 2002, 2005). The first study fed cats a diet containing either fish meal or CGM as the major protein source for three weeks. This study reported that fecal moisture of cats fed the CGM diet was about 10% lower than cats fed the fish meal diet (64 vs 73%, respectively), resulting in stiffer stool. However, DM digestibility was not different among diets at an average of 75%. Urine pH of cats was similar among dietary treatments at an average of 6.29 (Funaba et al., 2001). The authors then went on to compare the effects of a diet containing CGM or meat meal as the major protein source (80% of dietary

protein content) when fed to cats for three weeks. The CGM diet had higher cysteine, histidine, isoleucine, leucine, methionine, phenylalanine, threonine, and valine concentrations compared to the meat meal diet. However, the meat meal diet had higher concentrations of arginine, lysine, and tryptophan. The meat meal may have been more palatable compared to the CGM as daily food intake was higher. Dry matter digestibility of the meat meal diet was greater at 79% compared to the CGM diet at 72%. However, fecal moisture of cats was similar for both diets at an average of 64%. Urine pH of cats fed dietary treatments was similar at an average of 6.13 (Funaba et al., 2002). The third study compared CGM to meat meal and chicken meal as the major protein source in cat diets. Similar to the previous study, cysteine, histidine, isoleucine, leucine, phenylalanine, threonine, and valine were greater in the CGM diet compared to the meat meal and chicken meal diets. Whereas arginine, lysine, and tryptophan were greater in the meat meal and chicken meal diets compared to the CGM diet. All diets were equal in methionine and taurine. The CGM diet resulted in lower DM digestibility at 78% compared to the chicken meal and meat meal diets at 80 and 83%, respectively. Fecal moisture of cats fed the CGM diet was greater than cats fed the chicken meal diet (56 vs 52%, respectively). Urine pH of cats fed the CGM diet was lowest at 7.08 and greatest for cats fed the meat meal diet at 7.99 (Funaba et al., 2005). Based on these three studies, CGM was comparable to fish meal but inferior to meat meal and chicken meal regarding DM digestibility. Whereas, in terms of fecal consistency, CGM had similar results to meat meal when fed to cats. In addition, cats fed CGM had similar or decreased urine pH compared to animal-based meals. Of note, the arginine content in the CGM diets was slightly below AAFCO minimum levels for cat maintenance. Therefore, arginine supplementation may be required when feeding CGM as the primary protein source for cats.

Corn Gluten Feed (CGF)

Corn gluten feed (CGF) is produced by the same process as CGM. However, after separation of the fiber from the protein and starch slurry, the fiber is mixed with the steep liquor to create CGF (RFA, 2023). Therefore, approximately 2/3 of CGF is composed of fibrous residue where the other 1/3 is dried steep liquor (Blasi et al., 2001). The composition of CGF is influenced by the proportion of steep liquor which contains more energy and protein than the bran (Scott et al., 1997). On average, CGF contains 20-25% CP, less than 4% fat, and 6-10% crude fiber on a DM basis. The protein composition of CGF is mostly germ proteins, albumins and globulins (Shukla and Cheryan, 2001). Compared to CGM, CGF contains elevated fiber levels (> 6% crude fiber) which is likely to have a greater impact on nutrient digestibility and stool quality when fed to both dogs and cats. Due to the addition of sulfur dioxide during the wet milling process to aid in the extraction of starch, the sulfur content of CGF ranges between 0.33 to 0.73% on a DM basis. Therefore, sulfur levels need to be considered during diet formulation to prevent toxicity. However, like CGM, increased sulfur levels could be beneficial for urinary tract health. Another consideration for CGF utilization in pet food is that it contains lactic acid, produced during processing, which may impact palatability and fecal pH (Bernalier et al., 1999; Rausch and Belyea, 2006).

Kawauchi et al. (2011) conducted two studies to evaluate varying inclusion levels of CGF in dog diets. The CGF evaluated contained 22% CP, 4% fat, and 38% total dietary fiber (TDF) on a DM basis. The first study used a difference method to calculate digestibility in which 30% of a reference diet was replaced with CGF. Overall, there was a decrease in nutrient digestibility with the 30% inclusion of CGF. For example, the DM digestibility of the CGF diet was 74% whereas the DM digestibility of the reference diet (0% CGF) was 83%. Feces of dogs fed the

CGF diet were less dry and firm compared to dogs fed the reference diet. The second study used a regression method to calculate digestibility including a basal diet (0% CGF) and diets containing either 7, 14, or 21% CGF. Inclusion of CGF resulted in a linear reduction for DM, organic matter (OM), CP, fat, and gross energy (GE) digestibility. The digestibility of TDF had a quadratic reduction with CGF inclusion. Fecal output of dogs increased linearly from 56 to 107 g/dog/day with increased CGF. There was a linear reduction for fecal DM, fecal score, and fecal pH of dogs fed increasing levels of CGF. Total short chain fatty acid (SCFA) production increased quadratically with CGF, mainly due to a linear increase in propionate. Urine pH of dogs decreased linearly with inclusion of CGF (Kawauchi et al., 2011).

Distillers Dried Grains with Solubles (DDGS)

Distillers dried grains with solubles (DDGS) are a co-product from the dry milling of corn to ethanol. They are produced by blending and drying the non-fermentable residues left after the fermentation of corn starch. After fermentation, the stillage is centrifuged to remove the insoluble solids, also known as wet distillers grains, and liquids. Water is then evaporated from the liquid portion, resulting in the condensed distillers solubles. The wet distillers grains and solubles are then dried in a rotary drum dryer to produce DDGS (Kingsly et al., 2010). Similar to CGM and CGF, the nutritional content and functionality of DDGS varies based on grain source and processing technique. However, on average, DDGS contains 31% CP, 11% fat, and 8% crude fiber on a DM basis.

Since DDGS are a product of fermentation, they contain a residual yeast component which contributes to their nutritional composition and may provide functional properties and health benefits when fed to animals. However, the composition and functional properties of yeast in ethanol co-products is not well established because of a lack of standardized analytical

methods to determine the dead yeast and its chemical components in these ingredients. Published estimates of the contributions of yeast to DDGS biomass and crude protein are extremely variable (Shurson, 2018). Bauernfeind et al. (1994) compared yeast cell counts in distillers dried solubles with the cell count in dried yeast, calculating that about 20% of the weight of dried solubles is yeast. This method assumes the proportion of solubles that is added to the grain fraction prior to manufacturing DDGS, which varies among ethanol plants (AAFCO definition 27.6). Ingledew (1999) used a mass balance approach and numerous assumptions involving ethanol production processes. Estimations from this study are unreliable due to differences in processes among plants and the continuous improvements being made to ethanol production (Shurson, 2018). Belyea et al. (2004) calculated the average ratio of amino acid concentrations in yeast and DDGS and suggested that about 50% of DDGS protein was derived from yeast. This method did not account for the protein contribution from corn or consider the nonessential amino acid content. Han and Liu (2010) developed a multiple linear regression model that included the relative percentages of amino acids throughout the entire production of DDGS. Based on this equation, it was estimated that yeast accounts for 20% of the crude protein content in DDGS while corn contributes to 80%. While none of these methods directly measures the yeast content in DDGS, the calculation of Han and Liu (2010) likely provides the most accurate estimate as it accounted for the amino acid profile in both corn and yeast, included all amino acids, and was based on relative percentages rather than absolute concentrations of amino acids (Shurson, 2018). However, amino acid profiles can vary among corn varieties, crop year, and yeast strains. Furthermore, new production processes will likely change amino acid concentrations (Shurson, 2018). Nevertheless, the yeast component contributes to the amino acid profile of DDGS. Yeast proteins have been reported to contain a high concentration of lysine and tryptophan, making

them a good complement for zein protein (Yamada and Sgarbieri, 2005). Therefore, DDGS are a more balanced and complete source of amino acids in comparison to other corn protein sources such as CGM (Belyea et al., 2004).

In addition to contributing to the amino acid profile, the yeast in DDGS may provide health benefits due to β-glucans, mannooligosaccharides (MOS), and nucleotides in their cell wall. Estimates of these components also vary among published studies. For example, Lim et al. (2009) estimated that DDGS contained 0.57% β-glucan whereas Kim et al. (2008) reported that the average total glucan content in DDGS was 21.2%. The discrepancies are likely due to differences in analytical procedures and the actual composition of the β-glucans measured (Shurson, 2018). Alizadeh et al. (2016) reported that wheat-corn DDGS contained 1.6% mannose and 0.13% nucleotides. β-glucans are glucose polymers that are present in cell walls of yeast, fungi, and some bacteria. They are also present in the endosperm cell walls of cereal grains such as oats and barley (Volman et al., 2008). The structure of β-glucans varies among sources affecting their physiological functions. The β-glucans in yeast cell walls primarily consist of β-1,3 linkages and β-1,6 linked branches (Novak and Vetvicka, 2008). Yeast β-glucans are reported to enhance the immune response by increasing cytokine production and resistance to infection (Suzuki et al., 1990). Mannooligosaccharides are non-digestible short chain branched carbohydrates composed of up to 10 mannose units linked by α-1,3 and α-1,6 bonds (Tungland, 2018). They are reported to bind and limit the colonization of pathogens in the gastrointestinal tract, improving the integrity of the intestinal mucosa and activity of the immune system (Spring, 2015). Nucleotides are a class of molecules that are linked together to form DNA and RNA. They are composed of a phosphate group; adenine, cytosine, guanine, and thymine or uracil bases; and a pentose sugar. Nucleotides have been reported to improve intestinal morphology

and function, immune response, and composition of intestinal microbiota (Sauer et al., 2011). In addition, the nucleotides and high glutamic acid concentration in yeast provide an umami, or meaty, aroma and taste (Nagodawithana, 1992; Ugawa and Kuihara, 1994), which may enhance palatability in dogs and cats. Furthermore, yeast cell walls contain chitin, a linear polysaccharide composed of β-1,4 linked N-acetylglucosamine residues (Bulik et al., 2003), which can act as a dietary fiber. Therefore, the yeast cell wall, along with the fiber fraction of corn, may provide substrate for microbial fermentation in the gut, serving as a prebiotic (Silva et al., 2016; Iram et al., 2020).

Multiple studies have evaluated the use of DDGS in pet food. Allen et al. (1981) conducted four trials consisting of varying inclusion levels of DDGS in dog diets. The first trial evaluated DDGS exchanged for corn at 0, 4, 6, and 8%. Dry matter digestibility was not impacted by DDGS inclusion. In addition, fecal DM and fecal output of dogs were similar among dietary treatments. In order to determine the inclusion level at which nutrient digestibility and stool quality would be impacted, DDGS was exchanged for corn and soybean meal (SBM) at increased levels (8.9 and 15.7%). Dry matter digestibility was similar for the 0 and 8.9% DDGS diets. However, DM digestibility decreased with the 15.7% inclusion. In addition, the feces of dogs fed 15.7% DDGS were drier than the feces of dogs fed the remaining treatments. Overall, the first two trials indicated that moderate levels of DDGS (≤ 8.9%) can successfully be incorporated into adult dog diets without altering nutrient digestibility or stool quality. To further determine the inclusion level at which DDGS impacted the results in the second experiment, DDGS were included in diets at 13.1 and 26.1%. Dry matter and energy digestibility decreased with 26.1% DDGS but was maintained with 13.1% inclusion. Crude protein digestibility was not affected by DDGS inclusion. Finally, a study was conducted to determine if similar results would

be observed in puppies by evaluating diets containing 0% or 14.1% DDGS. There appeared to be a greater impact of DDGS in puppies compared to adult dogs as DM and energy digestibility decreased with the 14.1% inclusion.

Silva et al. (2016) evaluated diets containing 0, 6, 12, or 18% DDGS fed to dogs. The DDGS evaluated in their study contained 30% CP, 9% fat, and 9% crude fiber on a DM basis. Increasing levels of DDGS linearly decreased DM, OM, CP, fat, and GE digestibility. Fecal score of dogs was not altered by DDGS inclusion. However, fecal pH of dogs was more acidic with DDGS inclusion. In the palatability assessment, dogs preferred the diet containing 18% DDGS compared to the diet without DDGS.

Risolia et al. (2019) compared dog diets containing either 0% or 20% DDGS. The DDGS contained 25% CP, 11% fat, and 32% TDF on a DM basis. There were no differences in density, size, expansion index, hardness, or uniformity of kibble with DDGS inclusion. The inclusion of DDGS decreased DM, OM, fat, and GE digestibility. However, CP digestibility was not affected by DDGS inclusion. The fecal pH of dogs decreased when fed the DDGS diet. There were no differences in fecal DM or fecal score of dogs fed DDGS. The inclusion of DDGS increased total SCFA, acetate, and propionate concentration in fecal samples. Whereas butyrate, isobutyrate, valerate, and isovalerate production were similar among diets. In the palatability evaluation, there was no preference between diets with or without DDGS based on first choice or intake of dogs.

The decreased nutrient digestibility with CGF and DDGS is likely explained by the elevated fiber content. Fiber creates a physical barrier preventing action of digestive enzymes, such as amylase, lipase, and proteases, decreasing digestion and absorption of nutrients (Vanderhoof, 1998). This is supported by previous studies which have reported a negative

correlation between dietary fiber and nutrient digestibility (Fahey et al., 1992, Earle et al., 1998; Cole et al., 1999). In addition, both CGF and DDGS contain elevated levels of insoluble fiber which increases the rate of food passage in the small intestine, and lowers digestibility and nutrient absorption (Probert et al., 1995). The increased fecal output and reduced fecal score for diets containing CGF and DDGS also reflects the increase in insoluble fiber which causes water retention in feces and increases bulk (Meyer and Tungland, 2001).

The decreased pH and increased SCFA concentration of feces from dogs fed CGF and DDGS indicates that these ingredients could be utilized as a prebiotic. Prebiotics are reported to decrease production of putrefactive compounds which are harmful to intestinal health and cause bad odors in dog feces (Yamka et al., 2006). Therefore, the inclusion of corn co-products could also benefit animal health. Further research is warranted to determine optimum inclusion level to balance nutrient digestibility and fermentation.

To offset the decrease in nutrient digestibility, some previous studies have evaluated the impact of dietary enzyme supplementation on the digestibility of DDGS in dogs. The inclusion of DDGS in poultry and swine diets is often associated with the addition of fiber-hydrolyzing enzymes (Gaines et al., 2007). These enzymes have been reported to reduce the variations in nutritional quality of diets, improve food digestion, and reduce fecal excretion of nutrients (Bedford, 1993). For example, xylanase can hydrolyze the xylan fraction of hemicellulose and reduce diet viscosity, facilitating access of endogenous enzymes to nutrients, whereas protease could enhance the release and solubilization of fiber-associated proteins, improving digestibility (Risolia et al., 2019). Silva et al. (2016) reported that xylanase increased digestibility of diets containing 12% DDGS or greater when fed to dogs. However, Risolia et al. (2019) reported no improvement in DDGS digestibility with supplementation of xylanase or protease when fed to

dogs. The differing enzyme effects between Silva et al. (2016) and Risolia et al. (2019) could be due to the difference in enzyme concentration and activity, which was greater in Silva et al. (2016). Further research is warranted to determine if enzyme supplementation is beneficial in dog and cat diets containing corn co-products.

Enhanced Dried Distillers Grains

The development of new technologies in the ethanol industry has allowed for increased protein and reduced fiber content of dried distillers grains making them more appropriate for pet food. These ingredients are produced using post-fermentation separation technologies to split the protein and yeast from fiber prior to drying. There are only two studies which have evaluated enhanced dried distillers grains in pet food (Kaelle et al., 2023; Smith and Aldrich, 2023). The DDGS evaluated in the previous studies in dogs contained 20 to 30% protein whereas the enhanced products evaluated in Kaelle et al. (2023) and Smith and Aldrich (2023) contained 45% and 54% protein, respectively.

High-Protein Dried Distillers Grains (HPDDG)

Kaelle et al. (2023) evaluated the effects of high-protein dried distillers grains (HPDDG) containing 45% protein, 14% fat, and 11% crude fiber on a DM basis when fed to dogs. Their experimental diets contained increasing levels of HPDDG at 0, 7, 14, and 21% in exchange for SBM, and results found no significant differences in nutrient digestibility, fecal DM, score, pH, and ammonia. The production of other SCFA and branched chain fatty acids (BCFA) did not differ among dietary treatments; but there was a linear increase in valeric acid in fecal samples of dogs fed increasing levels of HPDDG. For the fecal microbiota analysis, six genera out of the 127 bacteria genera identified presented different relative abundance among dietary treatments. The genera *Streptococcus* and *Megamonas* decreased linearly with graded HPDDG inclusion.

Whereas the *Blautia, Lachnospira,* and *Clostridiales* genera showed a quadratic increase with increasing inclusion of HPDDG. In addition, a quadratic response was observed in the genera *Prevotella* with increased HPDDG. For alpha diversity, there was a linear increase in the number of operational taxonomic units, a quadratic effect for the Shannon index, and a trend for a linear increase in the Chao-1 index with the inclusion of HPDDG. For the palatability assessment, there was no difference in first choice or intake ratio when comparing 0 vs 7% HPDDG. However, dogs consumed more of the 21% compared with the 0% HPDDG, with no difference detected for first choice preference.

In contrast to studies which have evaluated traditional DDGS in dogs where decreased nutrient digestibility and increased fecal output were found, HPDDG did not impact nutrient digestibility or stool quality. This may be due to the greater proportion of protein to fiber in HPDDG compared to traditional DDGS. In addition, the raw material composition and processing methods such as the presence of solubles in DDGS, but not in HPDDG, fermentation end-products, and residual yeast concentration may impact results (Liu, 2011). Furthermore, HPDDG did not appear to have as great of an effect on SCFA production compared to traditional DDGS, where the only difference observed was the increase in valerate with HPDDG. This is likely due to the fiber in HPDDG, as proportions of SCFA produced in the gut may change depending on dietary fiber sources and content (de Godoy et al., 2015; Harris et al., 2020). The shifts in the fecal microbiota support that the fiber fraction of HPDDG was fermented by the gut microbiota of dogs. The reduction in *Streptococcus* and the increase in *Blautia* relative abundance in dogs fed HPDDG may be related to the improvement in gut functionality (Suchodolski et al., 2012; Alshawaqfeh et al., 2017; Felix et al., 2022). In addition, the genus *Prevotella*, a well-known fiber fermenter and SCFA producer, has been associated with intestinal

health (Schmidt et al., 2018; Minamoto et al., 2019; Pilla and Suchodolski, 2020). The increase in *Clostridiales* with the 21% inclusion of DDGS is also a possible indicator of improved intestinal functionality. *Clostridiales* belongs to the Firmicutes phylum which has a crucial role in preventing permeable bowel syndrome, which occurs when the permeability of the intestinal barrier is altered, causing excessive inflammation (Suchodolski et al., 2012). In addition, a greater richness and diversity of microorganisms is one of the leading indicators of a healthy gut microbiota (Ziese and Suchodolski, 2021), which was observed in Kaelle et al. (2023) with the increase in alpha diversity indices with HPDDG. Therefore, HPDDG appeared to shift the dog microbiota to support intestinal functionality and health. The increased palatability with HPDDG is likely due to the yeast component which contains high concentrations of glutamic acid (Martins et al., 2014; Lin et al., 2019; Kaelle et al., 2022). The results from Kaelle et al. (2023) indicate that HPDDG could be a more suitable protein source for pet food when compared to traditional DDGS due to the maintenance of nutrient digestibility and stool quality when fed to dogs. The addition of HPDDG also indicated potential beneficial effects on intestinal functionality; however, the evaluation of traditional DDGS on the canine microbiome has yet to be conducted.

Corn Fermented Protein (CFP)

Smith and Aldrich (2023) have also evaluated a high protein dried distillers grain called corn fermented protein (CFP) in a pet food application. This study compared a 25% inclusion of CFP, which contained 54% CP, 4% fat, and 28% TDF on a DM basis, to CGM and SBM. To achieve a similar bulk density among all diets, preconditioner (PC) water input and the mass restriction valve (MRV) during production of the CFP diet were adjusted. The CFP diet required more PC water than the CGM diet. In addition, the MRV was most restricted for the CFP diet at

40% and least restricted for the CGM diet at 60%. Remaining processing inputs such as PC steam, extruder (EX) water, and EX RPM were consistent among all treatments. Processing outputs such as total mass flow (TMF), die temperature, percent load, specific mechanical energy (SME), and in-barrel moisture (IBM) were similar among all treatments. However, CFP had the greatest die pressure whereas CGM had the lowest. Kibble length, mass, volume, and piece density were similar for all dietary treatments. Specific length of kibble was also similar among dietary treatments indicating no difference in longitudinal expansion. However, kibble diameter was smallest for CFP, which was reflected in the sectional expansion index (SEI) of kibble. There were no differences in kibble hardness or toughness among dietary treatments. Dogs fed CGM had fewer defecations than dogs fed CFP or SBM. Dry fecal output was also lowest for dogs fed GGM with CFP resulting in 55% more fecal output among dogs. Fecal score of dogs fed CFP were firmer than dogs fed CGM. Reduced DM, OM, and fat digestibility was observed for CFP compared to CGM and SBM. The CFP diet had similar CP and TDF digestibility to SBM but was lower than CGM. Gross energy digestibility was greatest for CGM and lowest for CFP. For the palatability assessment, dogs preferred CGM over CFP based on intake but there was no preference for first approach. There were no differences based on intake or first approach for dogs when CFP and SBM were compared. Dogs preferred CGM over SBM based on first choice but had no preference based on intake. In contrast, cats preferred CFP and SBM over CGM based on intake. However, there was no preference between SBM and CFP for cats based on intake. There were no differences among any of the comparisons for first choice in cats.

The low starch content in CFP (2.7%) likely contributed to the adjustments that were required during processing to produce a similar bulk density to the other treatments (Smith and Aldrich, 2023). Previous studies have reported that decreased starch content resulted in

decreased expansion, requiring changes in processing parameters to produce a similar product (Chevanan et al., 2007; Stein and Shurson, 2009). The increased PC water with CFP was likely needed to aid in the gelatinization of starch (Tran et al., 2008). In contrast, CGM is well known for its starch content which readily gelatinizes (Beylea et al., 2014). Another way to aid in the gelatinization of starch and promote kibble expansion is by increasing mechanical energy. In order to do this, Smith and Aldrich (2023) closed the MRV diameter, causing more back pressure and friction behind the die plate. Therefore, the reduced flow in the EX barrel created more mechanical energy to further gelatinize the starch (Riaz, 2000). This also explains the increased die pressure during the production of the CFP diet. The smaller SEI for CFP was likely due to the fiber content as previous studies have reported that increased fiber content can decrease radial expansion. (Hsieh et al., 1989 and 1991). Overall, it was possible to create similar products using CFP with minimal changes to processing parameters when compared to CGM and SBM.

The higher fiber level in CFP compared to CGM and SBM likely resulted in the differences in daily defecations, fecal mass, fecal score, and nutrient digestibility when fed to dogs. The preference for CGM over CFP and SBM in dogs could have been due to the corn starch in CGM (Li et al., 2017), whereas the higher affinity to CFP in cats compared to dogs could be due to the yeast, which is highly palatable to cats, likely due to the higher presence of nucleotides (White and Boudreau, 1975; Swanson and Fahey, 2004).

The results from Smith and Aldrich (2023) indicate that CFP may be inferior to CGM regarding nutrient digestibility and stool quality when fed to dogs. However, the yeast component in CFP may enhance palatability for cats compared to CGM. Although not evaluated in Smith and Aldrich (2023), the elevated fiber and yeast in CFP compared to CGM may promote intestinal

health similar to HPDDG. Therefore, research is needed to determine the impact of CFP on animal health.

Conclusion

The utilization of corn co-products in pet food may provide value added opportunities while providing sustainable and cost-effective ingredients. Overall, the work which has evaluated traditional corn co-products in dogs and cats is dated. Only recently has there been increased interest in these ingredients regarding vegetarian diets and sustainability. However, to increase utilization of corn co-products enhanced ingredients such as HPDDG and CFP may be warranted to improve consumer perception of corn in pet food. Therefore, the objectives of this work were to determine: 1) the optimal inclusion level of CFP for use in pet food and 2) the contribution from the yeast component of CFP on overall nutrition and animal health. To achieve these objectives, the effects of CFP on extrusion processing, kibble quality, palatability, nutrient digestibility, stool quality, and the fecal microbiome were assessed when fed to both dogs and cats.

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Chapter 2 - Evaluation of graded levels of corn fermented protein (CFP) on stool quality, apparent nutrient digestibility, and palatability in healthy adult cats

Abstract

Dried distillers' grains, co-products from the ethanol industry, may provide sustainable ingredients for pet food. Due to new post-fermentation separation techniques, corn fermented protein (CFP) is higher in protein and lower in fiber compared to traditional dried distillers' grains, increasing its appeal for inclusion into pet food. Therefore, the objectives of this study were to determine the effects of increasing levels of CFP on stool quality, apparent total tract digestibility (ATTD), and palatability in adult cats. Four extruded diets were fed to 11 adult cats in an incomplete 4x4 replicated Latin square design. The control diet contained 15% soybean meal (0C) and CFP was exchanged for soybean meal at either 5%, 10%, or 15% (5C, 10C, 15C). Cats were fed each dietary treatment for 9-d adaption followed by 5-d total fecal collection. Feces were scored on a 1-5 scale, with 1 representing liquid diarrhea and 5 representing hard pellet-like (Carciofi et al., 2008). A fecal score of 3.5-4 was considered ideal. Titanium dioxide was added to all diets (0.4%) as a marker to estimate digestibility. Data were analyzed using a mixed model in SAS (version 9.4, SAS Institute, Inc., Cary, NC) with treatment as a fixed effect and cat and period as random effects. Fecal dry matter percent and dry fecal output were greater ($P < 0.05$) at elevated levels of CFP. Stool scores were maintained ($P > 0.05$) throughout treatments (average; 4). Dry matter, organic matter, crude protein, and gross energy ATTD decreased when cats were fed 15C. There was no difference in ATTD of fat or total dietary fiber among treatments. For palatability assessment, cats preferred 5C over 0C but had no preference

with increased CFP inclusion. These results suggest that CFP is comparable to SBM but there may be a maximum inclusion level of 10% when fed to cats.

Introduction

Sustainability has become a demand in all industries as the overuse of resources has become a concern (Global Sustainability Study, 2021). The global pet food market is valued at almost $95 billion dollars annually and is expected to continue growing (Global Pet Food Market Size & Share Report, 2021). Due to its substantial size, a shift in the pet food industry to more sustainable products and production systems could have a significant impact. However, optimizing the sustainability of pet food is challenging as they are often formulated to exceed nutrient requirements, use ingredients that compete directly with the human food supply, and (or) are overconsumed by pets resulting in food wastage (Swanson et al., 2013).

The primary concerns regarding sustainability within the pet food industry are ingredient selection and nutrient composition (Acuff et al., 2021; Swanson et al., 2013). Specifically, protein is the most expensive and ecologically demanding macronutrient (Berardy et al., 2019; Nijdam et al., 2012). However, protein source and inclusion level are key drivers for pet owner selection of pet food (Acuff et al., 2021). Therefore, pet foods are often formulated to exceed nutritional requirements for protein and may contain protein sources which directly compete with the human food supply. On average, commercially available dry dog and cat foods contain 31% crude protein (DM basis; Hill et al., 2009), which exceeds the minimum recommendations set by the Association of American Feed Control Officials (AAFCO) at 18% for adult dogs and 26% for adult cats (DM basis). In addition, meat or muscle tissue is perceived by pet owners to be of higher nutritional quality for dogs and cats compared to protein co-products with animal-based sources preferred over plant-based sources (Okin, 2017; Association for Pet Obesity Prevention,

2018). However, crude protein digestibility, corrected for endogenous losses, of both sources has been reported to be similar when fed to dogs and cats (Golder et al., 2020). The amino acid profile of plant-based sources can meet that of animal-based proteins with the use of complementary ingredients (Li and Wu, 2020). Of note, taurine concentration must be especially considered with increased inclusion of plant-based protein sources. Substitution of meat for plant-based co-products could support environmental and economic sustainability by using fewer natural resources and providing competitively priced alternatives resulting in a smaller carbon footprint (Knight and Leitsberger, 2016; Acuff et al., 2021).

Specifically, distiller's dried grains with solubles (DDGS), a major co-product from ethanol production, may be of interest. These DDGS have commonly been included in livestock feed due to their moderate levels of protein, fat, and fiber (Lodge et al., 1997; Batal and Dale, 2006). Previous studies have supported the use of conventional DDGS in pet food (Allen et al., 1981; Silva et al., 2016). However, the use of co-product ingredients in pet food has been limited due to consumer perception. Therefore, ethanol companies are developing new techniques to enhance the nutritional composition for use in pet food. These enhanced products are produced using post-fermentation separation technologies to split the protein and yeast from the fiber prior to drying. Therefore, they are higher in protein and lower in fiber compared to traditional DDGS, which should increase their appeal for inclusion into pet food. Corn fermented protein (CFP), an enhanced ethanol co-product, has already been evaluated in pet food and was reported to have comparable digestibility and palatability to soybean meal when fed to dogs (Smith and Aldrich, 2022). Based on this study, further research is needed to determine the optimum inclusion level in pet foods. Therefore, the objective of this study was to evaluate increasing levels of CFP on stool quality, nutrient digestibility, and palatability when fed to adult cats.

Materials and Methods

The feeding trial was conducted at Kansas State University Veterinary Medicine Complex (Coles Hall) under the Institutional Animal Care and Use Committee (IACUC) #4348 protocol. The palatability trial was conducted at Summit Ridge Farms (Susquehanna, PA) under protocols KSUPALF00420, KSUPALF00520, and KSUPALF00620.

Diet Formulation and Production

Four different diets with increasing levels of CFP (POET Bioproducts, Sioux Falls, SD), as a replacement for equal levels of soybean meal (SBM; Fairview Mills, Seneca, KS), were formulated. Soybean meal was chosen as the control protein source due to a previous study which reported similar digestibility of SBM and CFP when fed to dogs (Smith and Aldrich, 2022). In addition, the protein content of CFP is comparable to SBM. The analyzed chemical composition of experimental ingredients, SBM and CFP, are presented in **Table 2.1**. The control diet contained 15% SBM (0C) and CFP was exchanged at either 5% (5C), 10% (10C), or 15% (15C) for the SBM. The formulated diets met the AAFCO nutritional requirements of healthy adult cats. Titanium dioxide (0.40%) was added to serve as an indigestible marker to estimate apparent total tract nutrient digestibility. The dry raw materials, except for the CFP, SBM, and titanium dioxide, comprised the dry base ration and were purchased from a commercial mill (Fairview Mills, Seneca, KS) (**Table 2.2**).

Each diet was mixed and produced using a single screw extruder (model E525, Extru-Tech, Manhattan, KS). The cool and dry product was packaged in laminated bags and transferred to the laboratory at Kansas State University to be coated. Kibbles were coated with chicken fat protected with natural antioxidants and a dry powdered flavor designed for cats. Coated diets were stored in poly-lined Kraft paper bags until fed.

