INFLUENCE OF FOUR DIFFERENTLY PROCESSED DIETS ON PLASMA LEVELS OF ADVANCED GLYCATION END PRODUCTS (AGES), SERUM LEVELS OF RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS (RAGE), SERUM AND URINE METABOLOME, AND FECAL MICROBIOME IN HEALTHY DOGS

by

SIOBHAN BRIDGLALSINGH

(Under the Direction of Joseph W. Bartges)

ABSTRACT

Advanced glycation end products (AGEs) are formed during the Maillard reaction when cooking foods using high heat and low moisture. Pet food processing uses high temperatures to increase safety by reduction of microbial load and allow storage. Dietary AGEs have been implicated as a risk factor for development of obesity and other diseases in human beings suggesting that high dietary intake by dogs may be detrimental to their health. Soluble RAGE (sRAGE) acts as a decoy receptor that binds AGEs preventing cellular RAGE activation and inflammation.

We measured the AGEs carboxymethyllysine (CML), carboxyethyllysine (CEL) and methylglyoxal hydroimidazolone-1 (MG-H1) in four differently processed diets (two high heat – canned wet and dry kibble; two low heat – air-dried and mildly cooked/raw) and plasma levels of CML, CEL, MG-H1, glyoxal hydroimidazolone-1 (GH-1) and argpyrimidine (AP) via mass spectrometry, to determine the influence of dietary AGE intake on plasma levels. Serum

concentrations of sRAGE were determined by immunoassay. High heat processed diets contained higher total AGEs that influenced plasma AGEs while sRAGE levels did not change.

We proposed that differently processed diets containing varied amounts of AGEs will alter the serum and urine metabolome. Nuclear magnetic resonance (NMR) spectroscopy was used to determine serum and urine metabolic changes. We identified eight discriminatory metabolites in serum and six in urine, and observed lower metabolite concentrations with the dry kibble. Serum and urine metabolite profiles fluctuated minimally over the feeding period for each diet.

We hypothesized that the fecal microbiome would change in response to the four differently processed diets. Microbiome analysis via 16S rRNA sequencing showed a decrease in α-diversity with the dry kibble, changes in genera and higher microbial, but non-pathogenic, load of the low heat processed diets.

This work demonstrates how processing methods and associated dietary AGE levels, by way of its metabolic transit through the canine body, can predispose dogs to diseases as in human beings. Further investigations are recommended to provide additional information that can guide pet food processing and nutritional management of inflammatory and degenerative diseases in dogs related to dietary AGE intake.

INDEX WORDS: advanced glycation end products, Maillard reaction, pet food processing, canine metabolome, canine fecal microbiome.

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DEDICATION

I dedicate this work to my mother, Anne Marie Lall, who has always been my rock and support throughout life, never failing to remind me to pray and laugh as we endure our tribulations and triumphs; to my sister, Rhiannon Bridglalsingh, who prays and laughs with me as we take our separate journeys while staying grounded in home and family; and to my father, Kelvin Bridglalsingh (deceased), who never had a doubt that I would relentlessly pursue and achieve my goals. You three have lit the fire within me to live my best life!

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

The Maillard reaction, described in 1912 by the French chemist, Louis Camille Maillard, produces dark colored melanoidins at the final stage of the reaction. It was termed "browning" (Maillard, 1912) since this was the color acquired by bread crusts, coffee and meats during cooking (ALjahdali & Carbonero, 2017). The reaction is divided into early, intermediate, and late stages, resulting in formation of numerous compounds at each stage, before production of the final pigmented product. This non-enzymatic process occurs when a carbonyl group of a reducing sugar reacts with a free amine group of an amino acid, peptide or protein to produce a wide range of heterogeneous compounds (Lin, Wu, & Yen, 2018) under conditions of high heat and low moisture. The focus of the current study is on compounds formed at the late stage of the Maillard reaction known as dietary advanced glycation end products (AGEs) that have been implicated in diseases of human beings such as obesity, diabetes mellitus, cardiovascular disease and atherosclerosis, renal failure, Alzheimer's Disease, Parkinson's Disease and several inflammatory and degenerative conditions (Delgado-Andrade, 2014; Poulsen et al., 2013).