Feeding Trial

Eleven healthy adult (3.1 ± 1.7 years) American shorthair cats (10 males and 1 female) were enrolled in this study. The cats had an average body weight of 5.6 ± 1.7 kg, and food allowance was controlled to maintain their weight throughout the study. The daily metabolizable energy requirement was calculated for lean cats with $100*BW_{kg}^{0.67}$ (NRC, 2006). The body weight of cats was measured at the beginning, middle, and end of each period. The study was conducted as an incomplete replicated Latin square design. Each of the four periods were composed of 9 days for adaptation followed by 5 days of collection. In this model, each animal served as its own control, and each treatment had 11 total observations.

The cats were housed on a 12 h light cycle with lights off from 1900 to 0700. In the adaption period, the cats were group-housed but fed individually. Whereas in the collection period, the cats were individually housed in stainless steel cages. The cats received two feedings per day at 0700 and 1600 h with access to food for 1 h and water *ad libitum*. In case a cat refused to eat an experimental diet, an additional 0.5-1.0% flavor enhancer was added topically to the food. During the collection period, all feces and orts were collected daily. The fecal samples were weighed and scored on a 1–5 scale with 0.5 increments [1 – liquid diarrhea to 5 – dry hard pellets; (Carciofi et al., 2008)]. A score of 3.5-4.0 was considered ideal. In addition, the pH of a fresh fecal sample (within 15 minutes of defecation) was recorded in triplicate with a calibrated glass-electrode pH probe (FC240B, Hanna Instruments, Smithfield, RI). Fecal samples were stored in labeled whirl-pak bags in a freezer until further processed. The orts were dried and weighed to compute daily food intake.

Digestibility Calculations

After each collection period, feces from each cat were composited and dried at 55°C in a forced air oven until constant weight (24-48 h). Dried samples were ground to pass through a 1 mm screen in a laboratory fixed blade impact mill (ZM 200, Retsch, Verder Scientific, Haan, Germany). Titanium dioxide (TiO_2) concentration was measured in food and feces using a spectrophotometric plate reader (Gen5TM, Biotek® Instruments, Inc.Winooski, VT) at 410 nm (Myers et al., 2004). Apparent total tract digestibility (ATTD) was estimated by titanium dioxide using the following equation:

$$ATTD = \left[1 - \frac{\%\ \mathrm{TiO2}\ in\ food * \ \%\ nutrient\ in\ feces}{\%\ \mathrm{TiO2}\ in\ feces * \ \%\ nutrient\ in\ food}\right] * 100$$

Digestibility was calculated using both the total collection and titanium dioxide methods, which resulted in similar digestibility values and trends. However, the titanium dioxide method resulted in a lower standard error of the mean. Therefore, digestibility values from the titanium dioxide method were selected to report in this manuscript.

Nutrient Analysis

Food and partially-dried fecal samples were analyzed in duplicate for moisture (AOAC 930.15), ash (AOAC 942.05), fat by acid hydrolysis and hexane extraction (AOAC 960.39), gross energy (Parr 6200 Calorimeter, Parr Instrument Company, Moline, IL), and total dietary fiber (AOAC 991.43). Crude protein was determined by Dumas combustion (AOAC 990.03) using a nitrogen analyzer (FP928, LECO Corporation, Saint Joseph, MI).

Palatability Trial

Experimental treatments (5C, 10C, and 15C) were evaluated for palatability vs. the control diet (0C) by cat panels at a commercial kennel (Summit Ridge Farms, Susquehanna, PA). Each experiment was conducted as a split-plate test, in which two stainless steel bowls

containing 100 g of food were presented to animals for a total of 4 hours. Each comparison trial was repeated for two days, with a bowl position switched daily. Twenty animals were fed daily, providing 40 observations for each paired comparison test. Preference was determined based on animals' first choice and total food consumption. Data from consumption was represented as the following ratio:

$$Intake\ Ratio = \left(\frac{consumption\ of\ Diet\ A}{total\ consumption\ Diet\ A + Diet\ B}\right)$$

Statistics

The digestibility experiment was conducted as an incomplete 4x4 replicated Latin square design. Each of the 11 experimental units (cats) were assigned to treatment using the spreadsheet by Kim and Stein (2009). Data were analyzed using a GLIMMIX procedure in SAS (version 9.4, SAS Institute, Inc., Cary, NC) with treatment as a fixed effect and cat and period as random effects. Tukey's post hoc test was applied for the least-squares means separation, with significance considered at $P < 0.05$.

In the palatability experiment, the consumption ratio was analyzed using a t-test in a 2-way ANOVA and the first-choice preference was analyzed using a Chi^2 test. The twenty cats were considered the experimental units for analysis.

Results and Discussion

Diet Chemical Analyses

During production experimental diets were dried to around 5% moisture. On a dry matter basis, the experimental treatments were approximately 91% organic matter (OM), 36% crude protein (CP), 13% fat, and 4918 kcal/kg gross energy (**Table 2.3**). The total dietary fiber (TDF) ranged from 16.1% in the 15C to 13.8% in the 0C. This would be expected as the experimental ingredients, SBM and CFP, have similar protein content (53.4% and 52.6%, respectively), but

CFP contained 34.9% TDF while SBM contained 19.9% TDF (**Table 2.1**). Therefore, it would be expected that as CFP replaced SBM that TDF of the diets would rise accordingly.

Feed Intake and Fecal Characteristics

Feed intake was not different ($P > 0.05$) among dietary treatments at an average of 72.9 g/d (**Table 2.4**). This was to be expected as food intake was controlled to maintain body weight. In addition, there were no significant feed refusals among dietary treatments and cats consumed the diets readily. Fecal dry matter percent was greater for cats fed the 10C and 15C treatments (33.2% and 32.8%, respectively) compared to the 0C treatment at 30.7% ($P < 0.05$), while the 5C treatment was intermediate at 32.1% (**Table 2.4**). Allen et al. (1981) reported an increase in fecal dry matter percent for dogs fed a diet containing 15.7% DDGS relative to dogs fed to a diet containing 0% DDGS. However, in their study there was no difference in fecal dry matter percent for dogs fed diets containing up to 8.9% DDGS. Dry fecal output was greater for cats fed the 15C treatment (15.8 g/d) than those fed the 0C and 5C treatments (12.7 g/d and 13.3 g/d, respectively; $P < 0.05$). Dry fecal output of cats consuming the 10C treatment was intermediate at 14.5 g/d. The increase in fecal output with the 15% inclusion is likely due to the increased fiber content of the diet. Yamka et al. (2003) reported that an increase in dietary fiber may result in decreased digestion and greater fecal mass due to an increased rate of passage through the digestive system and decreased absorption. Likewise, Smith and Aldrich (2022) reported an increase in dry fecal output for dogs consuming a diet containing 25% CFP compared to SBM. Fecal defecation of cats was similar among dietary treatments at an average of 0.85 times per day ($P > 0.05$). Fecal score was not impacted ($P > 0.05$) by dietary treatment with an average of 4.0, which was considered near ideal (**Table 2.4**). Smith and Aldrich (2022) also reported no differences in fecal defecation or fecal score among dogs consuming diets containing CFP or

SBM. Fecal pH was lowest ($P < 0.05$) for cats consuming the 10C treatment. This result was interesting as differences were expected for the 15C treatment rather than the 10C treatment as previous studies reported increased fiber intake associated with lower fecal pH (Faruk et al., 2018).

Apparent Total Tract Digestibility

Cats fed the 0C, 5C, and 10C treatments resulted in a greater DM digestibility (80.6%, 80.1%, and 80.7%, respectively) compared to those fed the 15C treatment (78.9%) (**Table 2.5**). A previous study reported a similar DM digestibility of 79.9% for a diet containing 15.7% DDGS when fed to dogs (Allen et al., 1981). In addition, Allen et al. (1981) reported a decrease in DM digestibility when dogs were fed the 15.7% DDGS treatment but no difference with up to 8.9% DDGS inclusion when compared to a control. The OM digestibility was lowest ($P < 0.05$) for cats fed the 15C treatment. The CP digestibility was greater for cats fed the 0C, 5C, and 10C treatments (87.0%, 86.6%, and 86.9%, respectively) when compared to the 15C treatment (85.4%). Silva et al. (2016) reported a similar CP digestibility at 85% with an 18% DDGS inclusion fed to dogs. Gross energy (GE) digestibility followed the same trend with 15C reporting a lower value at 84.2% compared to the remaining treatments. The decrease in digestibility by cats fed the 15C treatment is likely due to the increased fiber content. Previous studies have reported a decrease in nutrient digestibility with increased fiber inclusion fed to cats (Fisher et al., 2012; Sunvold et al., 1995). Smith and Aldrich (2022) also reported a decrease in DM, OM, and GE digestibility when dogs were fed a diet containing 25% CFP compared to SBM. However, in the current study, fat and TDF digestibility were not affected by CFP inclusion ($P > 0.05$; **Table 2.5**). Overall, cats fed the 15C treatment resulted in approximately 1.5%-unit lower digestibility when compared to those fed the 0C, 5C, or 10C treatments.

Therefore, there appears to be a threshold between 10-15% CFP inclusion at which diet digestibility, likely due to the fiber content, is impacted when fed to cats.

Conversely, it is important to consider the possible health benefits that CFP could provide for companion animals, specifically its fiber component. The roles dietary fiber play in overall health can be split into the soluble and insoluble fractions. Soluble fiber has been reported to decrease gastric emptying, increase satiety, reduce rate of glucose uptake, lower blood cholesterol, and provide substrate for beneficial microbe growth in the digestive system (Jenkins et al., 2008; Brennan and Cleary, 2005; Tungland, 2003; German et al., 1996). While insoluble dietary fiber has been described to decrease gastric transit time, dilute caloric density of diets, increase fecal bulk and moisture, and aid in laxation (Wenk, 2001). In terms of its fiber composition, CFP would likely be best utilized in a weight management diet as it contains 31.4% insoluble dietary fiber and 3.6% soluble dietary fiber (**Table 2.1**). An in vitro study reported that a novel corn fiber acted as an insoluble fiber and may be a good replacement for Solka-Floc, a common ingredient used for laxation and body weight control in pet food (de Godoy et al., 2009). In addition, the phenolic compounds in corn could provide additional health benefits such as reducing the risk of colon cancer and providing an antioxidant effect (Adom and Liu, 2002). Therefore, CFP is a unique ingredient as it could be included in pet food as a protein source at moderate levels (≤10%) without altering digestibility, but also to support animal health at higher levels.

Palatability

In first choice evaluation, cats chose the 5C treatment first over the 0C treatment 30 out of 40 times ($P < 0.05$) (**Table 2.6**). In addition, cats consumed significantly more of the 5C treatment compared to the 0C treatment with an intake ratio of 0.97. These results indicate that

cats preferred the 5C treatment over the 0C treatment. This preference could be due to the yeast component of CFP. Previous studies have reported that yeast, likely due to the nucleotides, is highly palatable to cats (Swanson and Fahey, 2004; White and Boudreau, 1975). However, there was no preference between the 10C or the 15C treatments when compared to the 0C treatment as first choice and consumption of the CFP diets were almost equal to the 0C. These results were surprising as it was expected that increased yeast with each dose would improve palatability. Smith and Aldrich (2022) observed similar results in which cats appeared to have no preference when comparing a diet containing 25% CFP to a diet containing SBM. These results imply that there may be a maximum inclusion level of CFP for increased palatability. Of note, the palatability of the 10 and 15% inclusion was still well accepted by cats and comparable to a diet containing SBM.

Summary

The starch in corn is converted to ethanol during fermentation, resulting in co-products containing increased levels of protein and fiber. Furthermore, post-fermentation technologies, in which CFP is produced, further elevates the protein content but decreases the fiber content. The evaluation of CFP in pet food warrants further investigation as the phenolic compounds in corn may provide further health benefits in addition to its role as an insoluble fiber. Therefore, inclusion levels of CFP may vary depending upon marketing, diet digestibility, and animal health goals. A limitation of this study was the narrow scope of the work which could be expanded in future studies to evaluate the fiber component of CFP on overall health when fed to cats. In addition, further work is needed to elucidate the components of CFP (fiber vs yeast) to have a better understanding of ingredient functionality.

Conclusion

In conclusion, CFP could provide a novel protein source for pet food based on acceptable stool quality, digestibility, and palatability when fed to cats. However, if the goal of inclusion is to maintain these parameters when compared to a control diet containing SBM, there appears to be a maximum inclusion level of CFP at 10% due to its increased fiber content.

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Chapter 2 Tables

Table 2.1 Analyzed chemical composition of experimental ingredients, soybean meal (SBM) and corn fermented protein (CFP), reported on a dry matter basis

Nutrient, %	SBM	CFP
Moisture	11.97	5.13
Ash	8.14	2.84
Protein	53.44	52.62
Fat	2.71	5.60
Insoluble Dietary Fiber	16.36	31.41
Soluble Dietary Fiber	3.52	3.58
Total Dietary Fiber	19.88	34.89

Table 2.2 Ingredient composition of feline diets with increasing levels of corn fermented protein

	Treatment[1]			
Ingredient, %	0C	5C	10C	15C
Corn	37.97	38.11	38.26	38.41
Chicken Meal	20.86	20.23	19.59	18.96
Chicken Meal, Low Ash	11.11	11.72	12.33	12.95
Soybean Meal	15.00	10.00	5.00	-
Corn Fermented Protein	-	5.00	10.00	15.00
Chicken Fat	5.65	5.52	5.40	5.27
Beet Pulp	4.00	4.00	4.00	4.00
Fish Meal	3.00	3.00	3.00	3.00
Flavor	1.00	1.00	1.00	1.00
Titanium Dioxide	0.40	0.40	0.40	0.40
Salt	0.25	0.25	0.25	0.25
Potassium Chloride	0.25	0.25	0.25	0.25
Vitamin and Mineral Premix	0.25	0.25	0.25	0.25
Choline Chloride, 60% Dry	0.20	0.20	0.20	0.20
Natural Antioxidant	0.07	0.07	0.07	0.07

[1]0C, 0% corn fermented protein; 5C, 5% corn fermented protein; 10C, 10% corn fermented protein; 15C, 15% corn fermented protein.

Table 2.3 Analyzed chemical composition of feline diets with increasing levels of corn fermented protein reported on a dry matter basis

	Treatment[1]			
Nutrient	0C	5C	10C	15C
Dry Matter, %	95.24	95.76	95.09	94.65
Moisture, %	4.76	4.24	4.91	5.35
Organic Matter, %	90.81	90.72	91.20	91.63
Ash, %	9.19	9.28	8.80	8.37
Crude Protein, %	35.35	36.32	36.24	36.72
Fat, %	12.16	12.96	12.56	12.66
Gross Energy, kcal/kg	4854.66	4915.26	4932.77	4969.45
Insoluble Dietary Fiber, %	11.01	10.75	11.55	12.95
Soluble Dietary Fiber, %	2.65	3.35	2.45	3.19
Total Dietary Fiber, %	13.76	14.20	14.00	16.13

[1]0C, 0% corn fermented protein; 5C, 5% corn fermented protein; 10C, 10% corn fermented protein; 15C, 15% corn fermented protein.

Table 2.4 Food intake and stool quality parameters of cats fed diets with increasing levels of corn fermented protein

	Treatment[1]					
Parameter	0C	5C	10C	15C	SEM	*P*-value
Food Intake, g/d	69.67	72.04	74.61	75.11	2.145	0.0616
Wet Fecal Output, g/d	41.98	41.66	43.74	48.39	2.57	0.0523
Fecal Dry Matter, %	30.73[b]	32.08[a,b]	33.22[a]	32.80[a]	0.565	0.0009
Dry Fecal Output, g/d	12.69[b]	13.29[b]	14.54[a,b]	15.77[a]	0.744	0.0015
Defecations per Day	0.83	0.83	0.83	0.92	0.046	0.1279
Fecal Score	3.96	4.03	4.09	4.00	0.083	0.4617
Fecal pH	5.45[a]	5.45[a]	5.26[b]	5.41[a]	0.052	0.0027

[1]0C, 0% corn fermented protein; 5C, 5% corn fermented protein; 10C, 10% corn fermented protein; 15C, 15% corn fermented protein.
[a-b]Means within a row lacking a common superscript letter are different ($P < 0.05$).

Table 2.5 Apparent total tract digestibility of cats fed diets with increasing levels of corn fermented protein estimated by TiO_2 as a dietary marker

	Treatment[1]					
Nutrient, %	0C	5C	10C	15C	SEM	*P*-value
Dry Matter	80.63[a]	80.14[a]	80.71[a]	78.88[b]	0.342	<0.0001
Organic Matter	85.83[a]	85.44[a]	85.61[a]	83.94[b]	0.286	<0.0001
Crude Protein	87.04[a]	86.60[a]	86.85[a]	85.39[b]	0.340	0.0002
Fat	95.81	95.91	96.17	95.56	0.266	0.1661
Total Dietary Fiber	47.60	49.54	47.90	49.81	1.160	0.1499
Gross Energy	86.03[a]	85.78[a]	85.88[a]	84.18[b]	0.285	<0.0001

[1]0C, 0% corn fermented protein; 5C, 5% corn fermented protein; 10C, 10% corn fermented protein; 15C, 15% corn fermented protein.
[a-b]Means within a row lacking a common superscript letter are different ($P < 0.05$).

Table 2.6 First choice (FC) and intake ratio (IR) of cats fed diets with increasing levels of corn fermented protein

Diet Comparison (A vs. B)[1]	FC[2]	IR[3]
5C vs 0C	30*	0.970*
10C vs 0C	18	0.538
15C vs 0C	19	0.590

[1]0C, 0% corn fermented protein; 5C, 5% corn fermented protein; 10C, 10% corn fermented protein; 15C, 15% corn fermented protein.
[2]Number of first visits to bowl A out of 40 observations.
[3]IR = intake (g) of diet A/total intake (g) of diets A+B.
*Comparison differs ($P < 0.05$).

Chapter 3 - Evaluation of graded levels of corn fermented protein (CFP) on extrusion processing and diet utilization in healthy adult dogs

Abstract

There has been increased interest among pet owners to feed vegetarian diets to their pets. However, the primary protein sources used in pet food today are predominantly animal based, warranting a need to evaluate novel plant-based ingredients to meet protein requirements. Corn fermented protein (CFP), a co-product from ethanol production, may provide a plant-based alternative protein source for pet food. Therefore, the study objectives were to determine the effects of increasing levels of CFP on extrusion processing, stool quality, apparent total tract digestibility (ATTD), and palatability in dog diets. Four extruded diets were fed to twelve adult beagle dogs in a replicated 4 x 4 Latin Square design. The control diet contained 15% soybean meal (0C) and CFP was exchanged at either 5%, 10%, or 15% of soybean meal (5C, 10C, 15C). Dogs were fed each dietary treatment for 9-d adaption followed by 5-d total fecal collection. Feces were scored on a 1-5 scale, with 1 representing liquid diarrhea and 5 representing hard pellet-like. Titanium dioxide was added to all diets (0.4%) as an external marker to estimate digestibility. Data was analyzed using orthogonal contrasts in SAS (version 9.4, SAS Institute, Inc., Cary, NC). Dry bulk density of kibble decreased ($P < 0.05$) while kibble toughness increased ($P < 0.05$) with CFP inclusion. Fecal dry matter, dry fecal output, and defecations per day of dogs increased ($P < 0.05$) when fed increased levels of CFP. Dry matter and crude protein digestibility of CFP treatments were comparable ($P > 0.05$) to 0C. There was a decrease ($P < 0.05$) in organic matter, crude fat, gross energy, and total dietary fiber digestibility in the CFP

treatments compared to 0C. A cubic relationship ($P < 0.05$) was observed in digestibility of all nutrients except crude fat, indicating 10C resulted in the lowest digestibility. Dogs had no preference when comparing the 5C to the 0C treatment. Inclusion of CFP at 5, 10, and 15% still resulted in acceptable processing parameters, kibble characteristics, and utilization when fed to dogs.

Introduction

As the human population has become more concerned about health, animal welfare, and the environment, there has been an increase in the vegetarian lifestyle (Pribis et al., 2010; Stahler and Mangels, 2022). The increase of affluent nations has shifted diets from plant-based to diets high in animal products, which has been identified as a contributor to the rise in chronic disease (Popkin and Du, 2003; Walker et al., 2005). In addition, over 66 billion terrestrial animals are slaughtered for consumption (Schlatzer, 2010), which has given rise to animal welfare concerns. Climate change is also becoming one of the biggest environmental issues which is thought to be impacted by animal agriculture (Koneswaran and Nierenberg, 2008). It is estimated that 75 million people are vegetarian by choice, which is expected to rise as education and affluence spreads (Leahy et al., 2010).

Not surprisingly, an increase in the human vegetarian population has resulted in the concurrent demand for vegetarian diets for pets. However, shifting dogs and cats to a vegetarian lifestyle is more challenging, as they belong to the order Carnivora whose ancestors survived by consuming primarily or entirely captured prey animals. The domestic dog has evolved to become omnivorous, as they have increased gene expression for pancreatic amylase, the ability to convert maltose to glucose, and increased intestinal glucose uptake compared to wolves (Semp, 2014; Axelsson et al., 2013). Therefore, dogs can metabolize carbohydrates and endure on a

lower protein diet (Buff et al., 2014). However, often due to consumer perception, pet food today consists primarily of animal-based ingredients to mimic ancestral diets. For vegetarian pet food to be safe and nutritious, the development and evaluation of novel plant-based ingredients which are high in protein is warranted. Traditional ingredients such as corn gluten meal, soybean meal, and pea protein concentrate are currently available, but new options may be valuable for several reasons, including additional options for balancing requirements, cost, and increasing sustainability by using valuable co-products from other industries.

Ingredients like corn fermented protein (CFP) may be able to meet this demand. Corn fermented protein, a co-product from ethanol production, is produced using post fermentation separation technology which results in a high protein ingredient. The combination of zein and yeast protein results in an ingredient containing 50% protein, which is nearly double that of traditional distillers dried grains. Graded levels of CFP have already been evaluated in cats resulting in acceptable palatability, stool quality, and nutrient digestibility (Kilburn-Kappeler et al., 2022). Therefore, the objective of this study was to evaluate increasing levels of CFP on extrusion processing, stool quality, apparent total tract digestibility (ATTD), and palatability in adult dogs.

Materials and Methods

The digestibility trial was conducted at the Kansas State University Large Animal Research Center (LARC) under the Institutional Animal Care and Use Committee (IACUC) #4097 protocol. The palatability trial was conducted at Summit Ridge Farms (Susquehanna, PA) under protocols KSUPALC00120, KSUPALC00220, and KSUPALC00320.

Diet Formulation

Four different diets with increasing levels of corn fermented protein (CFP; POET Bioproducts, Sioux Falls, SD) as a replacer of equal levels of soybean meal (SBM; Fairview Mills, Seneca, KS) were formulated (**Table 3.1**). The control diet contained 15% SBM (0C) and CFP was exchanged for either 5% (5C), 10% (10C), or 15% (15C) of soybean meal. The formulated diets met the AAFCO nutritional requirements of adult dogs. Titanium dioxide was added at 0.40% to serve as an indigestible marker to estimate apparent total tract nutrient digestibility. The dry raw materials, except for the CFP, SBM, and titanium dioxide, comprised the base ration and were purchased from a commercial mill (Fairview Mills, Seneca, KS).

Diet Production

Each diet was produced using a single screw extruder (model E525, ExtruTech, Inc., Sabetha, KS, USA). The preconditioner (model ADP 145, ExtruTech, Inc., Sabetha, KS, USA) was configured with 12, 45° back and 57 neutral beaters on each of the two shafts. The extruder profile and barrel temperatures were based on a typical commercial pet food configuration. At the end of the extruder barrel there were two round die inserts with an interior diameter of 3 mm. Dry matrix feed rate (318 kg/h), pre-conditioner (PC) cylinder speed (185 rpm), extruder (EX) water (0 kg/hr), EX steam (0 kg/hr), and EX knife speed (1600 rpm) were kept constant during the processing of all treatments.

During processing, PC and EX parameters were collected from sensor readouts every 2 minutes to evaluate potential effects of CFP inclusion on the process. Output variables included PC discharge temperature, EX motor load, EX die temperature, total mass flow (TMF), specific mechanical energy (SME), and in-barrel moisture content (MC).

The TMF was calculated by adding the dry feed rate with water and steam injected in PC and EX, assuming that 80% of the water coming from the PC and EX steam is lost during flash-off as kibbles exit the die:

TMF = dry feed rate + PC water + (0.2 * PC steam) + EX water + (0.2 * EX steam)

SME was calculated using the following formula:

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

where, τ is the EX % torque or EX motor load, τ_0 is the EX no load % torque (25% at EX screw speed 425 rpm), N is the EX screw speed (rpm), N_r is the rated EX screw speed (425 rpm), P_r is the rated EX motor power (114 kW), and m is TMF (kg/s).

The in-barrel moisture content (MC) was also calculated using the following formula:

$$MC = \frac{m_f \times X_f + m_{ps} + m_{pw} + m_{es} + m_{ew}}{m_f + m_{ps} + m_{pw} + m_{es} + m_{ew}}$$

where m_f is the feed rate, X_f is the moisture content of the raw material, m_{ps} is the percentage of added steam in the preconditioner, m_{pw} is the percentage of added water in the preconditioner, m_{es} is the percentage of steam added into the extruder, and m_{ew} is the percentage of water added into the extruder. A moisture content of 10% was assumed for X_f.

After extrusion, kibble was pneumatically conveyed through an 8” clean air hood system and deposited onto an oscillating belt spreader. The kibble was dried on a 1.5 m wide single pass two zone dryer (model AFI, ExtruTech, Sabetha, KS, USA) to achieve a less than 10% moisture content. Kibble was dried at approximately 110°C for 22 minutes. Dried kibble was coated with chicken fat protected with natural antioxidants and a dry powdered flavor designed for dogs. Coated diets were stored in poly-lined Kraft paper bags until fed.

Physical Characteristics of Kibble

Wet and dry bulk density was measured off the extruder and off the dryer every 15 mins during the processing of each treatment. Bulk density was measured using a 1 L cup in which kibble was leveled and weighed on a digital scale with 0.1 g sensitivity. In addition, five randomly selected kibbles every 15 mins of each diet production off the extruder and off the dryer were measured for diameter and length using a digital caliper. Ten randomly selected kibbles off the dryer were also weighed using a digital scale with 0.0001 g sensitivity (EX324N; Ohaus Corporation, Parsippany, NJ, U.S.A.). The diameter, length, and mass measurements were used to determine sectional expansion index and specific length.

Sectional expansion index (SEI) was determined by comparing the squared diameter of the dried extruded kibbles by the squared die diameter of the extruder:

$$SEI = \frac{D^2}{d^2}$$

where D is the extrudate diameter and d is the extruder die diameter.

Specific length in mm/g was determined by the following equation:

$$Specific\ length = \frac{l}{m}$$

where *l* is the extrudate length and *m* is the extrudate mass.

A texture analyzer (model TA-XT2, Texture Technology Corp., Scarsdale, NJ, USA) with a 30 kg load cell was used to measure kibble texture. A cylindrical probe (25 mm diameter) was used to compress 30 kibbles within each treatment. The procedure was adapted from Dogan and Kokini (2007) with a test speed of 2 mm/s and strain level set at 80 %. Kibble hardness was considered to be the peak force in kg of the first major kibble breakage and the energy to compress the kibbles to 80% was computed as the area under the curve in kg mm for each

compressed kibble not accounting for the negative values. The compression energy was considered as kibble toughness.

Feeding Trial

For this study, 12 healthy adult (6.3 ± 0.45 years) beagle dogs (8 castrated males and 4 spayed females) were enrolled. The dogs had an average body weight of 11.4 ± 1.2 kg. The daily metabolizable energy requirement was calculated for laboratory kennel dogs [$130*BW_{kg}^{0.75}$; NRC (2006)] to determine the amount of food offered to each dog per day. However, it was adjusted to $105*BW_{kg}^{0.75}$ to maintain body weight of dogs. The body weight was measured at the beginning, middle, and end of each period. The experiment consisted of four periods, and each one was composed of 9 days of adaptation followed by 5 days of collection. Dogs were randomly assigned to each of the four treatments over the four periods. In this model, each animal served as its own control, and each treatment had 12 total observations.

The dogs were individually housed in pens (1.83 m x 1.20 m) equipped with an acrylic-coated mesh floor to allow for separation of urine and feces. Six animals were maintained per room in a temperature-controlled (23°C) modular building with a 12 h light cycle. The dogs received two feedings per day at 0800 and 1700 h with water provided *ad libitum*. During the collection period, all feces were collected periodically throughout each day to prevent contamination and disturbance. The fecal samples were weighed, scored on a 1-5 scale with 0.5 increments [1 – liquid diarrhea to 5 – dry hard pellets; Carciofi et al. (2008)]. A score of 3.5 – 4.0 was considered ideal. In addition, the pH of a fresh sample (within 15 minutes of defecation) was recorded in triplicate with a calibrated glass-electrode pH probe (FC240B, Hanna Instruments, Smithfield, RI). Fecal samples were stored in a labeled whirl-pak bag in a freezer until further processed.

Digestibility Calculations and Nutrient Analysis

After each collection period, feces from each dog were composited and dried at 55°C in a forced air oven until constant weight (24 – 48 h). Dried samples were ground to pass through a 1 mm screen in a laboratory fixed blade impact mill (ZM 200, Retsch, Verder Scientific, Haan, Germany). Titanium dioxide (TiO_2) concentration was measured in food and feces using a spectrophotometric plate reader (Gen5TM, Biotek® Instruments, Inc.Winooski, VT) at 410 nm (Myers et al., 2004). Apparent total tract digestibility (ATTD) was estimated by TiO_2 using the following equation:

$$ATTD = \left[1 - \frac{\%\ TiO2\ in\ food * \%\ nutrient\ in\ feces}{\%\ TiO2\ in\ feces * \%\ nutrient\ in\ food}\right] * 100$$

Food and partially dried fecal samples were analyzed in duplicate for moisture (AOAC 930.15), ash (AOAC 942.05), crude fat by acid hydrolysis and hexane extraction (AOAC 960.39), gross energy (Parr 6200 Calorimeter, Parr Instrument Company, Moline, IL), and total dietary fiber (AOAC 991.43). Crude protein was determined by Dumas combustion (AOAC 990.03) using a nitrogen analyzer (FP928, LECO Corporation, Saint Joseph, MI).

Palatability Trial

Experimental treatments (5C, 10C, and 15C) were evaluated for palatability vs the control diet (0C) by dog panels at a commercial kennel (Summit Ridge Farms, Susquehanna, PA). Each experiment was conducted as a split-plate test, in which two stainless steel bowls containing 400 g of food were presented to dogs for a total of 30 minutes. Each comparison trial was repeated for two days, with bowl position switched daily. Twenty dogs were fed daily, providing 40 observations for each paired comparison test. Preference was determined based on dogs' first choice and total food consumption. Data from consumption was represented as the following ratio:

$$Intake\ Ratio = \frac{consumption\ of\ Diet\ A}{total\ consumption\ of\ Diet\ A + Diet\ B}$$

Statistics

Least square means of data were estimated by ANOVA using the GLIMMIX procedure in SAS (version 9.4, SAS Institute INC, Cary, NC, USA) with Tukey correction. Contrasts comparing control (0C) vs treatments (5C, 10C, and 15C), and linear, quadratic, and cubic relationships among all diets were considered significant at $P < 0.05$. For each diet production, sampling was conducted at evenly spaced intervals which were considered replicates. The digestibility experiment was conducted as a replicated 4x4 Latin Square design with 3 dogs randomly assigned to each of the 4 diets in each period. Therefore, dog and period were considered random effects in the model for analysis of data from the digestibility trial.

In the palatability experiments, the intake ratio was analyzed using a t-test in a 2-way ANOVA and the first-choice preference was analyzed using a Chi^2 test. Individual dogs were considered the experimental units for analysis.

Results

Extrusion Processing and Kibble Characteristics

Preconditioner (PC) cylinder speed was kept constant across all treatments at 185 rpm (**Table 3.2**). However, PC steam, PC water, and extruder (EX) screw speed fluctuated among dietary treatments. There was slightly more steam and water added to the PC during the production of 0C compared to the CFP treatments ($P < 0.05$). The 0C treatment resulted in the fastest ($P < 0.05$) screw speed at 425 rpm compared to the CFP treatments at an average of 395 rpm.

The PC discharge temperature was lower ($P < 0.05$) in the 0C compared to the CFP treatments and resulted in a significant quadratic relationship among dietary treatments (**Table**

3.2). The motor load was also lower ($P < 0.05$) in the 0C compared to the CFP treatments and resulted in a significant cubic relationship among dietary treatments. The 0C treatment resulted in the lowest die temperature (109°C) compared to the CFP treatments (average, 110°C). There was also a significant quadratic relationship in die temperature among dietary treatments. The 0C treatment had a greater ($P < 0.05$) total mass flow (TMF) compared to the other treatments, there was also a quadratic relationship ($P < 0.05$) indicating T2 and T3 resulted in lower TMF compared to T1 and T4. Specific mechanical energy (SME) was lower ($P < 0.05$) for 0C at 135 kJ/kg compared to the other treatments at an average of 141 kJ/kg. There was also a cubic relationship ($P < 0.05$) among dietary treatments for SME, indicating that 10C had the greatest SME at 149 kJ/kg. The in-barrel moisture content (MC) was higher ($P < 0.05$) during the production of 0C compared to the other treatments. There was also a quadratic relationship ($P < 0.05$) for MC, showing that T1 and T4 resulted in higher MC compared to T2 and T3.

There was a significant quadratic relationship in wet bulk density, wet kibble diameter, and wet kibble length among dietary treatments (**Table 3.2**). Dry bulk density was greater ($P < 0.05$) for the 0C treatment at 337 g/L compared to the CFP treatments at an average of 320 g/L. Dry bulk density also decreased linearly ($P < 0.05$) as CFP increased. Dry kibble diameter was not affected ($P > 0.05$) by CFP inclusion. Dry kibble length was smaller for 0C compared to CFP treatments and increased linearly ($P < 0.05$) with CFP inclusion. Specific length or SEI of kibble were not affected ($P > 0.05$) by CFP inclusion.

Inclusion of CFP linearly increased ($P < 0.05$) toughness of kibble, ranging from 5.1 kg mm to 6.4 kg mm. However, kibble hardness did not result in a significant linear relationship. Instead, there was a cubic relationship ($P < 0.05$) indicating that the 0C and 10C treatments resulted in the hardest kibble (**Table 3.2**).

Diet Chemical Analyses

The diets were drier than target at an average of 5% moisture. Overall nutrient composition for dry matter, organic matter, crude fat, and gross energy were maintained among dietary treatments at 94.7%, 91.2%, 12.3%, and 4970.4 kcal/kg, respectively (**Table 3.3**). The average crude protein content of CFP treatments was 36.7% whereas the crude protein content of 0C was 38.4%. The total dietary fiber content was greatest for the 15C treatment at 16.1% and lowest for the 0C treatment at 13.8%.

Feed Intake and Fecal Characteristics

Food intake of dogs was lower for the 0C compared to the CFP treatments and resulted in a cubic relationship ($P < 0.05$) among dietary treatments (**Table 3.4**). Wet fecal output of dogs was maintained ($P > 0.05$) among dietary treatments (**Table 3.4**). Fecal dry matter percent was lower for dogs fed the 0C (32%) compared to the CFP treatments (average, 33%) and increased linearly ($P < 0.05$) as CFP increased. Dry fecal output of dogs increased linearly ($P < 0.05$) with CFP inclusion, ranging from 35 to 40 g/d. Defecations per day also increased linearly ($P < 0.05$) with CFP inclusion, ranging from 2.2 to 2.4 times per day. Fecal score was lower ($P < 0.05$) for dogs fed the 0C at 3.7 compared to the CFP treatments at an average of 3.9. Fecal score of dogs also had a quadratic relationship ($P < 0.05$) among dietary treatments. Fecal pH of dogs was maintained similar ($P > 0.05$) among dietary treatments.

Apparent Total Tract Digestibility

There was a cubic relationship ($P < 0.05$) in dry matter, organic matter, crude protein, gross energy, and total dietary fiber digestibility among dietary treatments, indicating that 10C resulted in the lowest digestibility (**Table 3.5**). Dry matter and crude protein digestibility were not different ($P > 0.05$) when comparing 0C to CFP treatments. However, organic matter

digestibility was higher ($P < 0.05$) for 0C (87.6%) compared to the CFP treatments (average, 86.7%). Crude fat digestibility was greater ($P < 0.05$) in the 0C (97.8%) compared to the CFP treatments (average, 97.5%). Crude fat digestibility also resulted in a quadratic relationship ($P < 0.05$), indicating greater digestibility for 0C and 15C compared to 5C and 10C. Gross energy digestibility was higher ($P < 0.05$) in the 0C treatment at 88.1% compared to the CFP treatments at an average of 87.2%. The 0C treatment also resulted in greater ($P < 0.05$) total dietary fiber digestibility compared to CFP treatments.

Palatability

There was no preference between the 5C and 0C treatments when offered to dogs indicated by the non-significant results in first choice and intake ratio (**Table 3.6**). However, dogs chose the 0C treatment first over the 10C treatment 27 of 40 times indicating a first-choice preference ($P < 0.05$). Conversely, dogs did not consume significantly more of 0C compared to 10C based on the intake ratio. When comparing 15C vs 0C, dogs consumed more ($P < 0.05$) 0C with no preference based on first choice.

Discussion

Extrusion Processing and Kibble Characteristics

The differences in PC steam, PC water, and PC discharge temperature were minimal and interpreted to be of no practical importance. On average, CFP treatments contained greater levels of soluble fiber compared to 0C which may have increased viscosity within the extruder barrel (Donadelli et al., 2021), resulting in an increase in motor load. However, the difference in soluble fiber was minimal. Therefore, the decrease in screw speed with the CFP treatments was likely the major contributor to the increase in motor load. As a decreased screw speed would result in increased barrel fill, increasing motor load (Unlu and Faller, 2002). Therefore, the

variation in input variables (EX screw speed) likely caused the increase in motor load not the CFP inclusion itself.

Surprisingly, the fluctuation in EX screw speed did not appear to affect the final product. It would be expected that a decrease in screw speed would result in less mechanical energy, decreasing material cook and expansion (Rokey, 2006). Therefore, the fastest screw speed should have produced the most expanded kibble. However, this was not the case as the 0C treatment had the fastest screw speed but the 15C treatment was the most expanded indicated by the lowest bulk density. This could be explained by the fact that a decreased screw speed would increase material retention time in the extruder barrel allowing for increased cook time (Yeh et al., 1992). This is supported by the increased TMF with the 0C treatment. Therefore, the fluctuations in screw speed could have counteracted resulting in a similar process among dietary treatments. In other words, the degree of cook in the 10C and 15C treatments could have been comparable to the 0C and 5C treatments with the faster screw speeds. This is supported by the increased SME for CFP treatments, specifically 10C, compared to 0C. Regardless, the differences in bulk density were not of practical concern as the average dry bulk density of dietary treatments (324 g/L) resembled that of typical commercial kibble which have densities between 280 and 400 g/L (Rokey, 2006). The increase in die temperature would also be expected to increase product expansion (Shukla et al., 2005). However, this was not observed in this study as the 5C and 10C treatments resulted in the highest die temperature but not the lowest bulk density. The differences in MC among dietary treatments were minimal (<0.1%) and unlikely to affect processing or final kibble characteristics.

It would have been expected that the CFP treatments, specifically 15C, would result in denser kibble due to the increase in dietary fiber. Previous studies have reported decreased kibble

expansion with dietary fiber (Monti et al., 2016; Alvarenga et al., 2018). According to the Guy Classification System, fibers are dispersed phase fillers and known to have very poor functionality in extrusion, meaning they lead to less expanded final products (Guy, 2001). Therefore, it was surprising that the 15C resulted in the most expanded kibble. However, protein is also considered a dispersed phase filler (Guy, 2001), which was greatest in the 0C treatment. Therefore, the higher protein content could help to explain the decreased expansion observed in 0C. In contrast to the current study, Shukla et al. (2005) reported an increase in bulk density with increased inclusion of traditional distillers dried grains with solubles (DDGS). This could indicate that CFP has less of an effect on expansion, regarding bulk density, compared to DDGS.

On average, the CFP treatments resulted in greater levels of insoluble fiber compared to 0C which may affect longitudinal expansion and radial expansion of kibble. Donadelli et al. (2021) reported a greater longitudinal expansion compared to radial expansion in kibble containing ingredients with a higher concentration of insoluble fiber. Monti et al. (2016) also reported an increase in kibble length with the addition of an insoluble fiber compared to a soluble fiber. In addition, Alvarenga et al. (2018) observed an increase in kibble length with a decrease in kibble diameter as insoluble fiber increased. In the current study, CFP inclusion did increase kibble length, but kibble diameter was not affected. However, even with the differences in kibble length, specific length and SEI of kibble were maintained among dietary treatments, indicating that overall expansion was not impacted by CFP inclusion. Previous studies have reported a decrease in radial expansion with inclusion of distillers dried grains (Satterlee et al., 1976; Breen et al., 1977; Walker, 1980; Anderson et al., 1981; Shukla et al., 2005). These results could indicate that traditional distillers dried grains have a greater effect on radial expansion than CFP.

Previous research has reported that kibble expansion has an impact on hardness and compression energy (Moraru and Kokini, 2003; Yannioties et al., 2007). Therefore, it would be expected that the densest treatment (5C) would result in the greatest hardness and toughness. However, this was not the case as the 10C treatment resulted in the greatest hardness while the 15C treatment resulted in the greatest toughness. The increased toughness in 15C could be explained by the increase in dietary fiber. This corresponds to a previous study which reported a higher cutting force in kibble containing sugarcane fiber compared to kibble containing wheat bran (Monti et al., 2016). In addition, Kantrong et al. (2018) reported a correlation of increased hardness in rice-based snacks with a decrease in screw speed which supports the results in the current study.

Due to the varying results, there does not appear to be a direct correlation between increased levels of CFP on processing conditions or kibble characteristics. Instead, there seems to be more of an effect from the variation in input processing conditions, specifically EX screw speed.

Diet Chemical Analyses

The lower than target moisture content was likely due to the small kibble size which would have required less dry time than the standard 22 minutes. The small kibble size was intentional, as diets were produced for both dogs and cats. Of note, the ideal moisture content of kibble is ≤ 10% to prevent mold growth (Gautam et al., 2018). Therefore, the low moisture content in experimental treatments was not of concern. The decrease in crude protein content in the CFP treatments compared to the control was unexpected as the test ingredients, SBM and CFP, are comparable in protein content on a dry matter basis at 53.4% and 52.6%, respectively. Therefore, the slight increase in protein for 0C could be due to the normal variation among

laboratory analysis. The increase in total dietary fiber (TDF) in the 15C treatment was expected as CFP contained 34.9% TDF while SBM contained 19.9% TDF. Nutrient composition of test ingredients (SBM and CFP) has been previously published (Kilburn-Kappeler et al., 2022).

Feed Intake and Fecal Characteristics

There should not have been any differences in food intake among dietary treatments as the amount of food offered was controlled to maintain body weight of dogs. In addition, all dogs readily consumed the entire portion offered each day. Of note, differences in food intake were minimal (< 7 g/d) and unlikely to affect stool quality or nutrient digestibility.

The increase in fecal dry matter of dogs consuming the CFP treatments explains the consistent wet fecal output and the increase in dry fecal output with CFP inclusion. The authors previously reported a similar relationship in fecal dry matter and fecal output of cats fed increased levels of CFP (Kilburn-Kappeler et al., 2022). The increase in fecal dry matter also resulted in firmer stool which was observed with the increase in stool quality score of dogs fed CFP treatments. In addition, an increase in dry fecal mass resulted in an increase in the number of defecations per day for dogs fed CFP. The differences in stool quality with increased CFP inclusion is likely due to the increased fiber content in CFP treatments, specifically 15C, compared to 0C. As previous studies have attributed an increase in fecal bulk to increased dietary fiber in dogs (Fahey et al., 1992; Sunvold et al., 1995). In terms of fiber profile, CFP would be considered more of an insoluble fiber type (Kilburn-Kappeler et al., 2022). In agreement to the current study, Wichert et al. (2002) reported that addition of cellulose (an insoluble fiber) increased dry matter content of feces and frequency of well-formed feces when fed to dogs. Fecal pH was not affected by CFP, indicating that the increase in dietary fiber did not alter

microbial fermentation. This is supported by results from the work of Wichert et al. (2002) who also observed that fecal pH of dogs was not impacted by an insoluble fiber source.

Apparent Total Tract Digestibility

The significant cubic relationships among dry matter, organic matter, crude protein, gross energy, and total dietary fiber digestibility with the 10C treatment having the lowest digestibility was surprising. Rather, it was expected that the 15C treatment would result in the lowest digestibility due to the increased dietary fiber content and greatest fecal output. Previous studies have reported the effects of dietary fiber on gastric emptying, digesta transit time, and nutrient digestibility in dogs (Burrows et al., 1982; Russell and Bass, 1985; Fahey et al., 1990). Russell and Bass (1985) concluded that an increase in dietary fiber content and viscosity resulted in slowed gastric emptying in dogs. However, Burrows et al. (1982) reported a decrease in intestinal transit time with added dietary fiber in dogs. Therefore, decreased transit time could explain a decrease in nutrient digestibility (Burrows et al., 1982). Fahey et al. (1990) reported that increased dietary fiber did not impact digesta mean retention time of dogs but still decreased dry matter and organic matter digestibility. The differing results in the current and previous studies indicate that fiber type, inclusion level, and diet matrix can impact the effect of fiber on nutrient digestibility. Of note, the decrease in organic matter, crude fat, and gross energy digestibility with CFP treatments compared to 0C was minimal (< 1%) and unlikely to be of practical concern.

The digestibility of diets containing increasing levels of CFP when fed to dogs differed relative to that observed in cats. Kilburn-Kappeler et al. (2022) reported that digestibility of diets containing 5 and 10% CFP was comparable to the control when fed to cats. However, a significant decrease in digestibility was observed when cats were fed diets containing 15% CFP.

The study in cats indicated a clear level of inclusion in which digestibility was affected, which was not observed in the current study with dogs. This could be explained by the fact that cats have a shorter digestive tract compared to dogs decreasing their ability to utilize fiber (Verbrugghe and Hesta, 2017).

Palatability

The palatability of CFP in dogs differed from that in cats. Wherein, Kilburn-Kappeler et al. (2022) observed that cats preferred a 5% inclusion of CFP compared to a control (0% CFP) but had no preference with increased inclusion levels (10 and 15% CFP). In the current study, dogs had no preference between the control and the low inclusion level (5% CFP) but appeared to prefer the control over the higher CFP inclusion levels of 10 and 15%.

In addition to ingredients, palatability may be affected by processing and final kibble characteristics (Koppel et al., 2015). Therefore, the preference for 0C over the 10C and 15C treatments could be due to product texture, not the increased CFP inclusion. Specifically, the increased kibble toughness observed with the 10C and 15C treatments may have limited palatability in dogs. Regardless, dogs willing consumed all treatments and no refusals were observed.

Application of CFP in the Pet Food Industry

Several studies have raised concerns about the nutritional adequacy of vegetarian diets for companion animals, specifically insufficient amino acids. However, it is important to remember that animals require specific nutrients rather than specific ingredients. Therefore, both dogs and cats can subsist on vegetation diets if adequate levels of nutrients are met. Petfood formulation is specific to age and species, and the exclusion or intended use of ingredients in the final dietary composition based on marketing and labeling directly affects formulas and

formulation matrices. Specifically, vegetarian diets for cats will need additional supplementation, such as taurine, as cats have an inability to meet their nutrient requirements when fed exclusively plant-based ingredients.

Conclusion

In conclusion, the acceptable processing parameters, stool quality, nutrient digestibility, and palatability indicate that CFP can be utilized as a plant-based alternative protein source for dogs. Surprisingly, many parameters evaluated in this study resulted in a quadratic or cubic relationship as CFP increased rather than an exclusive linear response as expected. The quadratic relationships may indicate an optimum inclusion level of CFP for specific parameters. Whereas the cubic relationships could reveal other factors which may have affected the results such as processing conditions and physical characteristics of kibble, not CFP inclusion.

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Chapter 3 Tables

Table 3.1 Ingredient composition of canine diets with increasing levels of corn fermented protein

	Treatment[1]			
Ingredient, %	0C	5C	10C	15C
Corn	37.97	38.11	38.26	38.41
Chicken Meal	20.86	20.23	19.59	18.96
Chicken Meal, Low Ash	11.11	11.72	12.33	12.95
Soybean Meal	15.00	10.00	5.00	-
Corn Fermented Protein	-	5.00	10.00	15.00
Chicken Fat	5.65	5.52	5.40	5.27
Other[2]	9.42	9.42	9.42	9.42

[1]0C, 0% corn fermented protein; 5C, 5% corn fermented protein; 10C, 10% corn fermented protein; 15C, 15% corn fermented protein.
[2]Other ingredients: beet pulp, fish meal, flavor, titanium dioxide, salt, potassium chloride, vitamin and mineral premix, choline chloride, natural antioxidant.

Table 3.2 Least squares means and contrasts (0C vs 5C-15C (T), linear (L); quadratic (Q); cubic (C) level of corn fermented protein) for processing parameters and physical characteristics of diets with increasing levels of corn fermented protein

	Treatment[1]								
Parameter	0C	5C	10C	15C	SEM	0C vs T	L	Q	C
Preconditioner									
Cylinder Speed, rpm	185.00	185.00	185.00	185.00	0.000	1.000	1.0000	1.0000	1.000
Steam Flow, kg/hr	47.94	47.67	47.58	46.62	0.194	0.0460	0.0862	0.2596	0.9268
Water Flow, kg/hr	57.57	57.36	57.40	57.54	0.046	0.0005	0.7206	<0.0001	0.3807
Discharge Temperature, °C	89.09	90.37	90.84	90.02	0.179	<0.0001	<0.0001	<0.0001	0.3854
Extruder									
Screw Speed, rpm	425.00	415.74	375.00	394.44	9.220	0.0001	<0.0001	0.0299	0.0022
Motor Load, amps	70.56	72.04	76.41	72.81	0.842	<0.0001	<0.0001	<0.0001	<0.0001
Die Temperature, °C	108.70	110.68	111.23	108.42	0.354	<0.0001	0.7834	<0.0001	0.0839
TMF[2], kg/h	385.33	385.08	385.09	385.24	0.061	0.0001	0.1728	<0.0001	0.4731
SME[3], kJ/kg	135.35	140.49	149.37	133.62	2.345	0.0031	0.6211	<0.0001	0.0002
MC[4], %	32.41	32.34	32.33	32.36	0.032	0.0067	0.0825	0.0225	0.7755
Bulk Density, g/L	344.00	351.60	333.40	321.00	6.110	0.0967	0.0005	0.0353	0.1147
Kibble Diameter, mm	4.99	4.94	4.87	5.07	0.085	0.7299	0.5053	0.0383	0.2513
Kibble Length, mm	4.18	4.14	4.31	4.57	0.093	0.0555	<0.0001	0.0286	0.6209
Dryer									
Bulk Density, g/L	336.65	337.10	316.03	308.03	6.670	0.0308	0.0019	0.4008	0.1301

Kibble Diameter, mm	4.85	4.83	4.77	4.99	0.093	0.8559	0.2305	0.0774	0.2823
Kibble Length, mm	3.66	3.74	3.99	4.12	0.113	0.0023	<0.0001	0.7694	0.4357
Specific Length, mm/g	133.01	136.92	138.99	138.43	4.034	0.1253	0.1546	0.4356	0.9499
SEI[5], mm^2/mm^2	2.63	2.61	2.54	2.77	0.100	0.8691	0.2398	0.0742	0.2601
Hardness, kg	2.45	2.22	2.49	2.24	0.158	0.3213	0.4708	0.8745	0.0435
Toughness, kg mm	5.46	5.06	6.12	6.43	0.368	0.1749	0.0009	0.1795	0.0605

[1]0C, 0% corn fermented protein; 5C, 5% corn fermented protein; 10C, 10% corn fermented protein; 15C, 15% corn fermented protein.
[2]TMF = total mass flow.
[3]SME = specific mechanical energy.
[4]MC = in-barrel moisture content.
[5]SEI = sectional expansion index.

Table 3.3 Analyzed chemical composition of canine diets with increasing levels of corn fermented protein on a dry matter basis

	Treatment[1]			
Nutrient	0C	5C	10C	15C
Dry Matter, %	93.50	95.47	95.20	94.61
Moisture, %	6.50	4.53	4.80	5.39
Organic Matter, %	90.50	91.09	91.24	91.87
Ash, %	9.50	8.91	8.76	8.13
Crude Protein, %	38.44	36.52	36.63	36.87
Crude Fat, %	12.88	12.15	11.39	12.79
Insoluble Dietary Fiber, %	11.01	10.75	11.55	12.95
Soluble Dietary Fiber, %	2.65	3.35	2.45	3.19
Total Dietary Fiber, %	13.76	14.20	14.00	16.13
Gross Energy, kcal/kg	4992.05	4959.87	4933.84	4995.75

[1]0C, 0% corn fermented protein; 5C, 5% corn fermented protein; 10C, 10% corn fermented protein; 15C, 15% corn fermented protein.

Table 3.4 Least squares means and contrasts (0C vs 5C-15C (T), linear (L); quadratic (Q); cubic (C) level of corn fermented protein) for food intake and stool quality parameters of dogs fed diets with increasing levels of corn fermented protein

	Treatment[1]								
Parameter	0C	5C	10C	15C	SEM	0C vs T	L	Q	C
Food Intake, g/d	190.05	189.14	195.48	193.31	1.232	0.0151	0.0003	0.4757	0.0003
Wet Fecal Output, g/d	113.16	109.20	111.69	116.88	3.326	0.8349	0.2041	0.0611	0.7232
Fecal Dry Matter, %	31.59	32.43	33.59	34.00	0.296	<0.0001	<0.0001	0.3037	0.2640
Dry Fecal Output, g/d	35.65	35.35	37.41	39.61	1.184	0.0706	0.0008	0.1477	0.5585
Defecations per Day	2.25	2.18	2.30	2.42	0.080	0.4483	0.0200	0.1138	0.4726
Fecal Score	3.67	3.82	3.87	3.87	0.034	<0.0001	<0.0001	0.0043	0.7839
Fecal pH	5.85	5.80	5.64	5.77	0.092	0.1271	0.1722	0.1579	0.1845

[1]0C, 0% corn fermented protein; 5C, 5% corn fermented protein; 10C, 10% corn fermented protein; 15C, 15% corn fermented protein.

Table 3.5 Least squares means and contrasts (0C vs 5C-15C (T), linear (L); quadratic (Q); cubic (C) level of corn fermented protein) for apparent total tract digestibility, estimated by titanium dioxide as a dietary marker, of diets with increasing levels of corn fermented protein

	Treatment[1]								
Nutrient, %	0C	5C	10C	15C	SEM	0C vs T	L	Q	C
Dry Matter	82.87	83.16	81.68	82.83	0.420	0.3623	0.2344	0.1557	0.0024
Organic Matter	87.59	87.46	86.01	86.56	0.332	0.0021	0.0002	0.1594	0.0037
Crude Protein	88.32	88.81	87.92	88.89	0.285	0.3534	0.3674	0.2436	0.0012
Crude Fat	97.78	97.50	97.32	97.64	0.150	0.0198	0.1979	0.0073	0.3972
Gross Energy	88.06	87.95	86.49	87.17	0.300	0.0015	0.0001	0.0699	0.0009
Total Dietary Fiber	59.91	58.12	45.29	48.04	1.409	<0.0001	<0.0001	0.4465	<0.0001

[1]0C, 0% corn fermented protein; 5C, 5% corn fermented protein; 10C, 10% corn fermented protein; 15C, 15% corn fermented protein.

Table 3.6 First choice (FC) and intake ratio (IR) of dogs fed diets with increasing levels of corn fermented protein

Diet comparison (A vs B)[1]	FC[2]	IR[3]
5C vs 0C	22	0.471
10C vs 0C	13*	0.399
15C vs 0C	20	0.325*

[1]0C, 0% corn fermented protein; 5C, 5% corn fermented protein; 10C, 10% corn fermented protein; 15C, 15% corn fermented protein.
[2]Number of first visits to bowl A out of 40 observations.
[3]IR = intake (g) of diet A/total intake (g) of diets A+B.
*Comparison differs ($P < 0.05$).

Chapter 4 - Comparison of corn fermented protein (CFP) to distillers dried grains with solubles (DDGS) fed to healthy adult dogs

Abstract

Traditional distillers dried grains, co-products from the ethanol industry, can be utilized as sustainable ingredients for pet food; however negative consumer perception of co-products due to marketing campaigns prevents their widespread use. Corn fermented protein (CFP) is produced using post-fermentation separation technology resulting in a high protein ingredient, which may increase consumer appeal. Therefore, the study objective was to compare the effect of CFP on stool quality, nutrient digestibility, and palatability to traditional distillers dried grains when fed to dogs. The control diet contained 15% soybean meal (CON) and experimental diets contained either 3.5% brewer's dried yeast (BDY), 2.5% brewer's dried yeast plus 17.5% distillers dried grains with solubles (BDY+DDGS), or 17.5% corn fermented protein (CFP). Experimental diets were fed to adult dogs (n = 12) in a 4 x 4 replicated Latin square design. Dogs were adapted to diets for 9 days followed by a 5-d total fecal collection. Titanium dioxide (0.4%) was added to all diets as an external marker to estimate digestibility. Data was analyzed using a mixed model in SAS with treatment as a fixed effect and dog and period as random effects. Fecal output was greatest for dogs fed BDY+DDGS ($P < 0.05$). Feces were firmer for dogs consuming CFP compared to CON and BDY+DDGS ($P < 0.05$). Overall, nutrient digestibility was greatest for CON and BDY and lowest for BDY+DDGS with CFP intermediate. There were no differences in total short chain or branched chain fatty acid concentrations in fresh fecal samples of dogs fed dietary treatments ($P > 0.05$). However, percent propionate was greater in fecal

samples of dogs fed CON compared to BDY+DDGS ($P < 0.05$). Whereas percent valerate was greater in fecal samples of dogs fed CON compared to CFP ($P < 0.05$). In the palatability evaluation, dogs had no preference when CON was compared to BDY or BDY+DDGS. However, dogs appeared to prefer CON over CFP. Overall, CFP resulted in improved stool quality and nutrient digestibility when compared to DDGS, which could increase consumer appeal for inclusion into pet food. Whereas the impact of CFP on palatability warrants further investigation.

Introduction

Ethanol is a renewable fuel made from various plant materials collectively known as biomass. In 2022, the Inflation Reduction Act and EPA's Renewable Fuel Standard promoted the production and use of ethanol, increasing the U.S. annual production to more than 15.4 million gallons (Renewable Fuels Association, 2022). In the U.S., 94% of ethanol is produced from the starch in corn grain (EERE, 2023), which can be produced through the dry or wet milling process. Dry milling is most widely used which produces distillers dried grains with solubles (DDGS) as a co-product, whereas wet milling yields corn gluten meal (CGM), corn germ meal, corn gluten feed, and corn fiber as co-products. In 2022, 36.4 million metric tons of distillers dried grains, gluten feed, and gluten meal were generated from ethanol production (Renewable Fuels Association, 2022).

In the pet food industry, CGM has been the most utilized ethanol co-product and is frequently combined with soy products, such as soybean meal (SBM), to complement the amino acid profile. However, ethanol co-products have been traditionally used in ruminant and poultry nutrition. In 2022, 78% of distillers grains were consumed by cattle (Renewable Fuels Association, 2022). Even though previous studies have reported high digestibility for both

DDGS and CGM when fed to dogs (Allen et al., 1981; Yamka et al., 2004), the use of DDGS in monogastric nutrition is limited due to the fiber content. Furthermore, inclusion in pet food is decreasing due to consumer perception as low-quality ingredients.

In an effort to capture additional value from ethanol production, the ethanol industry has developed a new technology to enhance the nutrient profile of co-products. One of these enhanced co-products is corn fermented protein (CFP), which is produced using post-fermentation separation technology. This technology allows the protein and yeast to be separated from the fiber prior to drying, resulting in a higher protein, lower fiber ingredient compared to DDGS. This shift in nutrient composition could improve nutrient digestibility and consumer perception of distillers dried grains allowing for increased use in pet food. Corn fermented protein has been previously compared to SBM and CGM in both dogs and cats (Kilburn-Kappeler and Aldrich, 2023; Smith and Aldrich, 2023; Kilburn-Kappeler et al., 2022). However, CFP has not been compared to traditional distillers dried grains when fed to dogs. Therefore, the objective of this study was to compare the impact of CFP and DDGS on stool quality, nutrient digestibility, and palatability when fed to dogs.

Materials and Methods

The digestibility trial was conducted at the Kansas State University Large Animal Research Center (LARC) under the Institutional Animal Care and Use Committee (IACUC) #4097 protocol. The palatability trial was conducted at Summit Ridge Farms (Susquehanna, PA) under protocols KSUPALC00420, KSUPALC00520, and KSUPALC00620.