Dietary AGEs are derived exogenously while endogenous sources are formed during physiological glycation reactions. Both forms are indistinguishable from each other and together they contribute to the body's AGE circulating pool (Kellow & Coughlan, 2015). The precise pathophysiological role of AGEs has not yet been determined but mechanisms of action such as structural and functional alteration of proteins, localized reactive oxygen species formation leading to oxidative stress and most significantly, binding to the receptor for advanced glycation

end products (RAGE) (Bierhaus et al., 2005) to initiate and perpetuate sustained inflammation in cells (Ramasamy, Yan, & Schmidt, 2012), have been identified as the ways in which AGEs may contribute to inflammation and degeneration. An isoform of RAGE known as soluble RAGE (sRAGE) is not attached to the cell membrane but acts as a decoy receptor in circulation (Ciccocioppo et al., 2015). Once in the blood, sRAGE binds to AGEs preventing cellular RAGE activation and blocking inflammation and oxidative stress. Concentrations of sRAGE in diseases and in response to AGEs have been inconsistent for human beings (Prasad, 2019) while findings in dogs are limited to specific disease such as inflammatory bowel disease (IBD) (Heilmann et al., 2014).

Foods containing high amounts of AGEs include cooked red meats, high fat dairy products, processed foods, and drinks in addition to fried or roasted meals that are characteristic of the Western diet. This type of diet has been associated with similar diseases in human beings as described for AGEs (Bettiga et al., 2019). While dogs do not usually consume a Western diet, traditional commercial dog food is prepared using similar thermal processing conditions (van Rooijen et al., 2013) so AGEs may play a role in health and disease of dogs.

There are gaps in knowledge concerning the digestion, absorption, metabolism and excretion of AGEs. *In vivo* and *in vitro* studies in human beings and animals (Snelson & Coughlan, 2019) have provided more information about AGE metabolic transit but data in dogs is scarce. There is a need to trace the fate of dietary AGEs from digestion to elimination and to determine the biological effects of AGEs in fluids and tissues.

Measurement of AGEs in food and biofluids by mass spectrometry combined with liquid chromatography has produced reliable and repeatable results. Analysis of food items to create a database (Scheijen et al., 2016) and to determine plasma and urine AGE concentrations in

response to dietary AGEs in human beings (Scheijen et al., 2018) has been performed by liquid chromatography – mass spectrometry indicating that the same approach can be applied for similar investigations in dogs. Additionally, the main mechanism of action of AGEs is AGE-RAGE interaction driving cellular signaling for sustained inflammation, making it worthwhile to compare circulating AGE levels with sRAGE.

Determination of AGE levels in diet and biofluids offers information about intake but the biological fate of AGEs can be explored through study of biofluid metabolomics (Bouchard-Mercier, Rudkowska, Lemieux, Couture, & Vohl, 2013) and gastrointestinal microbiome metagenomics (Deng & Swanson, 2015). Dietary AGEs enter the digestive system as protein bound or free forms that take different paths from the gastrointestinal system to enter circulation or be excreted. Free AGEs are low molecular weight (LMW) substances that are absorbed directly by diffusion or transported by peptide transporters into circulation. Protein bound AGEs are high molecular weight (HMW) compounds that are too large to be absorbed by diffusion allowing them to reach the large intestine as potential substrates for colonic microbiota whose products are absorbed across the intestinal barrier to ultimately enter the circulation. Any unchanged fecal HMW AGEs are excreted in the feces while free or peptide bound AGEs are transported in the circulation to tissues and organs until eventual elimination in the urine (Poulsen et al., 2013). These pathways through the body suggest that investigation into the changes in the serum and urine metabolic profile as well as changes in the canine colonic microbiome may provide insights into the metabolic fate and effect of AGEs. To the authors' knowledge, no studies have investigated AGE levels in differently processed diets in dogs or how feeding on these diets influences AGEs in the digestive tract or circulation.