Diet Formulation and Production

Dietary treatments consisted of a control diet containing 15% soybean meal (CON) and experimental diets containing either 3.5% brewer's dried yeast (BDY), 2.5% brewer's dried

yeast plus 17.5% distillers dried grains with solubles (BDY+DDGS), or 17.5% corn fermented protein (CFP). Diets with ethanol co-products and/or yeast were formulated to have a similar nutrient profile. Also, it was assumed that CFP had 20% yeast and DDGS had 5.7% yeast (POET Bioproducts, Sioux Falls, SD); therefore, all treatments, except CON, were formulated to contain 3.5% yeast. The formulated diets met the AAFCO nutritional requirements for healthy adult dogs. Titanium dioxide (0.40%) was added to serve as an indigestible marker to estimate apparent total tract nutrient digestibility. Two base rations were purchased from a commercial mill (Fairview Mills, Seneca, KS). The amount of corn, chicken meal, and chicken fat were adjusted between base rations to maintain nutrient composition among dietary treatments and result in a complete formula (100%). The first base ration was used for CON and CFP treatments, and included all dry ingredients, except for the soybean meal (Fairview Mills, Seneca, KS), CFP (POET Bioproducts, Sioux Falls, SD), corn starch (Fairview Mills, Seneca, KS), corn gluten meal (Fairview Mills, Seneca, KS), and titanium dioxide (Fairview Mills, Seneca, KS). The second base ration was used for the BDY and BDY+DDGS treatments, and contained all dry ingredients except for soybean meal, DDGS (Fairview Mills, Seneca, KS), corn starch, corn gluten meal, BDY (Fairview Mills, Seneca, KS), and titanium dioxide. Soybean meal, corn gluten meal and/or corn starch were added to CON, BDY, and CFP to create similar nutrient profiles among all dietary treatments and to balance a 20% inclusion of experimental ingredients compared to BDY+DDGS (**Table 4.1**).

Each diet was mixed and produced using a single screw extruder (model E525, Extru-Tech, Manhattan, KS). The cool and dry product was packaged in laminated bags and transferred to the laboratory at Kansas State University to be coated. Kibble was coated with chicken fat protected with natural antioxidants (Nutrios, Springfield, MO) and a dry powdered flavor

designed for dogs (AFB International, St. Charles, MO). Coated diets were stored in poly-lined Kraft paper bags until fed.

Feeding Trial

For this study, 12 healthy adult (6.3 ± 0.45 years) beagle dogs (8 castrated males and 4 spayed females) were enrolled in a triplicated 4x4 Latin square design. The dogs had an average body weight of 11.4 ± 1.2 kg. The daily metabolizable energy requirement was calculated for laboratory kennel dogs [130*$BW_{kg}^{0.75}$; NRC, 2006]. However, it was adjusted to 105*$BW_{kg}^{0.75}$ to maintain body weight of dogs, which was measured at the beginning, middle, and end of each period. The experiment consisted of four periods, and each one was composed of 9 days of adaptation followed by 5 days of collection. In this model, each animal served as its control, and each treatment had 12 total observations.

The dogs were individually housed in pens (1.83 m x 1.20 m) equipped with an acrylic-coated mesh floor to allow for separate collection of urine and feces. Two rooms, with six animals each, were maintained in a temperature-controlled (23°C) modular building with a 12 h light cycle. The dogs received two feedings per day at 0800 and 1700 h with water *ad libitum*. During the collection period, all feces were collected periodically throughout the day to prevent contamination and disturbance. The fecal samples were weighed, scored on a 1 – 5 scale with 0.5 increments [1 – liquid diarrhea to 5 – dry hard pellets; Carciofi et al., 2008]. A score of 3.5 – 4.0 was considered ideal. All feces were scored by the same person for consistency. Feces were stored in labeled whirl-pak bags in a freezer until further processed. In addition, the pH of a fresh sample (within 15 minutes of defecation) was recorded in triplicate with a calibrated glass-electrode pH probe (FC240B, Hanna Instruments, Smithfield, RI), and 2-g aliquots were

transferred into three plastic microcentrifuge tubes using a spatula and stored at -80°C for short chain fatty acid (SCFA) and branched chain fatty acid (BCFA) analysis.

Digestibility Calculations

After each collection period, feces from each dog were composited and dried at 55°C in a forced air oven until constant weight (24 – 48 h). Dried samples were ground to pass through a 1 mm screen in a laboratory fixed blade impact mill (ZM 200, Retsch, Verder Scientific, Haan, Germany). Titanium dioxide (TiO_2) concentration was measured in food and feces using a spectrophotometric plate reader (Gen5TM, Biotek® Instruments, Inc.Winooski, VT) at 410 nm (Myers et al., 2004). Apparent total tract digestibility (ATTD) was estimated by titanium dioxide using the following equation:

$$ATTD = \left[1 - \frac{\%\ TiO2\ in\ food * \%\ nutrient\ in\ feces}{\%\ TiO2\ in\ feces * \%\ nutrient\ in\ food}\right] * 100$$

Digestibility was calculated using both the total collection and titanium dioxide methods, which resulted in similar digestibility values and trends. However, the titanium dioxide method resulted in a lower standard error of the mean. Therefore, digestibility values from the titanium dioxide method were selected to report in this manuscript.

Nutrient Analysis

Experimental ingredients, diets, and partially dried fecal samples were analyzed in duplicate for moisture (AOAC 930.15), ash (AOAC 942.05), fat by acid hydrolysis and hexane extraction (AOAC 960.39), gross energy (Parr 6200 Calorimeter, Parr Instrument Company, Moline, IL), and total dietary fiber (AOAC 991.43). Crude protein was determined by Dumas combustion (AOAC 990.03) using a nitrogen analyzer (FP928, LECO Corporation, Saint Joseph, MI).

Amino acid composition of experimental ingredients were analyzed at the University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO; AOAC 982.30 and 988.15). All amino acids, except methionine, cysteine, and tryptophan, were digested with 6 N HCl for 24 h at 110°C. The amino acids were then separated by ion-exchange chromatography and the concentration was determined with a Beckman 6300 amino acid analyzer (Beckman, Palo Alto, CA). Methionine and cysteine were first oxidized by performic acid to methionine sulfone and cysteic acid, respectively, prior to acid hydrolysis. Tryptophan was hydrolyzed in 3 M mercaptoethanesulfonic acid before analysis. Available lysine was determined (AOAC 975.44) and lysine availability (%) was calculated as the ratio of available lysine to total lysine.

Fecal Chemical Analysis

Fecal SCFA and BCFA concentrations were determined by gas-liquid chromatography (Erwin et al., 1961) using a capillary column (30 m x 0.25 mm internal diameter; 0.25 µm film thickness; Aligent Technologies, Santa Clara, CA). The system was equipped using helium as a carrier gas with a constant flow rate of 40 cm/s and utilizing a 25:1 split ratio injector with injection size of 0.5 µL. A flame ionization detector was configured with hydrogen as the makeup gas with a flow rate of 40 mL/min to clarify peak resolution. The detector and injector temperatures were set at 250°C, and the initial oven temperature was set to 80°C with a ramp rate of 10°C/min to 200°C. The peak area of chromatograms was analyzed using integrative software (GC solution version 2.42.00, Shimadzu, Kyoto, Japan). Concentrations of SCFA (acetate, propionate, and butyrate) and BCFA (isobutyrate, valerate, 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) and correcting for fecal dry matter content.

Palatability Trial

Experimental treatments (BDY, BDY+DDGS, and CFP) were evaluated for palatability versus the control diet by dog panels at a commercial kennel (Summit Ridge Farms, Susquehanna, PA). Each experiment was conducted as a split-plate test, in which two stainless steel bowls containing 400 g of food were presented to dogs for a total of 30 minutes. Each comparison trial was repeated for two days, with bowl position switched daily. Twenty dogs were fed daily, providing 40 observations for each paired comparison test. Preference was determined based on dogs' first choice and total food consumption. A detailed description of the palatability method can be found in Aldrich and Koppel (2015). Data from consumption was represented as the following ratio:

$$Intake\ Ratio = \frac{consumption\ of\ Diet\ A}{total\ consumption\ of\ Diet\ A + Diet\ B}$$

Statistics

The digestibility experiment was conducted as a 4x4 replicated Latin square design. Each of the 12 experimental units (dogs) were assigned to treatment using the spreadsheet by Kim and Stein (2009). Data were analyzed using a GLIMMIX procedure in SAS (version 9.4, SAS Institute, Inc., Cary, NC) with treatment as a fixed effect and dog and period as random effects. Tukey's post hoc test was applied for the least-squares means separation, with significance considered at $P < 0.05$.

In the palatability experiment, the consumption ratio was analyzed using a t-test in a 2-way ANOVA and the first-choice preference was analyzed using a Chi^2 test. The twenty dogs were considered the experimental units for analysis.

Results

Nutrient Analysis

Among experimental ingredients, the protein content of distillers dried grains with solubles (DDGS) was lowest at 32% while corn gluten meal (CGM) was highest at 70% on a dry matter basis (**Table 4.2**). Soybean meal (SBM) and corn fermented protein (CFP) both contained 53% protein while brewer's dried yeast (BDY) contained 47% protein. Fat content was highest in DDGS at 12% and lowest for SBM and BDY at 3%. Fat levels in CGM and CFP were intermediate at 7 and 6%, respectively. Total dietary fiber (TDF) was greatest for DDGS at 45% and lowest for CGM at 3%. All ingredients except BDY had a greater proportion of insoluble dietary fiber (IDF) compared to soluble dietary fiber (SDF).

The amino acid composition of experimental ingredients varied (**Table 4.3**). Corn gluten meal and DDGS had the lowest concentration of taurine at 0.12 and 0.11%, respectively, while BDY had the greatest taurine concentration at 0.22%, and SBM and CFP were intermediate at 0.19 and 0.16%, respectively. For cysteine, CGM and CFP had the greatest concentrations at 1.15 and 1.01%, respectively, while BDY and DDGS had the lowest at 0.49 and 0.54%, respectively. Soybean meal was intermediate at 0.80% cysteine. The concentration of methionine was highest for CGM and CFP at 1.47 and 1.18%, respectively. Distillers dried grains with solubles had the lowest concentration of methionine at 0.54% while SBM and BDY were intermediate at 0.73 and 0.70%, respectively. Soybean meal and BDY had the greatest concentration of lysine at 3.40 and 3.22%, respectively Lysine concentration was lowest for DDGS at 0.99% with CGM and CFP intermediate at 1.16 and 1.73%, respectively. The same trend was seen in available lysine. Lysine availability was greatest for SBM and BDY at an

average of 97% and lowest for CFP at 90%, while CGM and DDGS were intermediate at 96 and 94%, respectively.

When comparing nutrient composition of dietary treatments, dry matter was similar at an average of 95% (**Table 4.4**). The CFP treatment contained 92% organic matter whereas the remaining treatments contained an average of 91% organic matter. Protein content was greatest for the CON and BDY treatments at 41% while BDY+DDGS and CFP treatments contained 38% protein on a dry matter basis. Fat content was highest for the BDY+DDGS treatment at 15% compared to the remaining treatments at an average of 13%. The BDY+DDGS treatment contained the greatest amount of TDF at 18% while CON and BDY treatments contained the lowest at an average of 13%. The TDF content of the CFP treatment was intermediate at 15%. The same pattern among dietary treatments was observed in IDF levels. However, the lowest SDF content was observed in the CFP treatment. All diets had a greater proportion of IDF compared to SDF. Gross energy content of dietary treatments was similar around 5000 kcal/kg.

Feed Intake and Fecal Characteristics

Food intake of dogs was maintained among dietary treatments ($P > 0.05$; **Table 4.5**). Whereas all stool quality parameters were significantly different among dietary treatments (**Table 4.5**). Wet fecal output was greater for dogs fed BDY+DDGS at 125 g/d compared to the remaining treatments at an average of 105 g/d ($P < 0.05$). Feces were wetter for dogs fed CON at 34% dry matter compared to the remaining treatments at 36% dry matter ($P < 0.05$). Dry fecal output was greatest for dogs fed the BDY+DDGS treatment at 45 g/d and lowest for dogs fed the CON at 35 g/d ($P < 0.05$). Dry fecal output of dogs fed BDY and CFP were intermediate at 36 and 39 g/d, respectively. Dogs defecated more often ($P < 0.05$) when fed BDY+DDGS at 2.6 times per day compared to dogs fed CON and BDY at an average of 2.2 times per day, with CFP

intermediate at 2.4 defecations per day. Feces of dogs fed CFP were firmer ($P < 0.05$) than CON and BDY+DDGS, with BDY intermediate. Fecal pH of dogs fed BDY was higher ($P < 0.05$) at 5.8 compared to dogs fed BDY+DDGS at 5.6, with CON and CFP intermediate at 5.7.

Apparent Total Tract Digestibility

Dry matter digestibility was greater ($P < 0.05$) for CON and BDY at an average of 81% compared to BDY+DDGS and CFP at an average of 77% (**Table 4.6**). Organic matter digestibility was greatest ($P < 0.05$) for CON and BDY at 87% and lowest for BDY+DDGS at 82%. Organic matter digestibility of CFP was intermediate at 83%. Protein digestibility was greatest ($P < 0.05$) for CON at 88% and lowest for BDY+DDGS and CFP at an average of 85%, while BDY was intermediate at 87%. Fat digestibility was lower ($P < 0.05$) for BDY+DDGS at 96% compared to the remaining treatments at 98%. The digestibility of total dietary fiber was highest ($P < 0.05$) for CON and BDY and lowest for CFP. Gross energy digestibility was highest ($P < 0.05$) for CON and BDY at 88% and lowest for BDY+DDGS at 83% with CFP intermediate at 84%.

Fecal Chemical Analysis

Total short chain fatty acid concentration in fecal samples of dogs fed dietary treatments ranged from 351 to 421 μmol/g DM feces but no significant differences were observed (**Table 4.7**). There were also no significant differences among dietary treatments in percent acetate or butyrate with averages of 65 and 10%, respectively. However, percent propionate was greater ($P < 0.05$) in fecal sample of dogs fed CON at 28% compared to fecal samples of dogs fed BDY+DDGS at 24%, BDY and CFP were intermediate at 25%. No significant differences among dietary treatments were observed in total branched chain fatty acid concentration, ranging from 14 to 16 μmol/g DM feces. Percentage of isovalerate and iosobutyrate in fecal samples

were maintained ($P > 0.05$) among dietary treatments with averages of 56 and 37%, respectively. However, valerate percentage was greater ($P < 0.05$) in fecal samples of dogs fed CON at 9.3% compared to dogs fed CFP at 4.4%, with BDY and BDY+DDGS intermediate at 6.2 and 8.3%, respectively.

Palatability

When comparing CON to BDY or BDY+DDGS, dogs had no preference indicated by the non-significant first choice and intake ratios (**Table 4.8**). However, dogs appeared to prefer CON over CFP indicated by the significant first choice and intake ratio. Dogs chose CFP first only 13 out of 40 observations and consumed less CFP compared to CON ($P < 0.05$).

Discussion

Nutrient Analysis

As new ingredients for pet food are emerging, it is important to evaluate their amino acid composition to ensure complement ingredients are utilized within a dietary matrix to meet nutrient requirements. Dogs and cats require ten essential amino acids including arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. In addition, cats have an additional dietary requirement for taurine. Taurine, cysteine, methionine, and lysine are often the amino acids of most concern due to their low concentration in ingredients utilized in pet food, their roles within the body, and/or their degradation during processing.

Taurine, a sulfur-containing amino acid, is mainly found in cardiac and skeletal muscle. However, low concentrations of taurine are commonly found in some plant ingredients (Spitze et al., 2003; Donadelli et al., 2019). This free amino acid is known to have a role in the conjugation of bile acids, maintenance of normal retinal and myocardial function, and the osmoregulation

and modulation of calcium flux within cells (Huxtable, 1992; Sanderson, 2006). Retinal degeneration and cardiomyopathy have been linked to low blood or plasma taurine concentrations in strict carnivores (Hayes et al., 1975; Pion et al., 1987; Morris et al., 1990). Therefore, it is considered an essential amino acid in cats. However, previous studies have linked low blood and plasma taurine concentrations to the development of dilated cardiomyopathy (DCM) in dogs (Kittleson et al., 1997; Fascetti et al., 2003). These studies as well as the 2018 FDA warning about a possible relationship between DCM in dogs and the consumption of grain-free diets have heightened the importance of adequate methionine, cysteine, and taurine levels in pet food.

During the processing of most commercial pet food a thermal treatment, such as extrusion, is used to improve the safety and nutritive value of the product. However, the thermal process can negatively impact protein quality. Specifically, the Maillard reaction, which contributes to the desired flavor and color of the product, decreases the bioavailability of essential amino acids. During the Maillard reaction, a reducing sugar binds to a free reactive amino group of an amino acid. Due to the reactive Ɛ-amino group, lysine is often the amino acid involved in the Maillard reaction. Previous research has indicated that up to 62% of the lysine in pet food contains a bound Ɛ-amino group, likely due to the Maillard reaction (Williams et al., 2006; Rutherfurd et al., 2007). This complex, also referred to as early Maillard reaction products (MRP), may be absorbed from the gastrointestinal tract but cannot be utilized by the animal (Hurrell and Carpenter, 1981; Moughan, 2003; Finot, 2005). As lysine is the first or second limiting essential amino acid in commercial pet food (NRC, 2006), a reduced utilization decreases the nutritive value of the food.

Due to the removal of starch during processing, the concentration of most amino acids in DDGS is 3 to 4 times greater than that of corn (Stein et al., 2006; Han et al., 2010). However, heat treatments during processing have been reported to decrease the availability of amino acids in DDGS, specifically lysine (Cromwell et al., 1993). In addition, due to differences in crop sourcing and processing methods, the amino acid composition of DDGS varies (Cromwell et al., 1993; Spiehs et al., 2002; Stein et al., 2006). However, the amino acid composition of the DDGS used in the current study is comparable to previous reports (NRC, 1998; Spiehs et al., 2002; Fastinger and Mahan, 2006; Stein et al., 2006).

Compared to the DDGS evaluated in the current study, CFP contains higher levels of taurine, cysteine, methionine, and lysine. A previous study reported higher concentrations of lysine in yeast compared to DDGS (Han and Liu, 2010). In addition, the lysine and taurine concentration of the CFP evaluated in the current study was greater than that of a corn protein concentrate (Donadelli et al., 2019). Corn fermented protein also contained greater taurine compared to the CGM evaluated in this study and Donadelli et al. (2019). Since all ingredients originate from corn protein, the main differentiator between the ingredients is the yeast component. Therefore, these results indicate that the substantial yeast component in CFP may contribute to its amino acid profile, specifically lysine and taurine, when compared to DDGS, CGM, or a corn protein concentrate. As a result, CFP inclusion in pet food could be beneficial to help meet amino acid requirements, such as taurine for cats.

The maintenance of dry matter content among dietary treatments was expected as drying temperature and time was controlled during the processing of all treatments. In addition, the ash content of CFP was lower than SBM, BDY, and DDGS ingredients resulting in increased organic matter of the CFP treatment compared to the remaining treatments. The lower ash content of

CFP compared to DDGS is likely because CFP does not contain solubles due to its unique processing method, reducing its phosphorus content (Loy and Lundy, 2019). However, the ash content of CGM was the lowest among experimental ingredients indicating that CFP may have a greater mineral content than CGM. The mineral composition of CFP warrants further investigation. The decreased protein content in the BDY+DDGS treatment was expected as DDGS contained the lowest amount of protein among experimental ingredients. However, the decreased protein content of the CFP treatment was unexpected as CFP contains more protein than BDY and a similar protein content to SBM. A possible explanation could be the high protein content of CGM which likely increased the protein content of CON and BDY treatments even at the low inclusion levels. The increased fat content of BDY+DDGS compared to the other treatments was expected as DDGS contained the highest amount of fat among experimental ingredients. In addition, the fiber content of dietary treatments was of no surprise due to the fiber content of experimental ingredients. The greatest difference in nutrient composition among dietary treatments was the fiber content which is likely to have the greatest impact on diet utilization by dogs.

Feed Intake and Fecal Characteristics

Comparable food intake by dogs among dietary treatments was expected, as food offered per day was intentionally fed at levels to maintain body weight of dogs. In addition, all dogs consumed the entire portion offered each day.

The increase in fecal output with the BDY+DDGS treatment is likely due to the increased fiber content of the diet. Similar to Yamka et al. (2003) who reported that an increase in dietary fiber resulted in decreased digestion and greater fecal mass due to an increased rate of passage through the digestive system and decreased absorption. Previous studies have also reported

increased fecal output in dogs consuming CFP compared to SBM due to an increase in dietary fiber (Smith and Aldrich, 2023; Kilburn-Kappeler and Aldrich, 2023). However, in the current study, fecal output of dogs fed CFP was comparable to CON and/or BDY whereas a higher fecal output was observed in the BDY+DDGS treatment. This indicates that the higher fiber level in DDGS had a greater effect on fecal mass compared to CFP when fed to dogs.

The increased fecal dry matter percent of dogs fed BDY+DDGS and CFP was likely due to the decrease in nutrient digestibility as a result of the increased dietary fiber content. Allen et al. (1981) reported an increase in fecal dry matter percent for dogs fed a diet containing 15.7% DDGS relative to dogs fed a diet containing 0% DDGS. A previous study also reported increased fecal dry matter in dogs fed CFP compared to a control containing SBM (Kilburn-Kappeler and Aldrich, 2023). Therefore, the fiber levels in both DDGS and CFP appear to impact the percent dry matter of feces when fed to dogs.

The increased number of defecations per day for dogs fed BDY+DDGS compared to dogs fed CON and BDY was likely due to the increased fiber content in the BDY+DDGS treatment. In agreement with the current study, Smith and Aldrich (2023) reported no difference in fecal defecation among dogs consuming diets containing CFP or SBM which contained 18 and 15% total dietary fiber, respectively. These results indicate, that when compared to DDGS, CFP had less of an effect on the number of defecations per day when fed to dogs.

The similar fecal scores of dogs fed BDY+DDGS and CON is consistent with previous studies (Silva et al., 2016; Risolia et al., 2019). In agreement with the current study, Kilburn-Kappeler and Aldrich (2023) reported increased fecal scores for dogs fed diets containing 5, 10, and 15% CFP compared to a SBM containing control diet. In contrast, no difference in fecal scores were noted for dogs consuming diets containing 25% CFP or SBM (Smith and Aldrich,

2023). Even with statistical differences in fecal score among dietary treatments, all remained within ideal ranges.

Compared to the BDY treatment, dogs fed the BDY+DDGS treatment had a lower fecal pH. A previous study also reported a reduced fecal pH in dogs fed DDGS, indicating a possible prebiotic effect (Risolia et al., 2019). Silva et al. (2016) also observed a decreased fecal pH due to DDGS consumption in dogs, which was attributed to an increase in SCFA production (Kawauchi et al., 2011). In agreement with the current study, Kilburn-Kappeler and Aldrich (2023) reported no difference in fecal pH when dogs were fed increasing levels of CFP resulting in diets up to 16% total dietary fiber. Therefore, the increased fiber content in DDGS compared to CFP may have a greater effect on fecal pH.

Apparent Total Tract Digestibility

The decreased digestibility for BDY+DDGS and CFP compared to CON and BDY is likely due to the fiber content of dietary treatments. Previous studies evaluating DDGS have attributed a decrease in nutrient digestibility to increased dietary fiber in dogs (Allen et al., 1981; Corbin et al., 1984; Silva et al., 2016; Risolia et al., 2019). Silva et al. (2016) and Risolia et al. (2019) reported reduced nutrient digestibility in dry matter, organic matter, fat, and gross energy when dogs were fed DDGS. Allen et al. (1981) and Corbin et al. (1984) reported decreased dry matter digestibility in dogs with dietary addition of DDGS. Silva et al. (2016) also reported decreased protein digestibility when DDGS were fed to dogs. However, some studies have reported no difference in protein digestibility with DDGS inclusion in dogs (Allen et al., 1981; Corbin et al., 1984; Risolia et al., 2019). Whereas the current study observed decreased digestibility in all nutrients with BDY+DDGS.

Previous studies have reported a decreased digestibility in dogs fed diets containing CFP compared to SBM (Smith and Aldrich, 2023; Kilburn-Kappeler and Aldrich, 2023). For example, Smith and Aldrich (2023) reported that a diet containing CFP resulted in lower ($P < 0.05$) dry matter digestibility at 78% when compared to a diet containing SBM at 81% when fed to dogs. In addition, Kilburn-Kappeler and Aldrich (2023) reported a linear decrease ($P < 0.05$) in organic matter digestibility with increased inclusion levels of CFP in exchange for SBM when fed to dogs. However, in the current study the BDY+DDGS treatment resulted in a greater decrease in digestibility compared to the CFP treatment. Therefore, inclusion of CFP in pet food could be beneficial compared to traditional distillers dried grains due to the improved nutrient digestibility.

Fecal Chemical Analysis

Short and branched chain fatty acids are microbial fermentation products which are influenced by undigested material in the large intestine. For example, carbohydrate fermentation yields SCFA including acetate, propionate, and butyrate which can reduce the pH of the lumen (Wong et al., 2006). Whereas protein fermentation yields the production of BCFA and ammonia (Herrin, 1940; Nery et al., 2012). Ammonia accumulation in the intestine has been shown to shorten the life of colonocytes (Lin and Visek, 1991) and has cytotoxic properties (Fung et al., 2013). Thus, an increase in SCFA and a decrease in pH, BCFA, and ammonia could be interpreted as a positive effect on intestinal health (Verbeke et al., 2015).

Due to the increased dietary fiber content in BDY+DDGS and CFP it would be expected that SCFA production would have increased. However, total SCFA concentrations were comparable among all dietary treatments. More surprisingly, the proportion of propionate decreased in BDY+DDGS compared to CON. In contrast to the current study, Risolia et al.

(2019) reported that DDGS increased total SCFA production and proportion of acetate and propionate when fed to dogs. The decreased valerate in CFP appeared to be due to the yeast component as a numerical decrease was also observed in BDY and BDY+DDGS compared to CON. Even with the shifts in propionate and valerate, total SCFA and BCFA were unaltered indicating overall fermentation was not impacted by dietary treatment.

Palatability

Previous studies have evaluated the palatability of DDGS in dogs. Corbin et al. (1984) and Silva et al. (2016) reported improved palatability when dogs were fed a diet containing DDGS. Whereas Risolia et al. (2019) reported similar palatability results to the current study with no preference based on first choice or intake ratio when comparing a control diet to a diet containing DDGS in dogs.

Previous studies have also compared the palatability of CFP to SBM in dogs with varying results. Smith and Aldrich (2023) reported no preference between diets containing a 25% inclusion of SBM or CFP when fed to dogs. Kilburn-Kappeler and Aldrich (2023) reported no preference when a 5% inclusion of CFP was compared to a control diet containing SBM. However, increased CFP inclusion levels at 10 and 15% appeared to decrease palatability when fed to dogs (Kilburn-Kappeler and Aldrich, 2023). In the current study, a 17.5% inclusion of CFP appeared to decrease palatability when compared to a control containing SBM. When including CFP in pet food, additional palatant could easily be added to prevent any chance of decreased palatability. However, the palatability results are interesting, as the highest CFP inclusion level (25%) did not appear to effect palatability whereas palatability decreased with lower inclusion levels. This may indicate that the entire diet matrix or individual dogs may

impact palatability results. Regardless, dogs willing consumed all treatments and no refusals were observed.

Conclusion

Overall, the inclusion of CFP compared to DDGS resulted in improved stool quality and nutrient digestibility when fed to dogs. Compared to DDGS, the inclusion of CFP in pet food could be beneficial due to its enhanced nutrient composition, likely due to its substantial yeast component. The improved amino acid profile and decreased fiber content of CFP compared to DDGS allows CFP to be better equipped to meet nutrient requirements and result in higher nutrient digestibility. However, inclusion of CFP may be limited regarding palatability. In conclusion, CFP may be utilized as a protein source for pet food which could increase consumer appeal of co-products from the ethanol industry resulting in more sustainable products.

This study provided valuable insight regarding the comparison of traditional distillers dried grains to CFP, a novel ingredient containing both yeast and distillers dried grains, when fed to dogs. However, it would be beneficial to conduct a future study utilizing CGM as the control ingredient instead of SBM, allowing for an evaluation of corn protein. In addition, the experimental ingredients should be exchanged equally without the addition of SBM, CGM, or corn starch. Finally, the major contributor impacting the results was likely the varying fiber content of dietary treatments. Therefore, the future study should also maintain fiber content among dietary treatments to exclusively evaluate the effect of experimental ingredients.

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Chapter 4 Tables

Table 4.1 Ingredient composition of canine diets containing yeast and ethanol co-products on an as-is basis

	Treatment[1]			
Ingredient, %	CON	BDY	BDY+DDGS	CFP
Corn	34.6	30.0	30.0	34.6
Chicken Meal	30.0	35.0	35.0	30.0
Soybean Meal	15.0	8.0	-	-
Distillers dried grains with solubles	-	-	17.5	-
Corn Fermented Protein	-	-	-	17.5
Brewer's Dried Yeast	-	3.5	2.5	-
Corn Starch	-	6.5	-	2.5
Corn Gluten Meal	5.0	2.0	-	-
Chicken Fat	6.0	5.6	5.6	6.0
Beet Pulp	4.0	4.0	4.0	4.0
Fish Meal	3.0	3.0	3.0	3.0
Flavor	1.0	1.0	1.0	1.0
Titanium Dioxide	0.40	0.40	0.40	0.40
Salt	0.25	0.25	0.25	0.25
Potassium Chloride	0.25	0.25	0.25	0.25
Choline Chloride	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
Natural Antioxidant	0.05	0.05	0.05	0.05

[1]CON, control; BDY, brewer's dried yeast; BDY+DDGS, brewer's dried yeast and distillers dried grains with solubles; CFP, corn fermented protein.
[2]Supplied the following minimum supplements per kilogram: vitamin E (79,887 IU), niacin (64,736 mg), calcium pantothenate (12,186 mg), vitamin A (17,162,998 IU), thiamin mononitrate (14,252 mg), pyridoxine hydrochloride (5,537 mg), riboflavin (4,719 mg), vitamin D3 (920,000 IU), biotin (70 mg), vitamin B12 (22 mg), and folic acid (720 mg).
[3]Supplied the following minimum supplements per kilogram: zinc sulfate (88,000 mg), ferrous sulfate (38,910 mg), copper sulfate (11,234 mg), manganous oxide (5,842 mg), sodium selenite (310 mg), and calcium iodate (1,584 mg).

Table 4.2 Nutrient composition of experimental ingredients on a dry matter basis

	Experimental Ingredients[1]				
Nutrient, %	SBM	CGM	BDY	DDGS	CFP
Dry Matter	88.03	93.31	92.72	90.14	94.87
Organic Matter	91.86	98.71	92.76	94.79	97.16
Ash	8.14	1.29	7.24	5.21	2.84
Crude Protein	53.44	70.00	47.00	31.87	52.62
Fat	2.71	6.80	3.21	11.75	5.60
Total Dietary Fiber	19.88	2.57	25.67	44.71	34.89
Insoluble Dietary Fiber	16.36	2.46	3.34	41.60	31.41
Soluble Dietary Fiber	3.52	0.11	22.32	3.11	3.58

[1]SBM, soybean meal; CGM, corn gluten meal; BDY, brewer's dried yeast; DDGS, distillers dried grains with solubles; CFP, corn fermented protein.

Table 4.3 Amino acid composition of experimental ingredients on a dry matter basis

	Experimental Ingredients[1]				
Amino Acid, %	SBM	CGM	BDY	DDGS	CFP
Taurine	0.19	0.12	0.22	0.11	0.16
Hydroxyproline	0.08	0.00	0.00	0.00	0.00
Aspartic Acid	6.04	4.15	4.26	1.86	3.68
Threonine	2.04	2.22	2.02	1.19	2.01
Serine	2.22	2.84	1.88	1.26	2.18
Glutamic Acid	9.60	14.51	5.25	3.51	8.35
Proline	2.76	6.56	2.04	2.22	4.11
Lanthionine	0.03	0.05	0.03	0.27	0.09
Glycine	2.19	1.78	2.05	1.07	1.99
Alanine	2.25	5.85	3.01	1.97	3.65
Cysteine	0.80	1.15	0.49	0.54	1.01
Valine	2.59	3.15	2.43	1.56	2.81
Methionine	0.73	1.47	0.70	0.55	1.18
Isoleucine	2.64	2.98	2.12	1.25	2.32
Leucine	4.17	11.46	3.02	3.51	6.17
Tyrosine	2.07	3.58	1.53	1.20	2.29
Phenylalanine	2.82	4.49	1.95	1.78	2.77
Hydroxylysine	0.00	0.00	0.00	0.00	0.00
Ornithine	0.03	0.06	0.25	0.03	0.02
Lysine	3.40	1.16	3.22	0.99	1.73
Histidine	1.40	1.38	0.98	0.81	1.38
Arginine	3.74	2.08	2.22	1.23	2.23
Tryptophan	0.65	0.41	0.58	0.21	0.45
Available Lysine	3.31	1.11	3.14	0.93	1.55
Lysine Availability	97.35	95.69	97.52	93.94	89.60

[1]SBM, soybean meal; CGM, corn gluten meal; BDY, brewer's dried yeast; DDGS, distillers dried grains with solubles; CFP, corn fermented protein.

Table 4.4 Analyzed chemical composition of canine diets containing yeast and ethanol co-products on a dry matter basis

	Treatment[1]			
Nutrient	CON	BDY	BDY+DDGS	CFP
Dry Matter, %	95.61	95.92	94.78	95.38
Organic Matter, %	90.54	90.44	90.62	91.78
Ash, %	9.46	9.56	9.38	8.22
Crude Protein, %	41.13	40.82	38.18	37.55
Fat, %	13.15	13.07	14.82	13.70
Total Dietary Fiber, %	13.58	13.16	18.39	15.07
Insoluble Dietary Fiber, %	10.03	10.02	14.28	12.41
Soluble Dietary Fiber, %	3.65	3.14	4.10	2.64
Gross Energy, kcal/kg	5008.71	4988.17	5073.11	5054.00

[1]CON, control; BDY, brewer's dried yeast; BDY+DDGS, brewer's dried yeast and distillers dried grains with solubles; CFP, corn fermented protein.

Table 4.5 Food intake and stool quality parameters of dogs fed diets containing yeast and ethanol co-products[1]

	Treatment[2]					
Parameter	CON	BDY	BDY+DDGS	CFP	SEM	*P*-value
Food Intake, g/d	186.32	189.02	185.52	185.91	2.755	0.5806
Wet Fecal Output, g/d	103.44[b]	101.38[b]	124.75[a]	111.54[b]	3.787	<0.0001
Fecal Dry Matter, %	33.67[b]	35.70[a]	35.65[a]	35.77[a]	0.423	<0.0001
Dry Fecal Output, g/d	34.67[c]	36.10[b,c]	44.47[a]	39.17[b]	1.269	<0.0001
Defecations per Day	2.23[b]	2.18[b]	2.60[a]	2.39[a,b]	0.112	0.0003
Fecal Score	3.85[b]	3.97[a,b]	3.86[b]	4.04[a]	0.049	0.0007
Fecal pH	5.73[a,b]	5.83[a]	5.56[b]	5.67[a,b]	0.083	0.0215

[1]A total of 12 dogs were enrolled in a 4x4 replicated Latin square design, resulting in 12 observations per treatment for each parameter.
[2]CON, control; BDY, brewer's dried yeast; BDY+DDGS, brewer's dried yeast and distillers dried grains with solubles; CFP, corn fermented protein.
[a-c]Means within a row lacking a common superscript letter are different ($P < 0.05$).

Table 4.6 Apparent total tract digestibility of dogs fed diets containing yeast and ethanol co-products estimated by titanium dioxide as a dietary marker on a dry matter basis[1]

	Treatment[2]					
Nutrient, %	CON	BDY	BDY+DDGS	CFP	SEM	*P*-value
Dry Matter	81.56[a]	81.14[a]	77.13[b]	77.80[b]	0.275	<0.0001
Organic Matter	86.78[a]	86.82[a]	81.69[c]	82.52[b]	0.236	<0.0001
Crude Protein	88.34[a]	86.97[b]	85.63[c]	84.99[c]	0.243	<0.0001
Fat	97.92[a]	98.04[a]	96.12[b]	97.55[a]	0.191	<0.0001
Total Dietary Fiber	56.02[a]	57.72[a]	49.55[b]	42.73[c]	1.105	<0.0001
Gross Energy	87.63[a]	87.52[a]	83.30[c]	83.94[b]	0.185	<0.0001

[1]A total of 12 dogs were enrolled in a 4x4 replicated Latin square design, resulting in 12 observations per treatment for each parameter.
[2]CON, control; BDY, brewer's dried yeast; BDY+DDGS, brewer's dried yeast and distillers dried grains with solubles; CFP, corn fermented protein.
[a-c]Means within a row lacking a common superscript letter are different ($P < 0.05$).

Table 4.7 Fecal chemical analysis of dogs fed diets containing yeast and ethanol co-products[1]

	Treatment[2]					
Parameter	CON	BDY	BDY+DDGS	CFP	SEM	*P*-value
Total SCFA[3], μmol/g DM feces	421.03	350.93	384.64	346.57	33.441	0.1154
Acetate, %	61.73	65.54	66.08	64.91	1.954	0.1301
Propionate, %	28.40[a]	25.13[a,b]	24.21[b]	24.72[a,b]	1.467	0.0323
Butyrate, %	9.87	9.33	9.71	10.37	0.701	0.5288
Total BCFA[4], μmol/g DM feces	15.04	13.68	16.45	15.14	1.844	0.5289
Isovalerate, %	54.92	58.00	54.53	58.29	2.580	0.3309
Isobutyrate, %	35.78	35.81	37.17	37.30	2.107	0.8149
Valerate, %	9.30[a]	6.19[a,b]	8.30[a,b]	4.41[b]	1.735	0.0370

[1]A total of 12 dogs were enrolled in a 4x4 replicated Latin square design, resulting in 12 observations per treatment for each parameter.
[2]CON, control; BDY, brewer's dried yeast; BDY+DDGS, brewer's dried yeast and distillers dried grains with solubles; CFP, corn fermented protein.
[3]Total SCFA (acetate + propionate + butyrate); individual SCFA is expressed as a percent of total SCFA.
[4]Total BCFA (isovalerate + isobutyrate + valerate); individual BCFA is expressed as a percent of total BCFA.
[a-b]Means within a row lacking a common superscript letter are different ($P < 0.05$).

Table 4.8 First choice (FC) and intake ratio (IR) of dogs fed diets containing yeast and ethanol co-products[1]

Diet Comparison (A vs B)[2]	FC[3]	IR[4]
BDY vs CON	17	0.359
BDY+DDGS vs CON	24	0.389
CFP vs CON	13*	0.245*

[1]A total of 20 dogs were fed each diet comparison over 2 days, resulting in 40 observations per comparison.
[2]CON, control; BDY, brewer's dried yeast; BDY+DDGS, brewer's dried yeast and distillers dried grains with solubles; CFP, corn fermented protein.
[3]Number of first visits to bowl A out of 40 observations.
[4]IR = intake (g) of diet A/total intake (g) of diets A+B.
*Comparison differs ($P < 0.05$).

Chapter 5 - Diet production and utilization of corn fermented protein (CFP) compared to traditional yeast in healthy adult cats

Abstract

The inclusion of yeast in pet food can provide health benefits and increase palatability. Corn fermented protein is a co-product from ethanol production which contains approximately 20-25% yeast. The objective of this study was to determine the effects of the yeast in CFP on diet production and utilization when fed to healthy adult cats. The four experimental diets included a control with 15% soybean meal (CON) and diets containing either 3.5% brewer's dried yeast (BDY), 2.5% brewer's dried yeast plus 17.5% distillers dried grains with solubles (BDY+DDGS), or 17.5% corn fermented protein (CFP). All treatments except CON were formulated to contain 3.5% yeast. Experimental diets were fed to adult cats (n = 11) in an incomplete 4 x 4 replicated Latin square design. Cats were adapted to diet for 9 days followed by a 5-d total fecal collection. Titanium dioxide (0.4%) was added to all diets as an external marker to estimate digestibility. Data were analyzed using a mixed model in SAS (version 9.4, SAS Institute, Inc., Cary, NC) with treatment as a fixed effect and cat and period as random effects. Preconditioner discharge temperature was greater ($P < 0.05$) for CON and BDY (average, 96°C) compared to BDY+DDGS and CFP (average, 91°C). Extruder screw speed, die temperature, kibble toughness, and kibble hardness were greatest ($P < 0.05$) for CFP. The bulk density of BDY+DDGS at 392 g/L was greater ($P < 0.05$) than BDY and CFP (average, 342 g/L). The sectional expansion index of kibble for CFP was greater ($P < 0.05$) than BDY+DDGS and smaller ($P < 0.05$) than CON but similar to BDY. Fecal output was greatest ($P < 0.05$) for cats fed BDY+DDGS. Nutrient digestibility was lowest ($P < 0.05$) for BDY+DDGS. The concentrations of short-chain and branched-chain fatty acids in fecal samples were not altered (*P*

> 0.05) by dietary treatment. Cats had no preference ($P > 0.05$) when comparing CON to BDY or BDY+DDGS. However, cats consumed significantly less CFP compared to CON. The significant differences for bulk density, fecal output, and nutrient digestibility among dietary treatments are likely due to a greater fiber effect of DDGS compared to CFP. Therefore, the yeast component in CFP may provide greater kibble expansion and nutrient utilization compared to DDGS when fed to cats.

Introduction

Many commercial pet foods contain yeast as an ingredient, with brewer's dried yeast (BDY) as the most common source. Pet food can also contain yeast extract, yeast cell walls, or yeast culture. Yeast was originally included in pet food as a source of B vitamins, but its function has evolved due to the supplementation of B vitamins in pet food today (Beynen, 2019). In the current market, nutritional yeast supplements claim to control fleas and promote healthy skin and coat (Beynen, 2019). Whereas BDY is often added at a 1% inclusion to improve the palatability of kibble (Swanson and Fahey, 2007). In addition, extracts from yeast cell walls such as mannan oligosaccharides (MOS) and beta-glucans are reported to support gut and immune health in dogs (Swanson et al., 2002; Pawar et al., 2017; Rummell et al., 2022; Fries-Craft et al., 2023) and cats (Santos et al., 2018; Calabrò et al., 2020).

Brewer's yeast is the dried product of the slurry that remains after beer and ale fermentation. Typically, beer is derived from malted barley which is fermented slowly at 10-20°C and produced using a batch fermentation process resulting in approximately 6% alcohol. On average BDY contains 41% protein, 3% fat, and 6% ash on a dry matter basis (Swanson and Fahey, 2007). In addition to its use as a palatant, yeast has been reported to be an adequate protein source in dog diets (Reilly et al., 2021; Martins et al., 2013).

Corn fermented protein (CFP), a co-product from ethanol production, could be utilized as a novel yeast ingredient for pet food. Corn fermented protein contains 53% protein, 6% fat, and 3% ash on a dry matter basis (Kilburn-Kappeler et al., 2022). In addition, CFP contains a substantial yeast component at approximately 20-25%. In contrast to the brewing industry, ethanol is produced from corn using a rapid continuous fermentation at 35-38°C, yielding 9-12% alcohol. As a result, yeast co-products from the brewing and distilling industries differ in nutrient composition and organoleptic properties due to the difference in substrate and processing methods (Swanson and Fahey, 2007). Previous studies have reported CFP to be an acceptable protein source for both dogs and cats when compared to soybean meal and corn gluten meal (Kilburn-Kappeler et al., 2022; Smith and Aldrich, 2023; Kilburn-Kappeler and Aldrich, 2023), but the yeast component has yet to be evaluated in cats. Therefore, the objective of this study was to compare the effects of the yeast in CFP to BDY, a common yeast ingredient in pet food, on diet production and utilization in cats.

Materials and Methods

The feeding trial was conducted at Kansas State University Veterinary Medicine Complex (Coles Hall) under the Institutional Animal Care and Use Committee (IACUC) #4348 protocol. The palatability trial was conducted at Summit Ridge Farms (Susquehanna, PA) under protocols KSUPALF00720, KSUPALF00820, and KSUPALF00920.

Diet Formulation

Dietary treatments consisted of a control diet containing 15% soybean meal (CON) and experimental diets containing either 3.5% brewer's dried yeast (BDY), 2.5% brewer's dried yeast plus 17.5% distillers dried grains with solubles (BDY+DDGS), or 17.5% corn fermented protein (CFP). Diets with yeast and/or ethanol co-products were formulated to have a similar

nutrient profile. Also, it was assumed that CFP and DDGS were comprised of 20% and 5.7% yeast, respectively; therefore, all treatments, except CON, were formulated to contain 3.5% yeast. The formulated diets met the AAFCO nutritional requirements for adult cats. Two main dry rations were purchased from a commercial mill (Fairview Mills, Seneca, KS), which contained corn, chicken meal, beet pulp, fish meal, salt, potassium chloride, vitamin and mineral premix, choline chloride, and natural antioxidant. The inclusion of corn and chicken meal varied slightly among base rations while all other ingredients were maintained. One ration was used for CON and CFP treatments while the other was used for BDY and BDY+DDGS treatments in attempt to maintain nutrient composition among all diets. The remaining ingredients, soybean meal (Fairview Mills, Seneca, KS), CFP (POET Bioproducts, Sioux Falls, SD), DDGS (Fairview Mills, Seneca, KS), BDY (Fairview Mills, Seneca, KS), corn starch (Fairview Mills, Seneca, KS), and corn gluten meal (Fairview Mills, Seneca, KS), were added to the base rations during diet production. Soybean meal, corn gluten meal and/or corn starch were added to CON, BDY, and CFP to create similar nutrient profiles among all dietary treatments and to balance a 20% inclusion of experimental ingredients compared to BDY+DDGS. Titanium dioxide (Fairview Mills, Seneca, KS) was also added to all diets at 0.4% to serve as an indigestible marker to estimate apparent total tract nutrient digestibility (**Table 5.1**).

Diet Production

Each diet was produced using a single screw extruder (model E525, ExtruTech, Inc., Sabetha, KS). The preconditioner (model ADP 145, ExtruTech, Inc., Sabetha, KS) was configured with 12, 45° back and 57 neutral beaters on each of the two shafts. The extruder profile and barrel temperatures were based on a typical commercial pet food configuration. At the end of the extruder barrel there were two round die inserts with an interior diameter of 3 mm.

Dry matrix feed rate (318 kg/h) and pre-conditioner (PC) cylinder speed (185 rpm) were kept constant during the processing of all treatments.

During processing, PC and extruder (EX) parameters were collected from sensor readouts every 2 minutes to evaluate potential effects of CFP inclusion on the process. Output variables included PC discharge temperature, EX motor load, EX die temperature, total mass flow (TMF), specific mechanical energy (SME), and in-barrel moisture content (MC).

The TMF was calculated by adding the dry feed rate with water and steam injected in PC and EX, assuming that 80% of the water coming from the PC and EX steam is lost during flash-off as kibbles exit the die:

TMF = dry feed rate + PC water + (0.2 * PC steam) + EX water + (0.2 * EX steam)

SME was calculated using the following formula:

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

where, τ is the EX % torque or EX motor load, τ_0 is the EX no load % torque (25% at EX screw speed 425 rpm), N is the EX screw speed (rpm), N_r is the rated EX screw speed (425 rpm), P_r is the rated EX motor power (114 kW), and m is TMF (kg/s).

The in-barrel moisture content (MC) was also calculated using the following formula:

$$MC = \frac{m_f \times X_f + m_{ps} + m_{pw} + m_{es} + m_{ew}}{m_f + m_{ps} + m_{pw} + m_{es} + m_{ew}}$$

where m_f is the feed rate, X_f is the moisture content of the raw material, m_{ps} is the percentage of added steam in the preconditioner, m_{pw} is the percentage of added water in the preconditioner, m_{es} is the percentage of steam added into the extruder, and m_{ew} is the percentage of water added into the extruder. A moisture content of 10% was assumed for X_f.

After extrusion, kibble was pneumatically conveyed through an 8” clean air hood system and deposited onto an oscillating belt spreader. The kibble was dried on a 1.5 m wide single pass two zone dryer (model AFI, ExtruTech, Sabetha, KS) to achieve a less than 10% moisture content. Kibble was dried at approximately 105°C for 21 minutes. Dried kibble was coated with chicken fat protected with natural antioxidants (Nutrios, Springfield, MO) and a dry powdered flavor designed for cats (AFB International, St. Charles, MO). Coated diets were stored in poly-lined Kraft paper bags in a warehouse for 3 months prior to feeding.

Physical Characteristics of Kibble

Bulk density was measured off the dryer every 15 minutes during the processing of each treatment. Bulk density was measured using a 1 L cup in which kibble was leveled and weighed on a digital scale with 0.1 g sensitivity. In addition, every 15 minutes five randomly selected kibbles from each diet production off the dryer were measured for diameter and length using a digital caliper. Ten randomly selected kibbles off the dryer were also weighed using a digital scale with 0.0001 g sensitivity (EX324N; Ohaus Corporation, Parsippany, NJ, U.S.A.). The diameter, length, and mass measurements were used to determine sectional expansion index and specific length.

Sectional expansion index (SEI) was determined by comparing the squared diameter of the dried extruded kibbles by the squared die diameter of the extruder:

$$SEI = \frac{D^2}{d^2}$$

where D is the extrudate diameter and d is the extruder die diameter.

Specific length in mm/g was determined by the following equation:

$$Specific\ length = \frac{l}{m}$$

where *l* is the extrudate length and *m* is the extrudate mass.

A texture analyzer (model TA-XT2, Texture Technology Corp., Scarsdale, NJ) with a 30 kg load cell was used to measure kibble texture. A cylindrical probe (25 mm diameter) was used to compress 30 kibbles within each treatment. The procedure was adapted from Dogan and Kokini (2007) with a test speed of 2 mm/s and strain level set at 80%. Kibble hardness was considered to be the peak force in kg of the first major kibble breakage and the energy to compress the kibbles to 80% was computed as the area under the curve in kg mm for each compressed kibble not accounting for the negative values. The compression energy was considered as kibble toughness.

Feeding Trial

Eleven healthy adult (3.1 ± 1.7 years) American shorthair cats (10 males and 1 female) were enrolled in an incomplete 4x4 triplicated Latin square design comprised of four experimental treatments and four 14 d periods. Each of the four periods were composed of 9 days for diet adaptation followed by 5 days of fecal collection, which has been reported to be more than sufficient for stabilizing the gut to dietary intervention (Lin et al., 2023). Cats had an average body weight of 5.6 ± 1.7 kg. The daily metabolizable energy requirement was calculated for lean cats with $100*BW_{kg}^{0.67}$ (NRC, 2006). The food allowance of individual cats was adjusted during each adaptation period if needed to maintain body weight.

The cats were housed on a 12 h light cycle with lights off from 1900 to 0700. During the adaptation period, the cats were group-housed but fed individually. Whereas in the collection period, the cats were individually housed in stainless steel cages. The cats received two feedings per day at 0800 and 1700 h with access to food for 1 h and water *ad libitum*. During the collection period, all feces and orts were collected daily. All feces were weighed and scored on a

1 – 5 scale in 0.5 increments with a score of 3.5 – 4.0 considered ideal [1 – liquid diarrhea to 5 – dry hard pellets; (Carciofi et al., 2008)]. All feces were scored by a single person for consistency. However, this individual was not blinded to treatments. Feces were stored in labeled whirl-pak bags in a freezer until further processed. In addition, pH of a fresh sample (within 15 minutes of defecation) was recorded in triplicate with a calibrated glass-electrode pH probe (FC240B, Hanna Instruments, Smithfield, RI), and 2-g aliquots were transferred into three plastic microcentrifuge tubes using a spatula and stored at -80°C for short-chain fatty acid (SCFA) and branched-chain fatty acid (BCFA) analysis.

Digestibility Calculations

After each collection period, all feces from each cat were composited and dried at 55°C in a forced air oven until constant weight (24 – 48 h). Dried samples were ground to pass through a 1 mm screen in a laboratory fixed blade impact mill (ZM 200, Retsch, Verder Scientific, Haan, Germany). Titanium dioxide (TiO_2) concentration was measured in food and feces using a spectrophotometric plate reader (Gen5TM, Biotek® Instruments, Inc.Winooski, VT) at 410 nm (Myers et al., 2004). Apparent total tract digestibility (ATTD) was estimated by TiO_2 using the following equation:

$$ATTD = \left[1 - \frac{\%\ \mathrm{TiO2}\ in\ food * \ \%\ nutrient\ in\ feces}{\%\ \mathrm{TiO2}\ in\ feces * \ \%\ nutrient\ in\ food}\right] * 100$$

Digestibility was calculated using both the total collection and titanium dioxide methods, which resulted in similar digestibility values and trends. However, the titanium dioxide method resulted in a lower standard error of the mean. Therefore, digestibility values from the titanium dioxide method were selected to report in this manuscript.

Nutrient Analysis

Food and partially dried fecal samples were analyzed in duplicate for moisture (AOAC 930.15), ash (AOAC 942.05), fat by acid hydrolysis and hexane extraction (AOAC 960.39), gross energy (Parr 6200 Calorimeter, Parr Instrument Company, Moline, IL), and total dietary fiber (AOAC 991.43). Crude protein was determined by Dumas combustion (AOAC 990.03) using a nitrogen analyzer (FP928, LECO Corporation, Saint Joseph, MI).

Fecal Chemical Analysis

Fecal SCFA and BCFA concentrations were determined by gas-liquid chromatography (Erwin et al., 1961) using a capillary column (30 m x 0.25 mm internal diameter; 0.25 µm film thickness; Aligent Technologies, Santa Clara, CA). The system was equipped using helium as a carrier gas with a constant flow rate of 40 cm/s and utilizing a 25:1 split ratio injector with injection size of 0.5 µL. A flame ionization detector was configured with hydrogen as the makeup gas with a flow rate of 40 mL/min to clarify peak resolution. The detector and injector temperatures were set at 250°C, and the initial oven temperature was set to 80°C with a ramp rate of 10°C/min to 200°C. The peak area of chromatograms was analyzed using integrative software (GC solution version 2.42.00, Shimadzu, Kyoto, Japan). Concentrations of SCFA (acetate, propionate, and butyrate) and BCFA (isobutyrate, valerate, 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) and correcting for fecal dry matter content.

Palatability Trial

Experimental treatments (BDY, BDY+DDGS, and CFP) were evaluated for palatability versus the control diet (CON) by cat panels at a commercial kennel (Summit Ridge Farms,

Susquehanna, PA). Each experiment was conducted as a split-plate test, in which two stainless steel bowls containing 100 g of food were presented to animals for a total of 4 hours. Each comparison trial was repeated for two days, with a bowl position switched daily. Twenty animals were fed daily, providing 40 observations for each paired comparison test. Preference was determined based on animals' first choice and total food consumption. Data from consumption was represented as the following ratio:

$$Intake\ Ratio = \frac{consumption\ of\ Diet\ A}{total\ consumption\ Diet\ A+Diet\ B}$$

Statistics

Processing and digestibility data were analyzed using a GLIMMIX procedure in SAS (version 9.4, SAS Institute, Inc., Cary, NC). Tukey's post hoc test was applied for the least-squares means separation, with significance considered at $P < 0.05$. For each diet production, sampling was conducted at evenly spaced intervals which were considered replicates. The digestibility experiment was conducted as an incomplete 4x4 replicated Latin square design. Each of the 11 experimental units (cats) were assigned to treatment using the spreadsheet by Kim and Stein (2009). Dietary treatment was the fixed effect and cat and period were the random effects within the model.

In the palatability experiment, the consumption ratio was analyzed using a t-test in a 2-way ANOVA and the first-choice preference was analyzed using a Chi^2 test. The twenty cats were considered the experimental units for analysis.

Results and Discussion

Extrusion Processing and Kibble Characteristics

Preconditioner (PC) cylinder speed was maintained at 185 rpm for production of all dietary treatments (**Table 5.2**). Steam flow to the PC was greatest ($P < 0.05$) during production

of CON and BDY at an average of 47 kg/hr and lowest ($P < 0.05$) for CFP at 37 kg/hr. Steam flow for BDY+DDGS was intermediate at 41 kg/hr. Preconditioner water flow was greater ($P < 0.05$) during production of CON and BDY at an average of 57 kg/hr compared to BDY+DDGS and CFP at an average of 49 kg/hr. Preconditioner discharge temperature was greater ($P < 0.05$) for BDY+DDGS and CFP at 96°C compared to CON and BDY at 91°C.

Extruder (EX) screw speed was fastest ($P < 0.05$) for CFP at 475 rpm compared to the remaining treatments at an average of 446 rpm (**Table 5.2**). Extruder steam flow was greater ($P < 0.05$) for BDY+DDGS at 21 kg/hr compared to BDY and CON at 9 and 0 kg/hr, respectively. Extruder steam flow for CFP was intermediate BDY+DDGS and BDY at 14 kg/hr. Water flow to the EX was greatest ($P < 0.05$) for BDY+DDGS at 8 kg/hr and lowest ($P < 0.05$) for CON and BDY at 0 kg/hr, with CFP intermediate at 4 kg/hr. Extruder motor load was greatest ($P < 0.05$) for CON compared to the remaining treatments. Total mass flow (TMF) was maintained ($P > 0.05$) among dietary treatments at an average of 385 kg/h. Specific mechanical energy (SME) was greater ($P < 0.05$) for CON at 124 kJ/kg compared to the other treatments at an average of 106 kJ/kg. In-barrel moisture content (MC) was greatest ($P < 0.05$) for BDY+DDGS at 34% and lowest ($P < 0.05$) for CFP at 32%, CON and BDY were intermediate at an average of 33%. Die temperature was greatest ($P < 0.05$) for CFP at 114°C compared to the remaining treatments at an average of 110°C. Extruder knife speed was fastest ($P < 0.05$) for BDY+DDGS and CFP at 2000 rpm and slowest ($P < 0.05$) for CON at 1600 rpm.

Bulk density was greater ($P < 0.05$) for BDY+DDGS at 392 g/L compared to BDY and CFP at an average of 342 g/L, while CON was intermediate at 363 g/L (**Table 5.2**). Kibble diameter was largest ($P < 0.05$) for CON at 6.8 mm and smallest ($P < 0.05$) for BDY+DDGS at 4.6 mm. Kibble diameter for BDY and CFP was intermediate at an average of 5.5 mm. Kibble

length was longer ($P < 0.05$) for CFP at 4.5 mm compared to CON and BDY+DDGS at 4.1 and 3.4 mm, respectively. Kibble length of BDY was intermediate CFP and CON at 4.4 mm. Kibble length was smallest ($P < 0.05$) for BDY+DDGS. Specific length of kibble was largest ($P < 0.05$) for BDY+DDGS at 151 mm/g and smallest ($P < 0.05$) for CON at 106 mm/g, BDY and CFP were intermediate at an average of 135 mm/g. Sectional expansion index (SEI) was greatest ($P < 0.05$) for CON. The SEI of CFP was greater ($P < 0.05$) than BDY+DDGS, with BDY intermediate. Kibble hardness and toughness was greatest ($P < 0.05$) for CFP compared to the remaining treatments. In addition, kibble hardness for CON was greater ($P < 0.05$) than BDY+DDGS with BDY intermediate. Whereas kibble toughness for CON was greater ($P < 0.05$) than BDY with BDY+DDGS intermediate.

The increase in PC discharge temperature for CFP compared to BDY was likely due to the fluctuations in input steam and water. The increased EX screw speed, EX steam flow, and EX water flow with CFP compared to BDY was due to the extruder operator and not the dietary matrix. It would be expected that an increase in screw speed would result in more mechanical energy, increasing material cook and expansion (Rokey, 2006). Therefore, the fastest screw speed would be expected to produce the most expanded kibble, which was observed in this study as CFP had the lowest bulk density numerically. However, the SME results were surprising as CON had the greatest SME rather than CFP. Regardless, CON and CFP resulted in similar bulk density. The motor load is related to screw speed, degree of fill, and viscosity of the feed material in the screw channel (De Pilli et al., 2021). Therefore, the comparable motor load for BDY and CFP indicates that varying ingredients did not impact viscosity. It would be expected that an increase in screw speed would decrease barrel fill, decreasing motor load (Unlu and Faller, 2002). However, screw speed did not appear to affect motor load in the current study,

which is supported by the consistent TMF among dietary treatments. The differences in MC among dietary treatments were minimal (< 2%) and considered to be of no practical importance. The increased die temperature of CFP compared to BDY was likely due to the increased EX screw speed. The increase in die temperature would also be expected to increase product expansion (Shukla et al., 2005). The increase in knife speed was also due to the extruder operator and not a direct effect of the dietary ingredients. The increased knife speed may have resulted in the deceased kibble length in BDY+DDGS. However, the kibble length of CFP was not affected.

A previous study reported that input processing parameters had to be adjusted to produce a similar product for a diet containing CFP compared to diets containing soybean meal (SBM) or corn gluten meal (CGM; Smith and Aldrich, 2023). That study observed that CFP required more PC water input and mechanical energy to result in a similar bulk density to the remaining treatments, which was attributed to the decreased starch content in CFP compared to SBM and CGM (Smith and Aldrich, 2023). Therefore, increasing PC water input and mechanical energy in the extruder barrel promoted gelatinization in the CFP treatment allowing for improved expansion and similar bulk density among dietary treatments. In the current study, the increased screw speed during the production of CFP would result in increased mechanical energy also promoting expansion. Even though CFP did not result in the greatest SME, the increased screw speed could have resulted in the similar SME of CFP to BDY.