Research objectives

The objectives of this study were:

1. To determine the influence of AGE quantities in four differently processed diets, each fed for four weeks, on plasma AGE levels and serum sRAGE levels in healthy dogs.

2. To determine canine serum and urine metabolic profiles in response to feeding four differently processed diets over four weeks.

3. To determine the composition and changes in the fecal microbiome of dogs in response to feeding four differently processed diets for four weeks.

CHAPTER 2

LITERATURE REVIEW

Thermal food processing in human beings is associated with the development of several inflammatory and degenerative disease conditions (Lin et al., 2018). Investigations into types of processed foods and their etiological role in diseases have identified advanced glycation end products (AGEs) as a feature of the high calorie and high-heat processed foods of the "Western diet" (Hull, Woodside, Ames, & Cuskelly, 2012). Conventional pet food processing employs similar high heat methods that are also associated with dietary sources of AGEs (van Rooijen et al., 2013) and possibly risk of the same diseases to which human beings are predisposed. This review examines the existing literature on AGE formation and their implication in diseases, dietary sources related to thermal processing of foods and measurement of AGEs as well as the receptor for AGEs (RAGE) as an indicator of inflammation. This discussion also includes current information about alterations in the metabolome and microbiome in response to dietary change and in chronic inflammatory diseases, to lay the foundation for investigation into the relationship among all components of this research: dietary AGEs, RAGE, the canine metabolome and microbiome.

2.1 The Maillard Reaction

Also known as glycotoxins, AGEs are a vast, heterogeneous range of complex compounds formed during Maillard (or browning) reactions (Uribarri et al., 2015). First discovered in 1912 by Louis Camille Maillard, the Maillard reaction (MR) is a spontaneous, non-enzymatic reaction that occurs between the carbonyl group of reducing sugars and the amino group of amino acids

during food processing. The MR forms various compounds necessary for aroma, flavor and color of food in addition to AGEs.

Carbonyl groups are present in reducing sugars, oxidized lipids, quinones or Vitamin C while amines are derived from free or bound amino acids, peptides or proteins. Reducing sugars such as monosaccharides (e.g. glucose and fructose), disaccharides (e.g. maltose and lactose), oligosaccharides (e.g. fructo-oligosaccharides and mannan-oligosaccharides), and polysaccharides (e.g. glycogen and chitin) are all involved in AGE formation (Zamora & Hidalgo, 2005). Any sugars in which a glycosidic bond can be cleaved can contribute to formation of AGEs (Ledl & Schleicher, 1990; Poulsen et al., 2013). The primary amino group of the lysine side chains is the most reactive precursor amine in proteins. The side chains of histidine and tryptophan (Moughan & Rutherford, 2008; Silvan, van de Lagemaat, Olano, & Del Castillo, 2006; van Rooijen et al., 2013), the arginine guanidine group, and any *N*-terminal amino group are also reactive. Secondary amines are less reactive than primary amines while tertiary amines are completely inactive (Poulsen et al., 2013).

The Maillard reaction is divided into early, intermediate, late/advanced and/or final stages (Fig.2.1). In the early stages, a condensation reaction occurs between the carbonyl group with an amine moiety to form a reversible Schiff base. The intermediate stage is characterized by an Amadori rearrangement of the Schiff base to form a ketoamine called an Amadori compound. In the advanced stages, the Amadori compounds degrade by rearrangement, condensation, oxidation, dehydration and hydration reactions to form advanced Maillard reaction products (MRPs). These condensation products are known as pre-melanoidins since they lead to the final stage of the MR where there is formation of low molecular weight (LMW) and high molecular weight (HMW) melanoidins (Poulsen et al., 2013). These final products belong to a group of

heterogeneous, insoluble, nitrogen-containing compounds that are polymerized to give color to foods such as coffee, cocoa, malt, honey and bread crust (ALjahdali & Carbonero, 2017). Temperature and time affect the molecular weight of the various melanoidins while pH affects their chemical structure (Wang, Qian, & Yao, 2011).

The significant precursors of AGEs formed during the MR are reactive dicarbonyl species. These are pro-oxidative compounds such as α-oxoaldehydes: glyoxal (GO), 1- and 3-deoxyglucosones as well as fission products such as methylglyoxal (MGO) which, together with another intermediate, ε-*N*-deoxyketosyllysine (Amadori compound) can react with proteins or lipids to generate oxidants such as N^{ε}-carboxymethyl-lysine (CML) or pentosidine (Anese, Manzocco, Nicoli, & Lerici, 1999; Anese, Nicoli, Massini, & Lerici, 1999; Liu, Yang, Jin, Hsu, & Chen, 2008; Nicoli, Anese, & Parpinel, 1999). Pyrraline and hydroxymethylfurfural (HMF) are other advanced MRPs derived from these reactions (Erbersdobler & Somoza, 2007). Pentosidine, pyrraline, CML, and HMF are the most common compounds of the advanced stage that are used as markers to indicate extent of the Maillard reaction in foods. In the final stage, MRPs react with free amino groups by antioxidant activity to produce melanoidins giving brown color to heated foods (Hurrell & Carpenter, 1981; van Rooijen et al., 2013; H. Y. Wang et al., 2011).

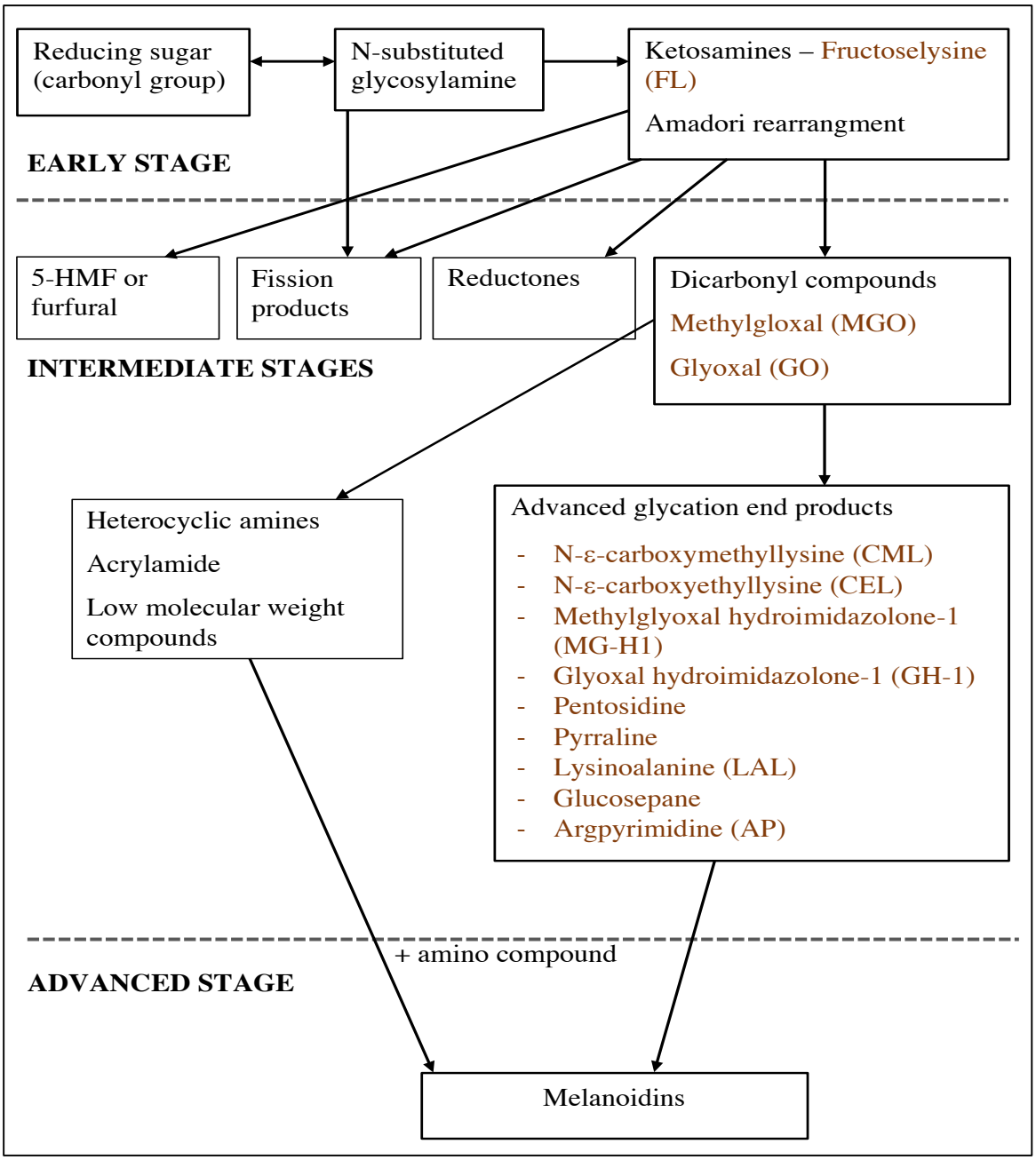

Fig. 2.1 Stages of the Maillard reaction and the more common products implicated in diseases.

2.1.1 Advanced glycation end products – endogenous formation and pool

Endogenously, AGEs are formed by *in vivo* glycation of proteins. Endogenous AGEs are produced via physiological processes such as protein degradation, monosaccharide autoxidation, Schiff's base fragmentation, fructosamine degradation and from α,β-dicarbonyl compounds formed via degradation of glycolytic intermediates and lipid peroxidation (N. Ahmed, Argirov, Minhas, Cordiero, & Thornalley, 2002). AGEs formed under physiologic conditions are the result of non-enzymatic reactions of glucose, α-oxoaldehydes and other saccharide derivatives with proteins, nucleotides and lipids (Poulsen et al., 2013; Thornalley, 1999).

Exogenous and endogenous AGEs contribute to the body's AGE pool. Dietary sources of AGEs are absorbed into circulation to join this pool and they remain structurally and functionally indistinguishable from endogenously formed AGEs (Uribarri et al., 2010).