Bulk density is a common and reliable quality control measurement used during the production of dry expanded pet food, which provides a quick and tangible measure of how well the product is cooked or expanded (Rokey, 2006). Bulk density and expansion have an inverse relationship meaning the lower the bulk density, the more expanded the product. The comparable bulk density, kibble diameter, kibble length, specific length, and SEI of BDY and CFP indicates

that the fiber content in CFP did not impact expansion. Whereas BDY+DDGS resulted in the greatest bulk density and smallest kibble diameter, length, and SEI likely due to the heightened dietary fiber content. As fiber is considered a dispersed phase filler during extrusion and is known to limit kibble expansion (Guy, 2001), which has been supported by previous studies (Hsieh et al., 1989, 1991; Monti et al., 2016; Alvarenga et al., 2018). Furthermore, Chevanan et al. (2004) and Kannadhason et al. (2010) reported that inclusion of DDGS decreased expansion of extruded aquaculture feed. Smith and Aldrich (2023) reported that CFP resulted in decreased kibble diameter and SEI compared to SBM and CGM. However, kibble length and specific length were maintained among dietary treatments (Smith and Aldrich, 2023). In the current study, kibble diameter and SEI were lower for CFP vs. CON. Kibble length and specific length were higher for CFP vs. CON.

Previous research has reported that kibble expansion has an impact on hardness and compression energy (Moraru and Kokini, 2003; Yannioties et al., 2007). The results for kibble hardness and toughness are interesting, as the diet with the least amount of expansion (BDY+DDGS) would be expected to result in greatest hardness and toughness. However, CFP resulted in the greatest kibble hardness and toughness in the current study. In contrast, Smith and Aldrich (2023) reported that a 25% inclusion of CFP did not impact kibble hardness or toughness when compared to a 25% inclusion of SBM or CGM. This could indicate that differences in processing parameters and ingredient matrices can impact the hardness and toughness of an extruded kibble.

It is important to note that the extruder operator may have a significant impact on processing conditions and subsequent product characteristics. Therefore, it is difficult to state any significant conclusions on the impact of ingredient matrices on final product characteristics.

Moving forward, it would be beneficial to maintain all input processing conditions or bulk density of treatments to better understand the effect of experimental ingredients.

Diet Chemical Analyses

Dry matter and organic matter contents of dietary treatments were maintained at averages of 95 and 91%, respectively (**Table 5.3**). The average crude protein content of CON and BDY was 41% whereas the average crude protein content for BDY+DDGS and CFP was 38%. The fat content was greatest for BDY+DDGS at 14% compared to the remaining treatments at 13%. The BDY+DDGS treatment resulted in the highest total dietary fiber at 18% followed by CFP at 15% then CON and BDY at 13%. The same pattern among dietary treatments was observed with insoluble dietary fiber content. However, CFP contained the lowest amount of soluble dietary fiber among dietary treatments. Gross energy was comparable among dietary treatments at an average of 5000 kcal/kg.

The maintenance of moisture content among dietary treatments was expected as drying conditions were controlled during processing. The decreased protein content in BDY+DDGS was also expected as DDGS contained the least amount of protein among experimental ingredients at 32% (Kilburn-Kappeler et al., 2023). However, the decreased protein content of CFP was surprising as CFP contains 53% protein which is comparable to SBM (53%) and greater than BDY (47%). The protein content of dietary treatments could have been impacted by the CGM and/or corn starch that was added to balance the 20% inclusion of experimental ingredients. The increased fat content in BDY+DDGS compared to the other treatments was expected as DDGS had the greatest fat content among experimental ingredients. In addition, DDGS had the greatest amount of total dietary fiber at 45% followed by CFP at 35% which can explain the increased fiber content in the BDY+DDGS and CFP treatments.

Feed Intake and Fecal Characteristics

Food intake of cats fed dietary treatments was greater ($P < 0.05$) for BDY+DDGS at 81 g/d compared to BDY at 78 g/d (**Table 5.4**). Food intake for CON and CFP was intermediate at an average of 80 g/d. Wet fecal output of cats was greatest ($P < 0.05$) for BDY+DDGS at 59 g/d compared to the remaining treatments at an average of 49 g/d (**Table 5.4**). Fecal dry matter increased ($P < 0.05$) for cats fed BDY at 35% compared to cats fed CFP and CON (average, 32%). Fecal dry matter of cats fed BDY+DDGS was intermediate to cats fed BDY and CFP at 34%. Dry fecal output of cats was greatest ($P < 0.05$) for BDY+DDGS at 20 g/d compared to the remaining treatments at an average of 16 g/d. The number of defecations per day was greater ($P < 0.05$) for cats fed BDY+DDGS compared to cats fed CON and CFP, with BDY intermediate. Feces were firmer ($P < 0.05$) when cats were fed BDY+DDGS compared to CON, whereas fecal scores of cats fed BDY and CFP were intermediate. Fecal pH of cats was comparable ($P > 0.05$) among all dietary treatments.

The minimal differences (< 3g) in food intake are not of practical concern as they are unlikely to affect stool quality or nutrient digestibility. In agreement with the current study, Kilburn-Kappeler et al. (2023) reported similar fecal output, defecations per day, fecal score, and fecal pH of dogs fed BDY compared to CFP. Previous studies have reported differences in stool quality when comparing yeast to traditional ingredients. For example, Reilly et al. (2021) reported increased fecal output of dogs consuming dried yeast compared to poultry by-product meal. Whereas Holt and Aldrich (2022) reported that fecal output on a dry matter basis of cats fed Torula yeast was comparable to cats fed chicken meal and SBM. However, defecations per day of cats were greater when fed a diet containing SBM than a diet containing Torula yeast, with defecations of cats consuming chicken meal intermediate (Holt and Aldrich, 2022). Even

with the differences in fecal output, Reilly et al. (2021) reported ideal fecal scores with no differences between dogs fed diets containing dried yeast or poultry by-product meal. Holt and Aldrich (2022), however, reported greater frequency of softer feces when cats were fed Torula yeast. Both studies also reported that fecal pH was not impacted by test ingredients (Reilly et al., 2021; Holt and Aldrich, 2022). The fecal dry matter percent of dogs fed CFP and BDY was comparable (Kilburn-Kappeler et al., 2023) whereas feces were dryer for cats fed BDY compared to CFP in the current study. Holt and Aldrich (2022) reported that consumption of Torula yeast decreased fecal dry matter percent of cats compared to SBM with no difference when compared to chicken meal. Based on the various results among the current and previous studies, it would be interesting to compare stool quality of cats fed BDY and CFP to an animal-based ingredient. However, the similar stool quality of cats fed BDY and CFP in the current study indicates that the greater dietary fiber content of CFP did not significantly impact stool quality when compared to BDY, which is supported by the previous study completed in dogs (Kilburn-Kappeler et al., 2023)

Apparent Total Tract Digestibility

There were significant differences in dry matter digestibility among all dietary treatments (**Table 5.5**). The dry matter digestibility was greatest ($P < 0.05$) for CON at 82% and lowest ($P < 0.05$) for BDY+DDGS at 75%. The dry matter digestibility of BDY and CFP were intermediate at 81 and 79%, respectively. Organic matter digestibility was greatest ($P < 0.05$) for CON and BDY at an average of 87% and lowest ($P < 0.05$) for BDY+DDGS at 81%, with CFP being intermediate at 84%. Crude protein digestibility was highest ($P < 0.05$) for CON at 89% and lowest ($P < 0.05$) for BDY+DDGS and CFP at an average of 84% with BDY being intermediate at 87%. Fat digestibility was greater ($P < 0.05$) for CON at 96% compared to BDY+DDGS and

CFP at 92 and 95%, respectively. In addition, fat digestibility was greater ($P < 0.05$) for BDY (96%) and CFP compared to BDY+DDGS. Total dietary fiber digestibility was greater ($P < 0.05$) for CON and BDY compared to BDY+DDGS and CFP. The digestibility of gross energy was greatest ($P < 0.05$) for CON and BDY at an average of 87% and lowest ($P < 0.05$) for BDY+DDGS at 82%, CFP was intermediate at 84%.

In agreement with the current study, Kilburn-Kappeler et al. (2023) reported increased dry matter, organic matter, crude protein, total dietary fiber, and gross energy digestibility of BDY compared to CFP when fed to dogs. In addition, fat digestibility of BDY and CFP was comparable in dogs (Kilburn-Kappeler et al., 2023). The decreased nutrient digestibility of the CFP treatment compared to the BDY treatment is likely due to the increased dietary fiber content of CFP. As increased dietary fiber has been known to decrease nutrient digestibility in both dogs and cats (Burrows et al., 1982; Fahey et al., 1990; Sunvold et al., 1995; Fisher et al., 2012). The TDF digestibility in the current study was higher than expected however values are similar to those reported for diets containing SBM and yeast fed to cats (Holt and Aldrich, 2022). In addition, previous studies have reported TDF digestibility of diets containing 15-25% CFP to be high at an average of 46.5% when fed to both dogs and cats (Kilburn-Kappeler et al., 2022; Smith and Aldrich, 2023; Kilburn-Kappeler and Aldrich, 2023; Kilburn-Kappeler et al., 2023). Previous studies have reported a decrease in dry matter, organic matter, and fat digestibility of a diet containing yeast compared to a diet containing an animal-based protein source in dogs and cats (Reilley et al., 2021; Holt and Aldrich, 2022). Whereas the digestibility of crude protein and total dietary fiber was comparable among the two diets in both studies (Reilly et al., 2021; Holt and Aldrich, 2022). Therefore, it would be interesting to compare the digestibility of BDY and CFP to an animal-based ingredient when fed to dogs and cats.

Fecal Chemical Analysis

Total SCFA concentrations in fecal samples of cats fed dietary treatments ranged from 516 to 545 μmol/g (**Table 5.6**). However, no significant differences in total SCFA were observed among dietary treatments. There were also no differences ($P > 0.05$) in percent acetate, propionate, or butyrate among dietary treatments with averages of 73, 21, and 7%, respectively. There were no significant differences observed in total BCFA concentrations among dietary treatments, ranging from 30 to 35 μmol/g. For BCFA, percent isovalerate, isobutyrate, and valerate were also comparable ($P > 0.05$) among dietary treatments at averages of 27, 16, and 57%, respectively.

In agreement with the current study, Kilburn-Kappeler et al. (2023) reported no significant differences in SCFA or BCFA concentrations in fecal samples of dogs fed BDY or CFP. These results indicate that CFP did not alter fermentation within the large intestine of dogs and cats when compared to BDY. Yet, Reilly et al. (2021) reported an increase in total SCFA as well as an increase in the proportion of acetate and butyrate in feces of dogs fed dried yeast compared to poultry by-product meal. However, the proportion of propionate in feces was comparable among the two diets (Reilly et al., 2021). In addition, total BCFA as well as the proportion of isobutyrate, isovalerate, and valerate were maintained in fecal samples of dogs fed diets containing dried yeast and poultry by-product meal (Reilly et al., 2021). Therefore, it appears that yeast may promote the beneficial production of SCFA compared to an animal-based ingredient. A future study would be warranted to determine if BDY and CFP have a similar effect when compared to an animal-based ingredient.

It is important to acknowledge that excreted fecal microbes may underestimate apparent nutrient digestibility if hindgut fermentation has been increased by dietary fiber levels and/or

yeast cell wall components. However, similar levels of total fecal SCFA and BCFA across treatments do not support an increase in hindgut fermentation in this study. Nevertheless, accounting for microbial contributions in fecal nutrient excretion could provide more accurate estimates of dietary nutrient digestibility.

Palatability

The palatability evaluation indicated no preference between the CON or BDY and BDY+DDGS treatments when fed to cats (**Table 5.7**), as first choice and intake ratios were comparable ($P > 0.05$) for each test. There was also no preference between CON or CFP based on first choice of cats. However, cats consumed more CON compared to CFP which is indicated by the significant intake ratio.

Yeast products have been used as palatability enhancers in pet food for many years (Swanson and Fahey, 2007). The increased palatability of yeast is attributed to nucleotides and high glutamic acid concentration which provides the umami, or meaty, aroma and taste (Nagodawithana, 1992; Ugawa and Kuihara, 1994). However, palatability results are variable among studies, indicating that the type of yeast and inclusion level may impact results. Swanson and Fahey (2007) reported that a 1% inclusion of brewer's yeast was more palatable than a 1% inclusion of corn wet milling yeast when fed to both dogs and cats. In the current study, cats preferred the CON to CFP based on the intake ratio but had no preference when comparing BDY to CON. However, CFP was not directly compared to BDY which would have provided interesting results. Kilburn-Kappeler et al. (2023) reported that dogs had no preference between CON and BDY but preferred CON over CFP based on first choice and intake ratio. Based on the amino acid profile reported by Kilburn-Kappeler et al. (2023), CFP contains more glutamic acid compared to BDY (8.4 vs 5.3%, respectively). Therefore, it would be expected that CFP would

have an intensified umami aroma and flavor, increasing palatability. However, these results indicate that distiller's yeast may be less palatable for dogs and cats compared to brewer's yeast. It is also important to consider the effects of processing and kibble texture on palatability (Koppel et al., 2015). As the CFP treatment resulted in the hardest and toughest kibble which may have caused decreased palatability. Regardless, cats willingly consumed all treatments and no refusals were observed during the digestibility study when choice was not a factor.

Conclusion

Compared to BDY, CFP required adjustments in processing parameters to achieve a similar bulk density of kibble. However, even with the similar bulk density, CFP resulted in a harder and tougher kibble compared to BDY. Surprisingly, the increased fiber content in CFP compared to BDY did not impact stool quality or SCFA and BCFA concentrations in fecal samples of cats. However, it likely decreased the nutrient digestibility of CFP compared to BDY. In addition, BDY appeared to be more palatable than CFP when compared to the control containing SBM. However, BDY and CFP were not directly compared in the palatability assessment and cats willing consumed all diets in the digestibility trial. To classify or utilize CFP as a yeast source, further research is warranted to evaluate if the yeast component of CFP has any potential benefit on animal health.

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Chapter 5 Tables

Table 5.1 Ingredient composition of feline diets containing yeast and ethanol co-products on an as-is basis

	Treatment[1]			
Ingredient, %	CON	BDY	BDY+DDGS	CFP
Corn	34.6	30.0	30.0	34.6
Chicken Meal	30.0	35.0	35.0	30.0
Soybean Meal	15.0	8.0	-	-
Distillers Dried Grains with Solubles	-	-	17.5	-
Corn Fermented Protein	-	-	-	17.5
Brewer's Dried Yeast	-	3.5	2.5	-
Corn Starch	-	6.5	-	2.5
Corn Gluten Meal	5.0	2.0	-	-
Chicken Fat	6.0	5.6	5.6	6.0
Other[2]	9.4	9.4	9.4	9.4

[1]CON, control; BDY, brewer's dried yeast; BDY+DDGS, brewer's dried yeast and distillers dried grains with solubles; CFP, corn fermented protein.
[2]Other ingredients: beet pulp, fish meal, flavor, titanium dioxide, salt, potassium chloride, vitamin and mineral premix, choline chloride, natural antioxidant.

Table 5.2 Processing parameters and physical characteristics of feline diets containing yeast and ethanol co-products

Parameter	Treatment[1]				SEM	*P*-Value
	CON	BDY	BDY+DDGS	CFP		
Preconditioner						
Cylinder Speed, rpm	185.00	185.00	185.00	185.00	0.000	1.000
Steam Flow, kg/hr	47.47^{a}	46.97^{a}	40.88^{b}	37.39^{c}	0.660	<0.0001
Water Flow, kg/hr	57.35^{a}	56.40^{a}	48.69^{b}	48.50^{b}	1.936	<0.0001
Discharge Temperature, °C	90.44^{b}	91.54^{b}	96.36^{a}	96.40^{a}	0.753	<0.0001
Extruder						
Screw Speed, rpm	450.00^{b}	450.00^{b}	438.16^{b}	475.00^{a}	4.785	<0.0001
Steam Flow, kg/hr	0.00^{c}	9.07^{b}	21.05^{a}	13.76^{a,b}	3.636	<0.0001
Water Flow, kg/hr	0.00^{c}	0.00^{c}	8.18^{a}	4.38^{b}	0.723	<0.0001
Motor Load, amps	67.22^{a}	64.43^{b}	64.06^{b}	64.06^{b}	0.661	<0.0001
TMF[2], kg/h	385.02	385.78	387.43	381.28	2.318	0.0989
SME[3], kJ/kg	123.55^{a}	106.11^{b}	101.50^{b}	111.77^{b}	4.387	<0.0001
MC[4], %	32.30^{a,b}	33.43^{a,b}	34.25^{a}	32.00^{b}	0.786	0.0267
Die Temperature, °C	110.97^{b}	110.76^{b}	109.68^{b}	114.21^{a}	0.807	<0.0001
Knife Speed, rpm	1600.00^{c}	1681.48^{b}	2000.00^{a}	2000.00^{a}	25.670	<0.0001
Dryer						
Bulk Density, g/L	363.07^{a,b}	343.13^{b}	392.23^{a}	339.97^{b}	14.760	0.0171
Kibble Diameter, mm	6.82^{a}	5.31^{b}	4.58^{c}	5.68^{b}	0.253	<0.0001
Kibble Length, mm	4.09^{b}	4.36^{a,b}	3.35^{c}	4.47^{a}	0.110	<0.0001
Specific Length, mm/g	105.76^{c}	135.81^{b}	150.57^{a}	133.31^{b}	4.101	<0.0001
SEI[5], mm^2/mm^2	5.32^{a}	3.17^{b,c}	2.36^{c}	3.64^{b}	0.345	<0.0001
Hardness, kg	2.43^{b}	2.26^{b,c}	2.02^{c}	2.84^{a}	0.136	<0.0001
Toughness, kg mm	8.28^{b}	6.59^{c}	7.23^{b,c}	9.85^{a}	0.564	<0.0001

[1]CON, control; BDY, brewer's dried yeast; BDY+DDGS, brewer's dried yeast and distillers dried grains with solubles; CFP, corn fermented protein.
[2]TMF = total mass flow.
[3]SME = specific mechanical energy.
[4]MC = in-barrel moisture content.
[5]SEI = sectional expansion index.
[a-c]Means within a row lacking a common superscript letter are different (P < 0.05).

Table 5.3 Analyzed chemical composition of feline diets containing yeast and ethanol co-products on a dry matter basis

	Treatment[1]			
Nutrient	CON	BDY	BDY+DDGS	CFP
Dry Matter, %	95.03	95.71	94.38	95.50
Moisture, %	4.97	4.29	5.62	4.50
Organic Matter, %	90.03	90.17	90.31	91.49
Ash, %	9.97	9.83	9.69	8.51
Crude Protein, %	41.59	40.58	38.50	37.22
Crude Fat, %	12.80	13.32	14.45	13.40
Total Dietary Fiber, %	13.66	13.19	18.46	15.05
Insoluble Dietary Fiber, %	10.09	10.04	14.34	12.40
Soluble Dietary Fiber, %	3.67	3.14	4.13	2.64
Gross Energy, kcal/kg	4951.82	4997.76	5065.36	5001.77

[1]CON, control; BDY, brewer's dried yeast; BDY+DDGS, brewer's dried yeast and distillers dried grains with solubles; CFP, corn fermented protein.

Table 5.4 Food intake and stool quality parameters of cats fed diets containing yeast and ethanol co-products[1]

	Treatment[2]					
Parameter	CON	BDY	BDY+DDGS	CFP	SEM	*P*-value
Food Intake, g/d	80.71[a,b]	77.90[b]	80.97[a]	78.90[a,b]	1.055	0.0186
Wet Fecal Output, g/d	51.80[b]	46.49[b]	59.22[a]	49.54[b]	2.316	<0.0001
Fecal Dry Matter, %	31.76[c]	35.29[a]	34.38[a,b]	32.91[b,c]	0.709	0.0002
Dry Fecal Output, g/d	16.36[b]	16.29[b]	20.24[a]	16.26[b]	0.585	<0.0001
Defecations per Day	0.95[b]	0.96[a,b]	1.11[a]	0.89[b]	0.055	0.0030
Fecal Score[3]	3.57[b]	3.73[a,b]	3.85[a]	3.77[a,b]	0.076	0.0095
Fecal pH	5.76	5.70	5.59	5.53	0.089	0.0645

[1]A total of 11 cats were enrolled in an incomplete 4x4 replicated Latin square design, resulting in 11 observations per treatment for each parameter.
[2]CON, control; BDY, brewer's dried yeast; BDY+DDGS, brewer's dried yeast and distillers dried grains with solubles; CFP, corn fermented protein.
[3]Scored on a 1 (liquid diarrhea) – 5 (dry hard pellets) scale with a score of 3.5 – 4.0 considered ideal.
[a-c]Means within a row lacking a common superscript letter are different ($P < 0.05$).

Table 5.5 Apparent total tract digestibility of diets containing yeast and ethanol co-products estimated by titanium dioxide as a dietary marker on a dry matter basis[1]

	Treatment[2]					
Nutrient, %	CON	BDY	BDY+DDGS	CFP	SEM	*P*-value
Dry Matter	82.29[a]	80.94[b]	75.37[d]	78.72[c]	0.513	<0.0001
Organic Matter	87.47[a]	87.08[a]	80.60[c]	83.61[b]	0.423	<0.0001
Crude Protein	88.91[a]	86.99[b]	84.44[c]	84.51[c]	0.483	<0.0001
Crude Fat	96.12[a]	95.64[a,b]	91.83[c]	94.97[b]	0.342	<0.0001
Total Dietary Fiber	61.20[a]	62.07[a]	49.47[b]	51.29[b]	1.208	<0.0001
Gross Energy	87.76[a]	87.19[a]	81.53[c]	83.81[b]	0.430	<0.0001

[1]A total of 11 cats were enrolled in an incomplete 4x4 replicated Latin square design, resulting in 11 observations per treatment for each parameter.
[2]CON, control; BDY, brewer's dried yeast; BDY+DDGS, brewer's dried yeast and distillers dried grains with solubles; CFP, corn fermented protein.
[a-d]Means within a row lacking a common superscript letter are different ($P < 0.05$).

Table 5.6 Chemical analysis of feces from cats fed diets containing yeast and ethanol co-products[1]

	Treatment[2]					
Parameter	CON	BDY	BDY+DDGS	CFP	SEM	*P*-value
Total SCFA[3], μmol/g DM feces	545.00	515.90	538.57	524.64	62.3286	0.9653
Acetate, %	75.33	72.25	71.56	71.72	2.0013	0.2151
Propionate, %	18.40	20.57	21.79	21.31	1.6787	0.2139
Butyrate, %	6.26	7.19	6.64	6.98	0.6029	0.4465
Total BCFA[4], μmol/g DM feces	33.23	35.19	30.09	31.17	4.8187	0.7263
Isovalerate, %	26.74	27.28	26.78	28.12	1.4172	0.7468
Isobutyrate, %	17.52	14.45	16.33	13.96	2.7396	0.5414
Valerate, %	55.74	58.27	56.89	57.92	2.7313	0.7903

[1]A total of 11 cats were enrolled in an incomplete 4x4 replicated Latin square design, resulting in 11 observations per treatment for each parameter.
[2]CON, control; BDY, brewer's dried yeast; BDY+DDGS, brewer's dried yeast and distillers dried grains with solubles; CFP, corn fermented protein.
[3]Total SCFA (acetate + propionate + butyrate); individual SCFA is expressed as a percent of total SCFA.
[4]Total BCFA (isovalerate + isobutyrate + valerate); individual BCFA is expressed as a percent of total BCFA.

Table 5.7 First choice (FC) and intake ratio (IR) of cats fed diets containing yeast and ethanol co-products[1]

Diet Comparison (A vs B)[2]	FC[3]	IR[4]
BDY vs CON	21	0.591
BDY+DDGS vs CON	23	0.350
CFP vs CON	24	0.256*

[1]A total of 20 cats were fed each diet comparison over 2 days, resulting in 40 observations per comparison.
[2]CON, control; BDY, brewer's dried yeast; BDY+DDGS, brewer's dried yeast and distillers dried grains with solubles; CFP, corn fermented protein.
[3]Number of first visits to bowl A out of 40 observations.
[4]IR = intake (g) of diet A/total intake (g) of diets A+B.
*Comparison differs ($P < 0.05$).

Chapter 6 - Evaluation of corn fermented protein (CFP) on the fecal microbiome of dogs

Abstract

Corn fermented protein (CFP), a co-product from the ethanol industry, is produced using post-fermentation technology to isolate the protein and yeast from fiber prior to drying. The study objective was to determine the effect of CFP compared to traditional ingredients on the fecal microbiome in dogs. The four experimental diets included a control with no yeast and diets containing either 3.5% brewer's dried yeast, 2.5% brewer's dried yeast plus 17.5% distillers dried grains with solubles, or 17.5% CFP. Experimental diets were fed to adult dogs (n = 12) in a 4 x 4 replicated Latin square design. Fresh fecal samples (n = 48) were analyzed by 16S Metagenomic Sequencing. Raw sequences were processed through Mothur (v.1.44.1). Community diversity was evaluated in R (v4.0.3, R Core Team, 2019). Relative abundance data was analyzed within the 50 most abundant operational taxonomic units using a mixed model of SAS (v9.4, SAS Institute, Inc., Cary, NC). Alpha and beta-diversity were similar for all treatments. There were no quantifiable shifts in predominant phyla among treatments ($P > 0.05$). However, nine genera resulted in differences in relative abundance among treatments. This data indicates that compared to traditional ingredients, CFP did not alter the overall diversity of the fecal microbiome of healthy adult dogs over a 14-d period.

Introduction

Distillers dried grains with (DDGS) or without solubles (DDG) can be utilized as alternative protein sources for pet food containing 25 to 43% crude protein (Spiehs et al., 2002; Salim et al., 2010). They also provide essential amino acids such as lysine (0.9 to 1.2%),

methionine (0.6 to 0.8%), and tryptophan (0.2%) (de Godoy et al., 2009; Rho et al., 2017). Furthermore, DDGS and DDG contain residual yeast protein in addition to corn protein, which contributes to the amino acid concentrations and profile (Belyea et al., 2004).

In addition, DDGS and DDG have a fiber component ranging from 30 to 55% total dietary fiber (Silva et al., 2016; Iram et al., 2020). This fiber component, in addition to the yeast cell wall, may serve as substrate for microbial fermentation in the gut, acting as a prebiotic (Silva et al., 2016; Iram et al., 2020). Therefore, the inclusion of DDGS and DDG into pet food could support intestinal health by modulation of the gut microbiome and production of short chain fatty acids (Tramontano et al., 2018).

A few studies have evaluated the effects of DDGS on diet digestibility and stool quality in dogs (Silva et al., 2016; Risolia et al., 2019; Kilburn-Kappeler et al., 2023). However, there are no studies which have evaluated the effects of traditional DDGS on the microbiome of dogs. Furthermore, there is only one study which has evaluated high-protein DDG (45% crude protein) and its impact on the canine microbiome (Kaelle et al., 2023).

Previous studies have evaluated the effects of corn fermented protein (CFP), an enhanced DDG, on diet digestibility and stool quality in dogs (Kilburn-Kappeler et al., 2023; Smith and Aldrich, 2023; Kilburn and Aldrich, 2023). This is the first study to evaluate CFP on the fecal microbiome of dogs. Corn fermented protein is produced using post-fermentation technology in which the protein and yeast are separated from the fiber prior to drying. This results in a higher protein, lower fiber ingredient compared to traditional DDGS at 53% crude protein and 35% total dietary fiber (Kilburn-Kappeler et al., 2023). In addition, CFP has a substantial yeast component at approximately 20 to 25% (POET Bioproducts, Sioux Falls, SD). Therefore, it was

hypothesized that the heighted yeast component in CFP compared to traditional DDGS would promote intestinal health even with the decreased fiber content.

Materials and Methods

Diet Formulation, Production, and Nutrient Composition

Dietary treatments consisted of a control diet containing 15% soybean meal (T1) and experimental diets containing either 3.5% brewer's dried yeast (T2), 2.5% brewer's dried yeast plus 17.5% distillers dried grains with solubles (T3), or 17.5% corn fermented protein (T4). It was assumed that corn fermented protein (CFP) had 20% yeast and distillers dried grains with solubles (DDGS) had 5.7% yeast; therefore, all treatments, except T1, were formulated to contain 3.5% yeast. It is important to note that the concentration of yeast in CFP, DDGS, and dietary treatments were an estimate and not analyzed. In addition, suppliers continue to develop methods to better quantify the yeast, therefore ingredients may vary in yeast content. The formulated diets met the AAFCO nutritional requirements for adult dogs. The amount of corn, chicken meal, and chicken fat were adjusted between base rations to maintain nutrient composition among dietary treatments and result in a complete formula (100%). The first base ration was used for T1 and T4, and included all dry ingredients, except for the soybean meal (Fairview Mills, Seneca, KS), CFP (POET Bioproducts, Sioux Falls, SD), corn starch (Fairview Mills, Seneca, KS), corn gluten meal (Fairview Mills, Seneca, KS), and titanium dioxide (Fairview Mills, Seneca, KS). The second base ration was used for T2 and T3, and contained all dry ingredients except for soybean meal, DDGS (Fairview Mills, Seneca, KS), corn starch, corn gluten meal, brewer's dried yeast (BDY; Fairview Mills, Seneca, KS), and titanium dioxide. Soybean meal, corn gluten meal and/or corn starch were added to T1, T2, and T4 to create

similar nutrient profiles among all dietary treatments and to balance a 20% inclusion of experimental ingredients compared to T3 (**Table 6.1**).

Each diet was mixed and produced using a single screw extruder (model E525, Extru-Tech, Manhattan, KS). The cool and dry product was packaged in laminated bags and transferred to the laboratory at Kansas State University to be coated. Kibble was coated with chicken fat protected with natural antioxidants (Nutrios, Springfield, MO) and a dry powdered flavor designed for dogs (AFB International, St. Charles, MO). Coated diets were stored in poly-lined Kraft paper bags until fed.

Diets were analyzed in duplicate for moisture (AOAC 930.15), ash (AOAC 942.05), fat by acid hydrolysis and hexane extraction (AOAC 960.39), gross energy (Parr 6200 Calorimeter, Parr Instrument Company, Moline, IL), and total dietary fiber (AOAC 991.43). Crude protein was determined by Dumas combustion (AOAC 990.03) using a nitrogen analyzer (FP928, LECO Corporation, Saint Joseph, MI). Nutrient composition of dietary treatments is presented in **Table 6.2**.

Feeding Trial

The feeding trial was conducted at the Kansas State University Large Animal Research Center (LARC) under the Institutional Animal Care and Use Committee (IACUC) #4097 protocol.

Twelve healthy adult (6.3 ± 0.45 years) beagle dogs (8 castrated males and 4 spayed females) with an average body weight of 11.4 ± 1.2 kg were individually housed in pens (1.83 m x 1.20 m) equipped with an acrylic-coated mesh floor to allow for separation of urine and feces. Dogs were kept in a temperature-controlled (23°C) modular building with a 12 h light cycle. Dogs received two feedings per day at 0800 and 1700 h with water ad libitum. Food quantities

were determined by calculating the daily metabolizable energy requirement (NRC, 2006) of each dog to maintain body weight.

Sample Collection

The study was conducted as a replicated 4x4 Latin square in which dogs were randomly assigned to diets. Each period consisted of 9 days for adaptation followed by 5 days of total fecal collection, which has been reported to be more than sufficient for stabilizing the gut to dietary intervention (Lin et al., 2023). During each collection period, a fresh fecal sample (within 15 minutes of defecation) from each dog was collected using a sterile Whirl-pak bag and 2 g aliquots were transferred with a spatula into plastic microcentrifuge tubes and stored at -80°C for DNA extraction.

Fecal DNA Extraction and Sequencing

The DNA was extracted from 200 mg of each stool sample (n = 48) using a QIAamp Power Fecal Pro DNA Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions (Handbook 02/2020). A bioanalyzer (NanoDrop 2000, Thermo Scientific, Waltham, MA) was used for quality control. DNA concentration was assessed on a Qubit fluorometer (Qubit 4.0, Invitrogen by Life Technologies, Carlsbad, CA). The 16S V3/V4 gene was amplified using the Illumina 16S Metagenomic Sequencing library prep protocol (Illumina, Inc., San Diego, CA) as specified by the manufacturer. Libraries were run on an Illumina Miseq system using 300x2 v3 paired end chemistry.

Data Analysis

Raw reads were trimmed for quality using CLC Genomics Workbench (Qiagen, v11.0.1) and then imported into mothur (v1.44.1) for further analysis (Schloss et al., 2009). The unique 16S reads were aligned to reference sequences from the SILVA rRNA database (Release 138)

for closed-reference observational taxonomic unit (OTU) assignment (Yilmaz et al., 2014). Near-identical sequences were merged using VSEARCH v2.15.1 (Rognes et al., 2016).

Beta-diversity was determined by a principal coordinate analysis (PCoA) in R (v4.0.3, R Core Team, 2019). Alpha-diversity was evaluated using observed unique sequences, Chao1, Shannon, and Simpson indices. Relative abundance data were analyzed within the 50 most abundant OTU using a mixed model of SAS (v9.4, SAS Institute, Inc., Cary, NC). Treatment was considered a fixed effect and dog and period as random effects. Results were considered significant at $P < 0.05$.

Results

Beta and Alpha-Diversity

The PCoA did not provide evidence of clustering among treatment groups, indicating that the beta-diversity was not different among dietary treatments (**Figure 6.1**). In addition, alpha-diversity measures were similar ($P > 0.05$) for all dietary treatments (**Figure 6.2**).

Phyla Relative Abundance

Predominant phyla within the 50 most abundant observational taxonomic units (OTU) were Firmicutes (73%), Bacteroidetes (15%), Fusobacteria (8%), and Actinobacteria (4%, **Table 6.3**). The relative abundance of Firmicutes was numerically lower for T3 at 69% compared to the remaining treatments at an average of 74%. The relative abundance of Bacteroidetes was 13% for T1, 14% for T2, and 16% for both T3 and T4. The relative abundance of Fusobacteria was numerically greater for T3 at 11% compared to the remaining treatments at an average of 8%. The relative abundance of Actinobacteria was numerically lower for T4 at 3% compared to the remaining treatments at an average of 5%. However, the shifts in predominant phyla among dietary treatments were not significant ($P > 0.05$).

Genera Relative Abundance

Among the 50 most abundant OTU, the relative abundance of 9 genera resulted in differences among dietary treatments (**Table 6.4**). The relative abundance of *Blautia* was greater ($P < 0.05$) for T1 at 12.4% compared to T3 and T4 at an average of 9.1% with T2 intermediate at 10.4%. The opposite was observed in *Candidatus Stoquefichus* with a greater ($P < 0.05$) relative abundance for T3 and T4 at an average of 1.7% compared to T1 at 0.4%, T2 was intermediate at 1.0%. A decreased ($P < 0.05$) relative abundance in *Collinsella* was observed for T4 at 2.4% compared to T1 and T2 at an average of 3.7%, T3 was intermediate at 3.2%. The relative abundance of *Erysipelatoclostridium* was greatest ($P < 0.05$) for T4 at 5.4% and lowest ($P < 0.05$) for T1 at 1.2%, T3 and T4 were intermediate at 3.8 and 4.7%, respectively. *Peptoclostridium* relative abundance was greater ($P < 0.05$) for T2 at 16.6% compared to T1 at 12.5% with T3 and T4 intermediate at an average of 15.2%. The relative abundance of *Phascolarctobacterium* was greater ($P < 0.05$) for T4 at 0.8% compared to T1 at 0.4% with T2 and T3 intermediate at 0.6%. *Romboutsia* relative abundance was greater ($P < 0.05$) for T3 at 8.3% compared to T1 at 4.6% with T2 and T4 intermediate at an average of 6.6%. The relative abundance of *Streptococcus* was greater ($P < 0.05$) for T1 at 9.7% compared to T3 and T4 at an average of 0.4%, T2 was intermediate at 5.5%. *Terrisporobacter* relative abundance was lower ($P < 0.05$) for T2 at 0.4% compared to T3 at 1.4%, with T1 and T4 intermediate at an average of 0.9%.

Discussion

Beta and Alpha-Diversity

One of the leading indicators of a healthy gut microbiota is an increased richness and diversity of microorganisms (Ziese and Suchodolski, 2021). Dogs with gastrointestinal disorders

have been reported to have lower diversity when compared to healthy dogs (Suchodolski et al., 2012; Isaiah et al., 2017; Felix et al., 2022; Diaz-Reganon et al., 2023). Therefore, the preservation of beta and alpha-diversity for dogs fed T4 indicates that CFP supported a healthy gut microbiome compared to traditional ingredients.

A previous study evaluated four diets containing increasing concentrations (0, 7, 14, and 21%) of high protein dried distillers grains (HPDDG), in exchange for soybean meal (SBM), on the fecal microbiome of dogs (Kaelle et al., 2023). In contrast to the current study where no differences in alpha-diversity were observed, the previous study reported a linear increase in the number of OTU, a quadratic effect for the Shannon index, and a trend for a linear increase in the Chao1 index with increased inclusion of HPDDG (Kaelle et al., 2023). This discrepancy is at least in part due to the variation in fiber content between the historical and current study (Kaelle et al., 2023). In addition, the variation in experimental ingredients among studies could affect the results.

Phyla Relative Abundance

The most abundant phyla in the current study are supported by previous studies which have identified that most bacterial sequences in the canine gastrointestinal tract belong to the phyla Firmicutes, Bacteroidetes, Fusobacteria, Actinobacteria, and Proteobacteria (Honneffer et al., 2017; Pilla and Suchodolski, 2020; Diaz-Reganon et al., 2023). For example, Firmicutes was reported to be the most abundant phylum in fecal samples of healthy dogs at 69% (Diaz-Reganon et al., 2023).

In addition to the reduced richness and diversity, the microbiome of dogs in disease states has been characterized by marked shifts in the relative abundance of phyla; specifically a decrease in Bacteroidetes and Firmicutes and an increase in Actinobacteria (Suchodolski et al.,

2012; Minamoto et al., 2015; Vazquez-Baeza et al., 2016; Minamoto et al., 2019). Therefore, dietary treatments did not negatively shift the relative abundance of phyla.

Genera Relative Abundance

The presence and relative abundance of bacterial genera can provide a more in-depth understanding of the microbial community. Similar to the shifts in the relative abundance of phyla, the presence or increased/decreased abundance of certain genera have been associated with disease. In addition, specific functions of some genera have been reported. Therefore, the type of dietary substrate available for microbial fermentation can be determined providing insight to the end products of microbial fermentation, such as short chain fatty acids (SCFA).

The *Blautia* genus has been reported to ferment many types of carbohydrates (Liu et al., 2008) and improve gut functionality (Suchodolski et al., 2012; Alshawaqfeh et al., 2017; Felix et al., 2022). In contrast to the current study in which a decrease in *Blautia* in DDGS and CFP diets was observed, *Blautia* increased with HPDDG inclusion in dog diets (Kaelle et al., 2023). Similar to alpha-diversity, the differing results among studies could be due to the larger variation in fiber content among dietary treatments in the previous study.

The genus *Candidatus Stoquefichus* has been previously associated with healthy dogs and is classified as commensal bacteria (Handl et al., 2011; Garcia-Mazcorro et al., 2012; Minamoto et al., 2015; Vazquez-Baeza et al., 2016). In addition, reduction of *Candidatus Stoquefichus* in dogs with inflammatory bowel disease (IBD) compared to healthy dogs has been reported (Diaz-Reganon et al., 2023). Therefore, the increased relative abundance of *Candidatus Stoquefichus* for T3 and T4 compared to T1 could indicate a beneficial shift.

An increase in the *Collinsella* genus has been observed in dogs with gastric dilation-volvulus (GDV), an acute life-threatening condition (Hullar et al., 2018). *Collinsella* has also

been associated with autoimmune diseases in humans and humanized mice (Chen et al., 2016). In addition, the abundance of *Collinsella* has been correlated to the production of proinflammatory cytokine IL-17A, as well as the alteration of gut permeability and disease severity (Chen et al., 2016). Therefore, the decrease in *Collinsella* for T4 compared to T1 and T2 may result in a healthier microbiome.

The genus *Erysipelatoclostridium* has been identified as a possible biomarker for major intestinal diseases such as Crohn's disease and *Clostridium difficile* infection in humans (Mancabelli et al., 2017). In addition, an increase in *Erysipelatoclostridium* was observed for dogs infected with canine parvovirus compared to healthy dogs (Wang et al., 2019). However, a significant reduction of *Erysipelatoclostridium* in dogs with acute diarrhea has also been reported (Soonthornsit et al., 2021). *Erysipelatoclostridium* is known to produce acetate and lactate by metabolizing proteins (Oliphant et al., 2019). Therefore, it could be expected that the treatments with the greatest protein content (T1 and T2) would result in greater relative abundance of *Erysipelatoclostridium.* In the current study, the opposite response was observed. In addition, the concentration of acetate in fecal samples of dogs fed dietary treatments did not differ (Kilburn-Kappeler et al., 2023).

The increase in *Peptoclostridium* has been related to obesity, metabolic syndrome, acute diarrhea, and IBD (Leung et al., 2013; Woting et al., 2014; Guard et al., 2015; Thomson et al., 2022). Therefore, the combination of distillers grains and yeast (T3 and T4) resulted in a more desirable abundance of *Peptoclostridium* than yeast alone (T2).

A previous study observed a decrease in the relative abundance of *Phascolarctobacterium* in dogs with IBD (Diaz-Reganon et al., 2023). Therefore, the increase in *Phascolarctobacterium* for T4 compared to T1 could indicate that CFP promoted intestinal

health. *Phascolarctobacterium* has been shown to be related to the synthesis of the SCFA propionate (Mackei et al., 2022). However, the propionate concentration in fecal samples of dogs fed dietary treatments was similar for T1 and T4 (Kilburn-Kappeler et al., 2023). A previous study reported a reduction in *Phascolarctobacterium* after dogs were fed a high protein diet compared to a medium or low protein diet (Ephraim et al., 2020). Therefore, the increased protein content in T1 compared to T4 could have impacted the relative abundance of *Peptoclostridium* in the current study.

Romboutsia has a broad range of metabolic capabilities including the utilization of carbohydrates and the fermentation of amino acids (Gerritsen et al., 2014). A previous study reported a decrease in *Romboutsia* when dogs were shifted to raw diets from kibble diets, indicating that *Romboutsia* might play an important role in carbohydrate utilization in the hindgut of dogs (Xu et al., 2021). Therefore, the increased fiber content in T3 may have resulted in the significant increase of *Romboutsia* for T3 compared to T1.

The *Streptococcus* genus has been classified as a heterofermentative bacteria that can produce lactic acid and has been previously associated in dogs with IBD (Suchodolski et al., 2012; Markel et al., 2012; Minamoto et al., 2015; Vazquez-Baeza et al., 2016; Diaz-Reganon et al., 2023). Furthermore, *Streptococcus* overgrowth has been considered a hallmark of canine dysbiosis (Vazquez-Baeza et al., 2016; Alshawaqfeh et al., 2017; White et al., 2017). Therefore, the decreased relative abundance of *Streptococcus* for T3 and T4 compared to T1 could be beneficial. A decrease was also reported in the genus *Streptococcus* when HPDDG was fed to dogs (Kaelle et al., 2023).

Previous studies have suggested that *Terrisporobacter* is a pathogenic bacterium (Ho et al., 2019; Lee et al., 2020; Li et al., 2021; Guo et al., 2023). For example, it was concluded that

Terrisporobacter might regulate enzymes in bile acid metabolism or lipid biosynthesis, eventually leading to higher serum lipid levels and dyslipidemia (Guo et al., 2023). *Terrisporobacter* has also been positively correlated with oxidative stress in humans and mice (Cai et al., 2019; Li et al., 2021). Therefore, a low relative abundance of *Terrisporobacter* would be beneficial. In addition, a negative correlation of *Terrisporobacter* with propionate and total SCFA production has been reported (Li et al., 2021). The highest relative abundance of *Terrisporobacter* was observed in T3 which correlated with a reduction in propionate. However, total SCFA concentrations were not impacted (Kilburn-Kappeler et al., 2023).

Taken together, the differences in the relative abundance of genera for dogs fed experimental treatments in this study indicate a healthier microbiome shift compared to dogs fed the control.

Conclusion

In conclusion, CFP and traditional ingredients containing yeast did not alter the overall diversity of the fecal microbiome of healthy adult dogs over a 14-d period. However, experimental ingredients did shift the canine microbiome on a genus level, likely due to the type of substrate in the gastrointestinal tract. The unique combination of fiber and yeast in CFP may promote intestinal health of dogs when compared to SBM.

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Chapter 6 Tables and Figures

Table 6.1 Ingredient composition of canine diets containing yeast and ethanol co-products on an as-is basis

	Treatment[1]			
Ingredient, %	T1	T2	T3	T4
Corn	34.6	30.0	30.0	34.6
Chicken Meal	30.0	35.0	35.0	30.0
Soybean Meal	15.0	8.0	-	-
Distillers Dried Grains with Solubles	-	-	17.5	-
Corn Fermented Protein	-	-	-	17.5
Brewer's Dried Yeast	-	3.5	2.5	-
Corn Starch	-	6.5	-	2.5
Corn Gluten Meal	5.0	2.0	-	-
Chicken Fat	6.0	5.6	5.6	6.0
Other[2]	9.4	9.4	9.4	9.4

[1]T1, control; T2, brewer's dried yeast; T3, brewer's dried yeast and distillers dried grains with solubles; T4, corn fermented protein.
[2]Other ingredients: beet pulp, fish meal, flavor, titanium dioxide, salt, potassium chloride, vitamin and mineral premix, choline chloride, natural antioxidant.

Table 6.2 Analyzed chemical composition of canine diets containing yeast and ethanol co-products on a dry matter basis

	Treatment[1]			
Nutrient	T1	T2	T3	T4
Dry Matter, %	95.61	95.92	94.78	95.38
Organic Matter, %	90.54	90.44	90.62	91.78
Ash, %	9.46	9.56	9.38	8.22
Crude Protein, %	41.13	40.82	38.18	37.55
Fat, %	13.15	13.07	14.82	13.70
Total Dietary Fiber, %	13.58	13.16	18.39	15.07
Insoluble Dietary Fiber, %	10.03	10.02	14.28	12.41
Soluble Dietary Fiber, %	3.65	3.14	4.10	2.64
Gross Energy, kcal/kg	5008.71	4988.17	5073.11	5054.00

[1]T1, control; T2, brewer's dried yeast; T3, brewer's dried yeast and distillers dried grains with solubles; T4, corn fermented protein.

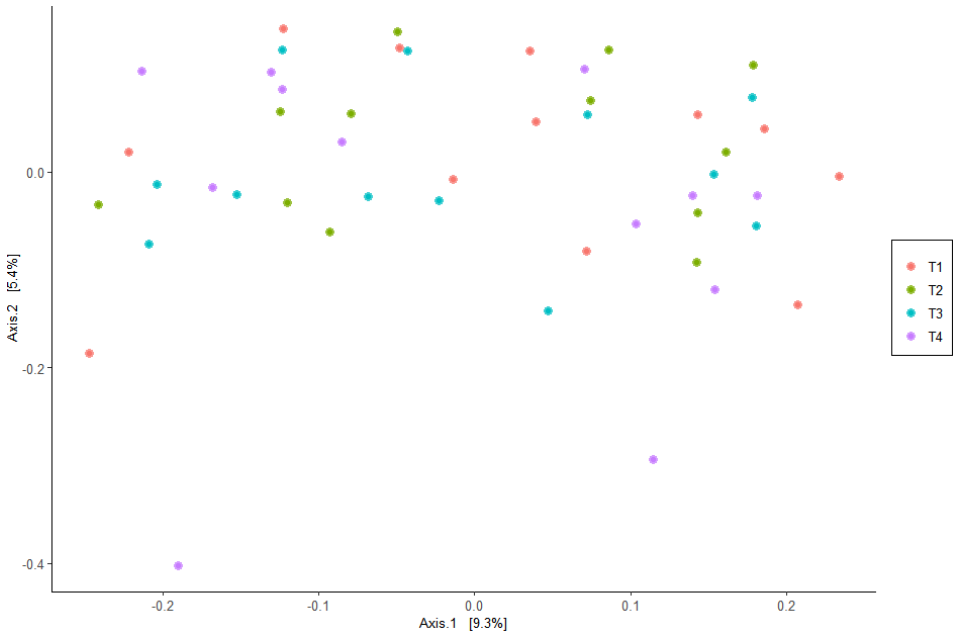

Figure 6.1. Principal coordinate analysis (PCoA) explaining 9.3% and 5.4% of the variability in operational taxonomic units (OTU) of Bray-Curtis UniFrac distances for fecal samples from dogs fed dietary treatments. Treatments: T1 = control; T2 = brewer's dried yeast; T3 = brewer's dried yeast and distillers dried grains with solubles; T4 = corn fermented protein.

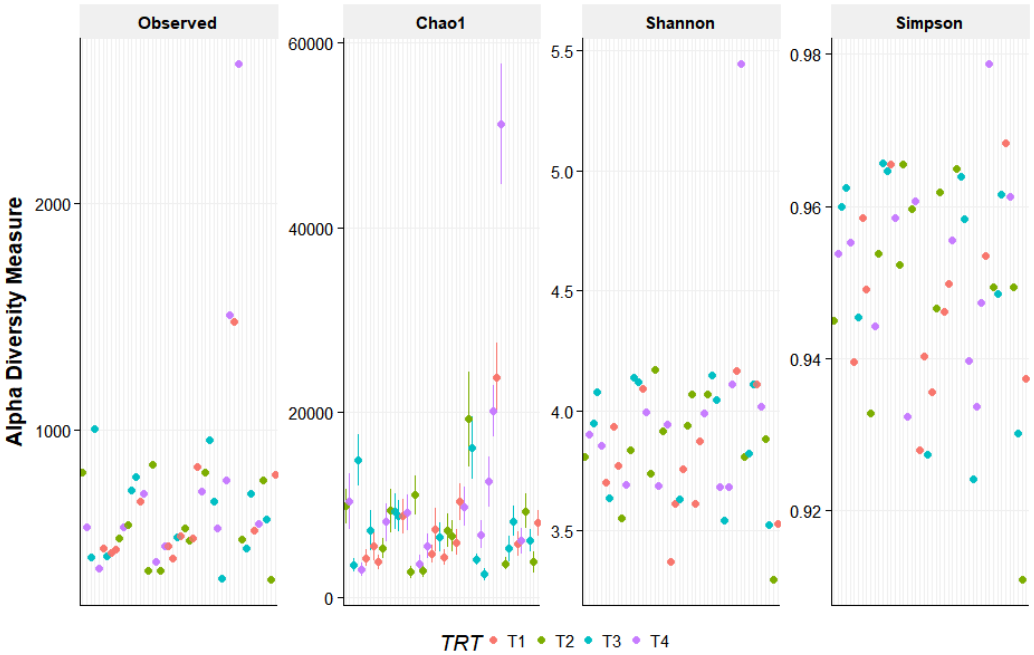

Figure 6.2. Alpha diversity measures for fecal samples from dogs fed dietary treatments. Treatments: T1 = control; T2 = brewer's dried yeast; T3 = brewer's dried yeast and distillers dried grains with solubles; T4 = corn fermented protein.

Table 6.3 Relative abundance of bacterial phyla among the 50 most abundant operational taxonomic units (OTU) in fecal samples from dogs fed dietary treatments

	Treatment[1]					
Phylum, %	T1	T2	T3	T4	SEM	*P*-value
Firmicutes	74.59	74.12	69.01	72.40	3.278	0.3310
Bacteroidetes	13.12	14.05	15.78	15.98	2.615	0.6459
Fusobacteria	7.34	7.67	10.60	8.34	1.379	0.1003
Actinobacteria	4.96	4.17	4.61	3.28	0.861	0.2563

[1]T1, control; T2, brewer's dried yeast; T3, brewer's dried yeast and distillers dried grains with solubles; T4, corn fermented protein.

Table 6.4 Relative abundance of bacterial genera among the 50 most abundant operational taxonomic units (OTU) in fecal samples from dogs fed dietary treatments

	Treatment[1]					
Genus, %	T1	T2	T3	T4	SEM	*P*-value
Allobaculum	3.35	2.54	1.63	2.76	0.791	0.2032
Alloprevotella	1.18	1.73	1.35	1.33	0.496	0.7216
Anaerovoracaceae ge	0.57	0.36	0.55	0.65	0.176	0.4185
Bacteroides	9.30	9.78	11.65	11.06	1.974	0.6110
Bifidobacterium	1.15	0.54	1.38	0.87	0.729	0.6881
Blautia	12.42^{a}	10.44^{a,b}	8.88^{b}	9.35^{b}	0.984	0.0056
Candidatus Stoquefichus	0.42^{b}	1.04^{a,b}	1.75^{a}	1.58^{a}	0.355	0.0032
Catenibacterium	0.66	0.36	0.50	0.48	0.200	0.5220
Clostridium sensu stricto 1	0.87	1.04	1.37	1.25	0.324	0.4392
Collinsella	3.81^{a}	3.62^{a}	3.23^{a,b}	2.41^{b}	0.407	0.0086
Dubosiella	1.38	0.37	1.89	1.62	1.443	0.7407
Erysipelatoclostridium	1.15^{c}	3.80^{b}	4.72^{a,b}	5.40^{a}	0.533	<0.0001
Erysipelotrichaceae UCG-003	1.95	1.58	1.15	2.08	0.666	0.5151
Faecalibacterium	2.56	2.55	2.50	3.14	0.524	0.5708
Faecalibaculum	0.56	0.61	1.83	2.40	0.824	0.0807
Fusobacterium	7.34	7.67	10.60	8.34	1.379	0.1003
Holdemanella	3.85	3.39	3.65	2.72	0.648	0.3384
Lachnospiraceae ge	0.95	1.35	1.12	0.94	0.221	0.2223
Lachnospiraceae unclassified	8.17	7.92	7.26	8.14	0.677	0.5131
Lactobacillus	0.86	0.94	0.02	2.09	1.355	0.5092
Peptoclostridium	12.53^{b}	16.62^{a}	15.32^{a,b}	15.09^{a,b}	1.388	0.0425
Peptococcus	0.57	0.57	0.35	0.30	0.157	0.2022
Peptostreptococcus	1.23	0.00	0.20	0.00	0.768	0.3394
Phascolarctobacterium	0.36^{b}	0.59^{a,b}	0.59^{a,b}	0.76^{a}	0.141	0.0662

Prevotella 9	2.05	2.03	2.15	3.17	0.920	0.5496
Prevotellaceae Ga6A1 group	0.59	0.51	0.63	0.42	0.284	0.8883
Romboutsia	4.60[b]	6.16[a,b]	8.28[a]	7.12[a,b]	1.096	0.0160
Streptococcus	9.69[a]	5.47[a,b]	0.13[b]	0.70[b]	2.200	0.0002
Terrisporobacter	0.44[a,b]	0.39[b]	1.35[a]	1.32[a,b]	0.345	0.0077
Turicibacter	5.44	6.02	3.98	2.51	1.320	0.0499

[1]T1, control; T2, brewer's dried yeast; T3, brewer's dried yeast and distillers dried grains with solubles; T4, corn fermented protein.
[a-c]Means within a row lacking a common superscript letter are different ($P < 0.05$).

Chapter 7 - Evaluation of corn fermented protein (CFP) on the fecal microbiome of cats

Abstract

Co-products from the ethanol industry, such as distillers dried grains with solubles (DDGS), can provide alternative protein sources for pet food. Corn fermented protein (CFP) is produced using post-fermentation technology to split the protein and yeast from fiber prior to drying. This results in a higher protein ingredient compared to DDGS, increasing its appeal for pet food. In addition, the substantial yeast component, at approximately 20-25%, may promote gut health. Therefore, the objective of this study was to determine the effects of the yeast in CFP on the fecal microbiome of cats. The four experimental diets included a control with no yeast (T1) and diets containing either 3.5% brewer's dried yeast (T2), 2.5% brewer's dried yeast plus 17.5% DDGS (T3), or 17.5% CFP (T4). All diets except T1 were formulated to contain 3.5% yeast. Diets were fed to adult cats (n = 11) in an incomplete 4 x 4 replicated Latin square design. Cats were adapted to diet for 9 days followed by a 5-d total fecal collection. During each collection period, fresh fecal samples from each cat were collected and stored at -80°C until analysis. Fresh fecal samples (n = 44) were analyzed by 16S Metagenomic Sequencing. Raw sequences were processed through mothur (v.1.44.1). Community diversity was evaluated in R (v4.0.3, R Core Team, 2019). Relative abundance was analyzed within the 50 most abundant operational taxonomic units (OTU) using a mixed model of SAS (v9.4, SAS Institute, Inc., Cary, NC). Diet was the fixed effect and cat and period were random effects. Results were considered significant at $P < 0.05$. Alpha-diversity indices (Observed, Chao1, Shannon, Simpson) and beta-diversity metric (principal coordinate analysis) were similar for all treatments. Predominant phyla were Firmicutes (66%), Bacteroidetes (25%), Actinobacteria (8%), Proteobacteria (0.64%), and

Desulfobacteria (0.54%). The relative abundance of Firmicutes and Actinobacteria was lower ($P < 0.05$) for T3 compared to T4 and T2, respectively. On a more specific phylogenic level, 17 genera resulted in differences ($P < 0.05$) among dietary treatments. Overall, this data indicates that CFP did not alter the overall diversity of the fecal microbiome of healthy adult cats over a 14-d period.

Introduction

Even though cats are strict carnivores with a short colon and nonfunctional cecum, they have a considerable fermentative capacity (Rochus et al., 2014). Therefore, the addition of prebiotics to feline diets can benefit intestinal and host health (Barry et al., 2010; De Godoy et al., 2013; Garcia-Mazcorro and Minamoto, 2013; Dos Santos et al., 2016). Yeast derivatives, such as beta-glucans and mannan oligosaccharides (MOS), are used as prebiotics in the pet food industry. These derivatives have been reported to lower intestinal pH, promote microbial diversity, and reduce protein fermentation products (Barry et al., 2010; Kanakupt et al., 2011).

The composition of the microbiome is highly influenced by diet. Therefore, a change in dietary substrate can shift the microbial population to promote host health. For example, one of the main benefits of intestinal microbiota modulation is increased production of short chain fatty acids (SCFA), which can be achieved by increasing dietary carbohydrate content (Mills et al., 1999; Wong et al., 2006; Barko et al., 2018). However, dietary substrate can also result in undesirable compounds such ammonia and phenols from microbial fermentation of protein (De Preter et al., 2010; Jackson et al., 2020; Ephraim et al., 2020). Therefore, a nutrient balance is required to not only meet animal requirements and consumer demands but to also support intestinal health.

Corn fermented protein (CFP) could help to achieve this nutrient balance in pet food. Corn fermented protein, a co-product of ethanol production, is produced using post-fermentation technology to split the protein and yeast from fiber prior to drying. This results in a higher protein ingredient compared to traditional distillers dried grains, increasing its appeal for pet food among consumers. In addition, the substantial yeast component, at approximately 20-25%, may promote gut health. Previous studies have evaluated the effect of high protein distillers dried grains on the fecal microbiome of dogs, indicating potential benefits for intestinal functionality (Kaelle et al., 2023; Kilburn-Kappeler et al., 2023b). Therefore, the objective of this study was to investigate the effects of CFP compared to traditional ingredients on the fecal microbiome of cats.

Materials and Methods

The feeding trial was conducted at Kansas State University Veterinary Medicine Complex (Coles Hall) under the Institutional Animal Care and Use Committee (IACUC) #4348 protocol.

Diet Formulation, Production, and Nutrient Composition

Dietary treatments consisted of a control diet containing 15% soybean meal (T1) and experimental diets containing either 3.5% brewer's dried yeast (T2), 2.5% brewer's dried yeast plus 17.5% distillers dried grains with solubles (T3), or 17.5% CFP (T4). It was assumed that CFP had 20% yeast and distillers dried grains with solubles (DDGS) had 5.7% yeast; therefore, all treatments, except T1, were formulated to contain 3.5% yeast. The formulated diets met the AAFCO nutritional requirements for adult cats. The amount of corn, chicken meal, and chicken fat were adjusted between base rations to maintain nutrient composition among dietary treatments and result in a complete formula (100%). The first base ration was used for T1 and

T4, and included all dry ingredients, except for the soybean meal (SBM, Fairview Mills, Seneca, KS), CFP (POET Bioproducts, Sioux Falls, SD), corn starch (Fairview Mills, Seneca, KS), corn gluten meal (Fairview Mills, Seneca, KS), and titanium dioxide (Fairview Mills, Seneca, KS). The second base ration was used for T2 and T3, and contained all dry ingredients except for SBM, DDGS (Fairview Mills, Seneca, KS), corn starch, corn gluten meal, brewer's dried yeast (BDY; Fairview Mills, Seneca, KS), and titanium dioxide. Soybean meal, corn gluten meal and/or corn starch were added to T1, T2, and T4 to create similar nutrient profiles among all dietary treatments and to balance a 20% inclusion of experimental ingredients compared to T3 (**Table 7.1**).

Each diet was mixed and produced using a single screw extruder (model E525, Extru-Tech, Manhattan, KS). The cool and dry product was packaged in laminated bags and transferred to the laboratory at Kansas State University to be coated. Kibble was coated with chicken fat protected with natural antioxidants (Nutrios, Springfield, MO) and a dry powdered flavor designed for cats (AFB International, St. Charles, MO). Coated diets were stored in polylined Kraft paper bags until fed.

Diets were analyzed in duplicate for moisture (AOAC 930.15), ash (AOAC 942.05), fat by acid hydrolysis and hexane extraction (AOAC 960.39), gross energy (Parr 6200 Calorimeter, Parr Instrument Company, Moline, IL), and total dietary fiber (AOAC 991.43). Crude protein was determined by Dumas combustion (AOAC 990.03) using a nitrogen analyzer (FP928, LECO Corporation, Saint Joseph, MI). Nutrient composition of dietary treatments is presented in **Table 7.2**.

Feeding Trial

Eleven healthy adult (3.1 ± 1.7 years) American shorthair cats (10 males and 1 female) were enrolled in an incomplete 4x4 triplicated Latin square design. Each of the four periods were composed of 9 days for diet adaptation followed by 5 days of fecal collection. Cats had an average body weight of 5.6 ± 1.7 kg, and food allowance was controlled to maintain their weight throughout the study. The daily metabolizable energy requirement was calculated for lean cats with $100*BW_{kg}^{0.67}$ (NRC, 2006). The cats received two feedings per day at 0800 and 1700 h with access to food for 1 h and water *ad libitum*. During the adaptation period, the cats were group-housed but fed individually. Whereas in the collection period, the cats were individually housed in stainless steel cages. During each collection period, a fresh fecal sample (within 15 minutes of defecation) from each cat was collected using a sterile Whirl-pak bag and 2 g aliquots were transferred with a spatula into plastic microcentrifuge tubes and stored at -80°C for DNA extraction.

Fecal DNA Extraction and Sequencing

The DNA was extracted from 200 mg of each stool sample (n = 44) using a QIAamp Power Fecal Pro DNA Kit (Qiagen, Hilden, Germany) and Qiacube Connect (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions (Handbook 02/2020). A nanodrop (NanoDrop 2000, Thermo Scientific, Waltham, MA) was used for quality control of nucleic acid purity. Extractions were quantified on a Qubit fluorometer (Qubit 4.0, Invitrogen by Life Technologies, Carlsbad, CA). The 16S V3/V4 gene was amplified using the Illumina 16S Metagenomic Sequencing library prep protocol (Illumina, Inc., San Diego, CA) as specified by the manufacturer. The size and quality of libraries and the pool were assessed with a 2100

Bioanalyzer (Agilent, Santa Clara, CA). Libraries were run on an Illumina MiSeq system using 300x2 v3 paired end chemistry.

Data Analysis

Raw reads were trimmed for quality using CLC Genomics Workbench (Qiagen, v11.0.1) and then imported into mothur (v1.44.1) for further analysis (Schloss et al., 2009). The unique 16S reads were aligned to reference sequences from the SILVA rRNA database (Release 138) for closed-reference operational taxonomic unit (OTU) assignment (Yilmaz et al., 2014). Near-identical sequences were merged using VSEARCH v2.15.1 (Rognes et al., 2016).

Beta-diversity was determined by a principal coordinate analysis (PCoA) in R (v4.0.3, R Core Team, 2019). One sample (T4, cat 8, period 4) was considered an outlier and removed from the data. Alpha-diversity was evaluated using observed unique sequences, Chao1, Shannon, and Simpson indices. Relative abundance data were analyzed within the 50 most abundant OTU using a mixed model of SAS (v9.4, SAS Institute, Inc., Cary, NC). Treatment was considered a fixed effect and cat and period as random effects. Tukey's post hoc test was applied for the least-squares means separation, with significance considered at $P < 0.05$.

Results and Discussion

Beta and Alpha Diversity

Beta diversity is a representation of the entire microbial community. Principal coordinate analysis (PCoA) plots relationships on a two-dimensional scatterplot, with the axes representing fractions of variability. Each point on the scatterplot represents a single sample, and the distance between points represents how compositionally different the samples are from each other (Koleff et al., 2003). Consequently, PCoA plots can be interpreted as samples with high similarity appearing as clusters, and samples with high dissimilarity appearing randomly dispersed

(Goodrich et al., 2014). Therefore, the lack of apparent clustering by treatment groups in the current study indicates that beta diversity was not altered by dietary treatments (**Figure 7.1**).

Alpha diversity represents the within-sample variation for each cat's microbiome. There are several computed indices that have been proposed to help describe the number of species in a sample (richness) and how dominant or rare each species is to the others (diversity; Wagner et al., 2018). For the current study, the Observed index (number of OTU), Chao1 index (species richness), Shannon index (species diversity), and Simpson index (species evenness) were evaluated. Alpha diversity indices were similar ($P > 0.05$) for all treatments (**Figure 7.2**), indicating that dietary treatments did not influence the species richness or diversity within samples.

A healthy microbiome is characterized by high species richness and diversity with a reduction often associated with disease. In contrast, cats with chronic enteropathy had decreased alpha diversity compared to healthy cats (Marsilio et al., 2019; Kathrani et al., 2022). In the current research, the overall diversity of the microbiome was maintained when cats were fed BDY, BDY+DDGS, or CFP compared to the control containing SBM suggesting the experimental treatments did not hamper the healthy ecosystem of the colon. In a similar fashion, beta and alpha diversity were also maintained in dogs fed similar dietary treatments to this study (Kilburn-Kappeler et al., 2023b).

Phyla Relative Abundance

Most studies have reported that the microbiome of healthy cats is dominated by Firmicutes, followed by Proteobacteria, Actinobacteria, Bacteroidetes, and Fusobacteria (Ritchie et al., 2008; Handl et al., 2011; Eshar et al., 2018; Ganz et al., 2022). The most abundant phyla among all samples in the current study were Firmicutes at 66%, Bacteroidetes at 25%,

Actinobacteria at 8%, Proteobacteria at 0.64%, and Desulfobacteria at 0.54% (**Table 7.3**). The predominant phylum in the current study is similar to the previous studies in healthy cats except for Desulfobacteria which were observed instead of Fusobacteria. Interestingly, Desulfobacteria, a phylum known for sulfate reduction and nitrogen fixation, has been correlated to Parkinson's disease in humans (Sayavedra et al., 2021; Zhang et al., 2022).

The relative abundance of Firmicutes for T3 at 61% was lower ($P < 0.05$) than that of T4 at 69%, with T1 and T2 intermediate at 66%. The relative abundance of Actinobacteria was lower ($P < 0.05$) for T3 at 7% compared to T2 at 10%, with T1 and T4 intermediate at an average of 8%. The comparison of the relative abundance of Bacteroidetes among dietary treatments resulted in a significant *P*-value, however, the Tukey adjustment eroded this difference ($P > 0.05$) among treatment means. Numerically, the relative abundance of Bacteroidetes was greater for T3 at 31% compared to the remaining treatments at an average of 23%. The relative abundance of Proteobacteria was also numerically greater for T3 at 0.8% compared to the remaining treatments at an average of 0.6%. The average relative abundance of Desulfobacteria for T1 and T3 was 0.7% and 0.4% for T2 and T4.

The microbiome is responsive to nutrients rather than individual dietary ingredients (Pilla and Suchodolski, 2021). Therefore, the differences in phyla relative abundance among dietary treatments are likely due to the differences in nutrient composition, specifically fiber and protein. For example, dietary fiber has been reported to increase Firmicutes in dogs (Middelbos et al., 2010; Beloshapka et al., 2013; Panasevich et al., 2015; Myint et al., 2017). Therefore, the decrease in Firmicutes for T3 was surprising as T3 contained the greatest amount of fiber among dietary treatments. In addition, the differences in Actinobacteria may be due to the type of fiber present in dietary treatments (Barry et al., 2012). Furthermore, Hooda et al. (2013) reported a

decrease in Actinobacteria in kittens fed a high protein/low carbohydrate diet compared to a moderate protein/moderate carbohydrate diet. Therefore, it could be expected that diets with higher protein content would result in lower relative abundance of Actinobacteria when fed to cats. However, the opposite response was observed in the current study. Of note, the relative abundance of Firmicutes, Bacteroidetes, and Actinobacteria was not impacted by dietary treatments when fed to dogs (Kilburn-Kappeler et al., 2023b). The variable results among studies could be due to differences in dietary matrices, animal population, environment, and/or specific taxa (e.g., genera) present in each phylum.

Genera Relative Abundance

When compared on a more specific phylogenic level, 17 genera out of the 50 most abundant OTU within samples resulted in significant differences among dietary treatments (**Table 7.4**). The relative abundance of *Acidaminococcus* was lower ($P < 0.05$) for T3 at 0.4% compared to T1 at 1.3% with T2 and T4 intermediate at an average of 1.0%. The relative abundance of *Allisonella* was highest ($P < 0.05$) for T4 at 0.5% and lowest ($P < 0.05$) for T1 and T2 at 0.3%, T3 was intermediate at 0.4%. The relative abundance of *Bifidobacterium* was lower ($P < 0.05$) for T3 and T4 at an average of 0.6% compared to T1 and T2 at an average of 2.5%. The relative abundance of *Blautia* was greater ($P < 0.05$) for T2 at 13.7% compared to T3 and T4 at an average of 10.7%, T1 was intermediate at 11.6%. *Catenibacterium* relative abundance was greatest ($P < 0.05$) for T4 at 5.4% compared to the remaining treatments at an average of 2.9%. The relative abundance of *Dialister* was greater ($P < 0.05$) for T4 at 4.6% compared to T1 at 2.3% and T2 at 3.0%, T3 was intermediate at 4.1%. The lowest ($P < 0.05$) relative abundance of *Erysipelatoclostridium* was observed for T1 at 0.1% compared to the remaining treatments at an average of 1.2%. *Fusicatenibacter* relative abundance was greater ($P < 0.05$) for T1 and T4 at an

average of 1.0% compared to T2 at 0.4% with T3 intermediate at 0.8%. The relative abundance of *Holdemanella* was greater ($P < 0.05$) for T3 and T4 at an average of 10.6% compared to T1 at 6.0% with T2 intermediate at 8.7%. *Megamonas* relative abundance was greater ($P < 0.05$) for T1 at 2.0% compared to T3 at 0.8% with T2 and T4 intermediate at an average of 1.2%. *Peptoclostridium* relative abundance was greater ($P < 0.05$) for T4 at 9.2% compared to T1 and T3 at 5.0 and 6.9%, respectively, T2 was intermediate at 7.3%. The relative abundance of *Peptococcus* was greater ($P < 0.05$) for T2 at 1.5% compared to T3 and T4 at an average of 1.1%, T1 was intermediate at 1.2%. The relative abundance of *Prevotella 9* was greater ($P < 0.05$) for T3 at 23% compared to T2 at 14.8% with T1 and T4 intermediate at an average of 15.9%. The relative abundance of *Solobacterium* was greater ($P < 0.05$) for T3 at 3.2% compared to T1 and T2 at 1.3% with T4 intermediate at 3.0%. *Streptococcus* relative abundance was greater ($P < 0.05$) for T1 at 8.5% compared to T3 and T4 at an average of 1.4% with T2 intermediate at 4.2%. The relative abundance of *Subdoligranulum* was greater ($P < 0.05$) for T1 and T2 at an average of 4.1% compared to T3 and T4 at an average of 1.3%. The genera classified as *uncultured* was greatest ($P < 0.05$) for T3 at 2.1% compared to the remaining treatments at an average of 0.7%.

Ganz et al. (2022) reported the genera with the highest relative abundance in healthy pet cats were *Prevotella*, *Bacteroides*, *Collinsella*, *Catenibacterium*, *Blautia*, *Faecalibacterium*, and *Megasphaera*. Each of these genera were among the 50 most abundant OTU in the current study. Additional studies have also reported *Bacteroides* and *Prevotella* to be the most frequent genera observed in the microbiome of healthy cats (Johnston et al., 2001; Alessandri et al., 2020). Hooda et al. (2013) also reported that *Faecalibacterium* was common in the fecal microbiome of healthy kittens fed a range of diets.

Many of these genera are associated with the production of short chain fatty acids (SCFA) and branched chain fatty acids (BCFA) by fermentation of carbohydrates, protein, and fiber in the gastrointestinal tract (Mead, 1971; Levine et al., 2013; Butowski et al., 2019). For example, *Prevotella*, *Catenibacterium*, and *Megasphaera* digest glucose or lactate producing propionate (Butowski et al., 2019). *Faecalibacterium* ferments carbohydrates producing acetate and can further convert this acetate to butyrate (Morrison et al., 2016). *Bacteroides*, *Blautia*, and *Collinsella* also ferment dietary carbohydrates to SCFA (Qin et al., 2019; Ziese and Suchodolski, 2021). Ganz et al. (2022) also reported prevalence of *Subdoligranulum* and *Fusicatenibacter* among healthy cats, which are positively associated with SCFA production (Takeshita et al., 2016; Van den Abbeele et al., 2020). The synthesis of SCFA is beneficial as butyrate is the preferred substrate used by the gut mucosa, propionate contributes to gluconeogenesis in the liver, and acetate is most concentrated in the blood (Morrison et al., 2016). Therefore, proportions of these genera and their end products can influence host health. However, the significant differences in relative abundance of genera did not impact the production of SCFA and BCFA among dietary treatments in the current study (Kilburn-Kappeler et al., 2023a).

Bacteroides, *Prevotella*, and *Faecalibacterium* have been reported to increase with increased dietary fiber content in dogs (Pilla and Suchodolski, 2021). Therefore, the increase in *Prevotella* for T3 could be explained by the increased dietary fiber content. In addition, Butowski et al. (2019) reported an increase in *Prevotella* with the addition of plant fiber to a raw diet fed to adult cats. An increase in *Blautia* was reported in kittens fed high protein/low carbohydrate diets compared to kittens fed medium protein/medium carbohydrate diets (Hooda et al., 2013). In contrast, a decrease in *Bifidobacterium* was reported in kittens fed high protein/low carbohydrate diets (Vester et al., 2009; Hooda et al., 2013). Therefore, the decrease

in *Blautia* and *Bifidobacterium* for T3 and T4 could be due to the decreased protein content in dietary treatments. Prebiotic fibers have also been reported to increase *Bifidobacterium* in cats (Bermingham et al., 2013; Hooda et al., 2013; Young et al., 2016). In addition, Beloshapka et al. (2013) reported an increase in *Bifidobacterium* in dogs fed diets containing yeast cell wall. The relative abundance of *Bifidobacterium* among dietary treatments in the current study indicates that different yeast sources may have varying effects on the fecal microbiome. The relative abundance of *Dialister* and *Acidaminococcus* among dietary treatments in the current study is interesting as decreased abundance was observed in kittens fed high protein/low carbohydrate diets (Hooda et al., 2013). Xu et al. (2021) reported a lower relative abundance of *Allisonella* and *Megamonas*, carbohydrate fermenters (Kieler et al., 2017; Butowski et al., 2019; Che et al., 2019), in dogs consuming a raw diet compared to a kibble diet. The relative abundance for both *Allisonella* and *Megamonas* was lower in T3 compared to T1, possibly indicating differences in dietary starch content. Schauf et al. (2018) reported a lower relative abundance of *Solobacterium* in dogs fed a high fat/low starch diet compared to a low fat/high starch diet. However, in the current study, the diet with the highest fat content (T3) resulted in a higher relative abundance of *Solobacterium*.

In contrast to the current study in cats, the relative abundance of *Bifidobacterium*, *Catenibacterium, Holdemanella*, *Peptococcus*, and *Prevotella* did not differ among dietary treatments when fed to dogs (Kilburn-Kappeler et al., 2023b). Furthermore, the relative abundance of *Collinsella* was impacted in dogs but not in cats fed dietary treatments. However, a similar effect in the relative abundance of *Blautia* was observed in both dogs and cats, with a significant decrease for T3 and T4. The effect of dietary treatments on the relative abundance of *Erysipelatoclostridium* was also similar for dogs and cats with the lowest abundance observed in

T1. The relative abundance of *Peptoclostridium* was lower for T1 in both dogs and cats fed dietary treatments. In addition, the same pattern in relative abundance of *Streptococcus* was observed among dogs and cats with T1 resulting in a greater abundance compared to T3 and T4 (Kilburn-Kappeler et al., 2023b).

Conclusion

This data indicates that CFP did not alter the overall diversity of the fecal microbiome of healthy adult cats over a 14-d period. The shifts in relative abundance of taxa appeared to be influenced by the type of dietary substrate available for microbial fermentation, specifically fiber and protein. In comparison to dogs, dietary treatments had a greater influence on the fecal microbiome of cats which was indicated by differences in the relative abundance of phyla as well as the increased number of genera with significant differences. Overall, CFP maintained animal health based on the fecal microbiome, but further investigation is warranted to determine potential effects as a prebiotic.

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Chapter 7 Tables and Figures

Table 7.1 Ingredient composition of feline diets containing yeast and ethanol co-products on an as-is basis

	Treatment[1]			
Ingredient, %	T1	T2	T3	T4
Corn	34.6	30.0	30.0	34.6
Chicken Meal	30.0	35.0	35.0	30.0
Soybean Meal	15.0	8.0	-	-
Distillers Dried Grains with Solubles	-	-	17.5	-
Corn Fermented Protein	-	-	-	17.5
Brewer's Dried Yeast	-	3.5	2.5	-
Corn Starch	-	6.5	-	2.5
Corn Gluten Meal	5.0	2.0	-	-
Chicken Fat	6.0	5.6	5.6	6.0
Other[2]	9.4	9.4	9.4	9.4

[1]T1, control; T2, brewer's dried yeast; T3, brewer's dried yeast and distillers dried grains with solubles; T4, corn fermented protein.
[2]Other ingredients: beet pulp, fish meal, flavor, titanium dioxide, salt, potassium chloride, vitamin and mineral premix, choline chloride, natural antioxidant.

Table 7.2 Analyzed chemical composition of feline diets containing yeast and ethanol co-products on a dry matter basis

	Treatment[1]			
Nutrient	T1	T2	T3	T4
Dry Matter, %	95.03	95.71	94.38	95.50
Organic Matter, %	90.03	90.17	90.31	91.49
Ash, %	9.97	9.83	9.69	8.51
Crude Protein, %	41.59	40.58	38.50	37.22
Fat, %	12.80	13.32	14.45	13.40
Total Dietary Fiber, %	13.66	13.19	18.46	15.05
Insoluble Dietary Fiber, %	10.09	10.04	14.34	12.40
Soluble Dietary Fiber, %	3.67	3.14	4.13	2.64
Gross Energy, kcal/kg	4951.82	4997.76	5065.36	5001.77

[1]T1, control; T2, brewer's dried yeast; T3, brewer's dried yeast and distillers dried grains with solubles; T4, corn fermented protein.

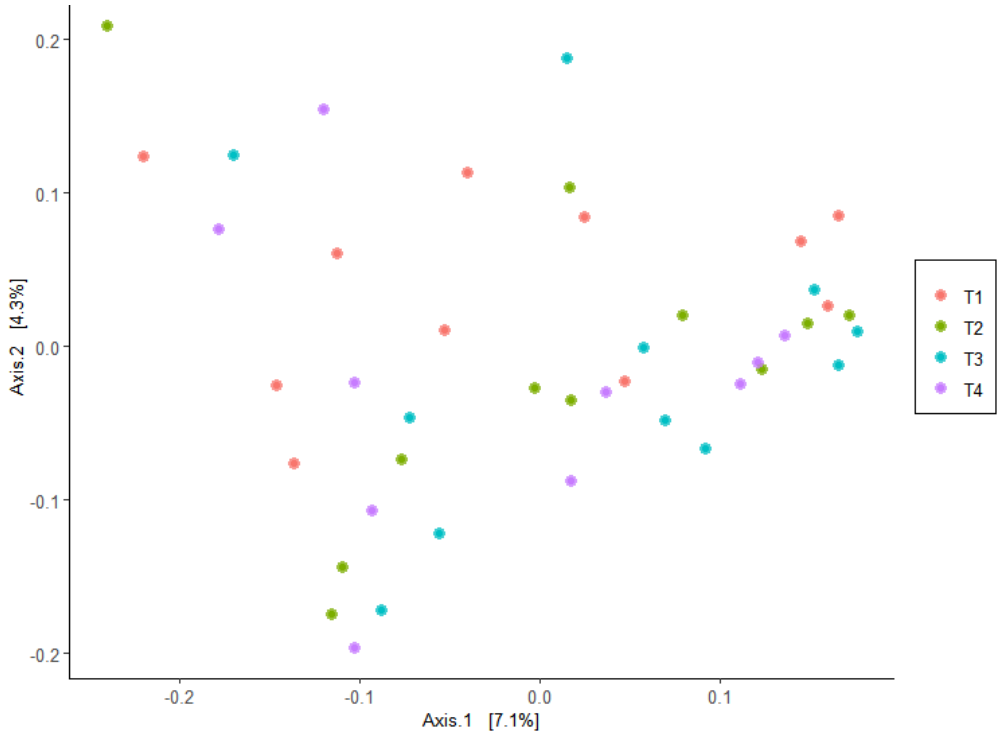

Figure 7.1 Principal coordinate analysis (PCoA) explaining 7.1% and 4.3% of the variability in operational taxonomic units (OTU) of Bray-Curtis UniFrac distances for fecal samples from cats fed dietary treatments. Treatments: T1 = control; T2 = brewer's dried yeast; T3 = brewer's dried yeast and distillers dried grains with solubles; T4 = corn fermented protein.

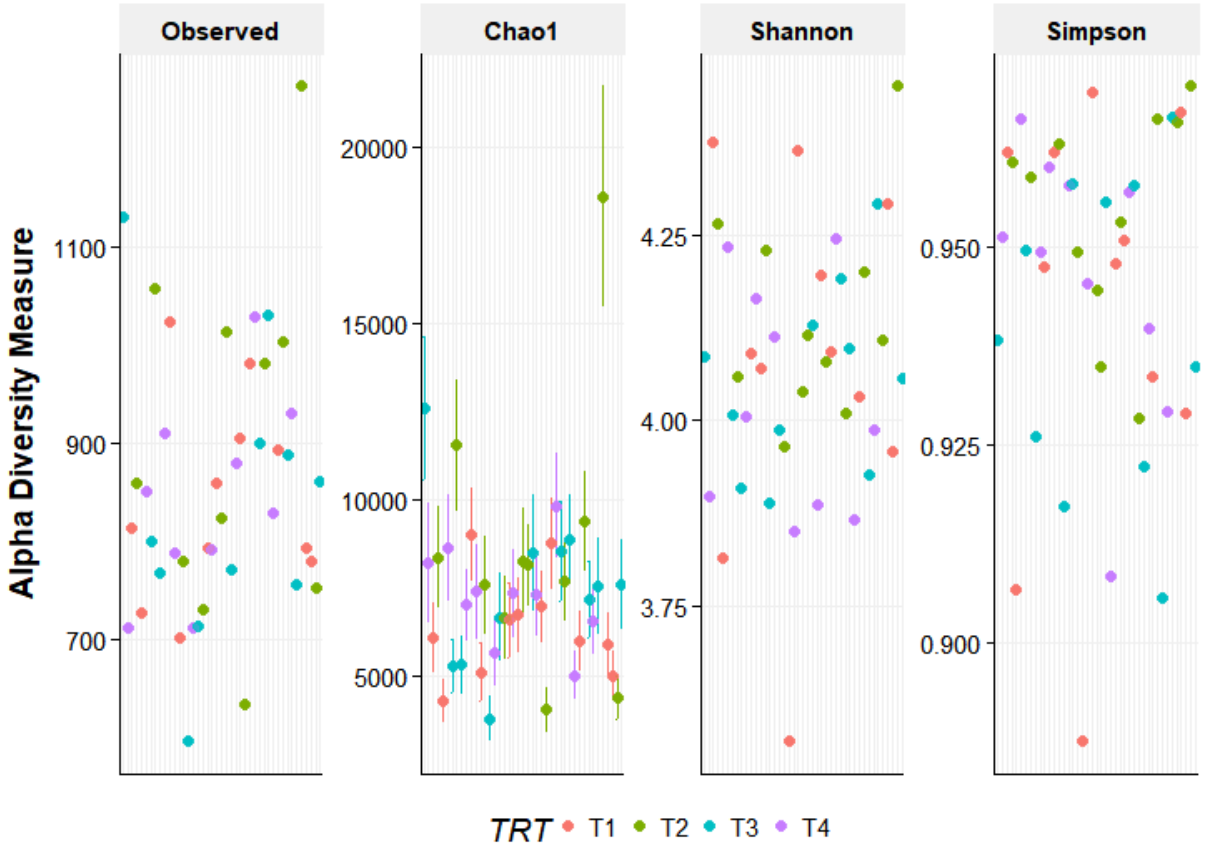

Figure 7.2 Alpha diversity measures for fecal samples from cats fed dietary treatments. Treatments: T1 = control; T2 = brewer's dried yeast; T3 = brewer's dried yeast and distillers dried grains with solubles; T4 = corn fermented protein.

Table 7.3 Relative abundance of bacterial phyla among the 50 most abundant operational taxonomic units (OTU) in fecal samples from cats fed dietary treatments

	Treatment[1]					
Phylum, %	T1	T2	T3	T4	SEM	*P*-value
Firmicutes	66.07[a,b]	66.71[a,b]	60.75[b]	69.39[a]	2.875	0.0423
Bacteroidetes	23.37	22.70	31.32	22.06	3.321	0.0294
Actinobacteria	9.15[a,b]	9.67[a]	6.50[b]	7.61[a,b]	1.183	0.0375
Proteobacteria	0.56	0.53	0.84	0.64	0.914	0.1756
Desulfobacteria	0.71	0.39	0.72	0.33	0.182	0.0755

[1]T1, control; T2, brewer's dried yeast; T3, brewer's dried yeast and distillers dried grains with solubles; T4, corn fermented protein.
[a-b]Means within a row lacking a common superscript letter are different ($P < 0.05$).

Table 7.4 Relative abundance of bacterial genera among the 50 most abundant operational taxonomic units (OTU) in fecal samples from cats fed dietary treatments

	Treatment[1]					
Genus, %	T1	T2	T3	T4	SEM	*P*-value
Acidaminococcus	1.33[a]	0.67[a,b]	0.43[b]	1.25[a,b]	0.299	0.0132
Allisonella	0.25[c]	0.25[c]	0.39[b]	0.52[a]	0.043	<0.0001
Alloprevotella	2.20	2.58	1.93	1.71	0.408	0.1950
Bacteroides	4.32	4.39	4.54	4.37	0.810	0.9934
Bifidobacterium	2.57[a]	2.39[a]	0.41[b]	0.77[b]	0.567	0.0007
Blautia	11.63[a,b]	13.68[a]	10.79[b]	10.53[b]	1.019	0.018
Catenibacterium	2.37[b]	3.56[b]	2.86[b]	5.38[a]	0.497	<0.0001
Catenisphaera	0.58	1.13	1.79	0.66	0.615	0.1973
Collinsella	6.13	6.93	5.87	6.04	0.762	0.5168
Coprococcus	0.94	0.59	0.78	0.87	0.215	0.3973
Desulfovibrio	0.71	0.39	0.72	0.33	0.182	0.0755
Dialister	2.28[c]	3.03[b,c]	4.13[a,b]	4.64[a]	0.523	0.0005
Erysipelatoclostridium	0.10[b]	0.93[a]	1.11[a]	1.41[a]	0.192	<0.0001
Faecalibacterium	1.28	1.30	2.29	2.06	0.439	0.0550
Fusicatenibacter	0.93[a]	0.43[b]	0.78[a,b]	1.03[a]	0.166	0.0068
Holdemanella	5.97[b]	8.73[a,b]	10.11[a]	11.14[a]	1.164	0.0010
Lachnospiraceae ge	2.17	2.00	2.32	2.36	0.221	0.3707
Lachnospiraceae unclassified	1.98	2.52	1.99	2.19	0.576	0.2151
Ligilactobacillus	4.63	3.32	2.16	3.46	2.843	0.8517
Megamonas	2.04[a]	1.53[a,b]	0.80[b]	0.92[a,b]	0.455	0.0384
Megasphaera	3.52	3.07	1.75	3.20	0.524	0.0097
Negativibacillus	1.28	1.31	1.36	0.90	0.191	0.1005
Olsenella	0.46	0.32	0.24	0.82	0.394	0.5068
Peptoclostridium	5.03[c]	7.31[a,b]	6.89[b,c]	9.18[a]	0.766	0.0002
Peptococcus	1.21[a,b]	1.52[a]	1.11[b]	1.04[b]	0.129	0.0047

Peptostreptococcaceae unclassified	1.72	1.06	0.65	1.18	0.591	0.3361
Prevotella 9	16.67[a,b]	14.83[b]	22.67[a]	15.03[a,b]	2.750	0.0250
Solobacterium	1.33[b]	1.31[b]	3.19[a]	2.96[a,b]	0.631	0.0050
Streptococcus	8.53[a]	4.24[a,b]	1.64[b]	1.19[b]	2.387	0.0173
Subdoligranulum	4.89[a]	3.31[a]	1.45[b]	1.08[b]	0.658	<0.0001
Sutterella	0.56	0.53	0.84	0.64	0.152	0.1756
uncultured	0.26[b]	0.92[b]	2.09[a]	0.98[b]	0.303	<0.0001

[1]T1, control; T2, brewer's dried yeast; T3, brewer's dried yeast and distillers dried grains with solubles; T4, corn fermented protein.
[a-c]Means within a row lacking a common superscript letter are different ($P < 0.05$).

Chapter 8 - Conclusion and future steps

Based on the first research objective, the optimal inclusion level of CFP in pet food is 10% to maintain stool quality, nutrient digestibility, and palatability compared to soybean meal. The optimum inclusion level in cats was clear at 10% as the 15% inclusion resulted in a decrease in nutrient digestibility and stool quality, whereas dogs appeared to be able to utilize greater inclusion levels. This could be explained by the fact that cats have a shorter digestive tract compared to dogs decreasing their ability to utilize fiber, which was elevated with the 15% CFP inclusion. Further research is warranted regarding the palatability of CFP as processing parameters and physical characteristics of kibble may have impacted results. Nevertheless, inclusion of CFP up to 15% resulted in acceptable stool quality, nutrient digestibility, and palatability when fed to both dogs and cats.

Based on the second research objective, the yeast component of CFP could provide better nutrient utilization compared to traditional DDGS when fed to both dogs and cats. This conclusion was supported by the increased nutrient digestibility and stool quality observed with the CFP treatment compared to the BDY+DDGS treatment. However, these results could have been confounded with the decreased dietary fiber in CFP compared to DDGS. In addition, the 17.5% inclusion of CFP did not alter hindgut fermentation in dogs or cats based on the maintenance of total SCFA and BCFA concentrations in fecal samples. Furthermore, CFP did not alter the overall diversity of the fecal microbiome when fed to both dogs and cats which was supported by similar beta and alpha diversity among dietary treatments. Further research is warranted to determine if CFP can be utilized as a prebiotic and at what inclusion level.

Moving forward, it would be beneficial to characterize the yeast component of CFP, which will require development of a standardized analytical method to determine dead yeast and

its chemical components. The characterization of the yeast could determine the potential of CFP to promote animal health. Specifically, the β-glucans present in the yeast cell wall could enhance the immune response when fed to dogs and cats. Therefore, a future study should be conducted to determine the effect of CFP on the immune system of dogs and cats which could provide an additional benefit for CFP utilization in pet food.

Corn fermented protein is a unique ingredient as it could be included in pet food as a protein source at moderate levels (≤10%) without altering nutrient digestibility or stool quality, but also to support animal health at higher levels. In conclusion, the utilization of CFP in pet food could provide a value-added opportunity by improving consumer perception of ethanol co-products while also providing a sustainable and cost-effective protein source.