2.1.2 Digestion, Absorption, Metabolism and Excretion

There are limited data concerning bioavailability and metabolic fate of dietary AGEs. The glycated amino acids in food are protein bound and cannot be absorbed in the gut until digestion occurs by gastric and intestinal peptidases to release them as bound to either free amino acids, di- or tri-peptides (Hellwig, Matthes, Peto, Lobner, & Henle, 2014; Kellow & Coughlan, 2015). Furthermore, since heat denatures proteins and reduces digestibility, any protein bound AGEs may remain undigested and trapped in the gastrointestinal tract (Poulsen et al., 2013). If digestion degrades AGEs into smaller peptide forms, they can reach the intestinal brush border to be further broken down and absorbed. All low molecular weight (LMW) AGEs in free amino acid or peptide structure may be absorbed by simple diffusion or via peptide transporter proteins (Hellwig et al., 2011). However, cross-linked LMW AGEs that cannot be digested as well as high molecular weight (HMW) AGEs, either cross-linked or aggregated, remain undigested in

the gut lumen to either be excreted in feces or used as a fermentation substrate by colonic microbial organisms (Tuohy et al., 2006). As much as 10 – 30% of dietary AGEs are absorbed in the intestines and enter circulation in rats and human beings (Faist & Erbersdobler, 2001; Koschinsky et al., 1997). Radioactively labeled AGEs localize in renal and hepatic tissue (Bergmann et al., 2001; He, Sabol, Mitsuhashi, & Vlassara, 1999) but whether this is different for each compound is unknown (Kellow & Coughlan, 2015; Poulsen et al., 2013). This current lack of knowledge regarding the fate of unabsorbed AGEs warrants investigation into colonic microbial fermentation to determine proportions excreted or used as substrates by microbes. Bacteria possess deglycating enzymes so the colonic microbiota can possibly use certain glycated amino acids as a source of carbon, nitrogen or energy (Hellwig et al., 2015; Monnier, 2005; Qu et al., 2017).

It is unlikely that AGEs participate in hepatic detoxification by phase 1 and 2 enzymes (Poulsen et al., 2013). Phase 1 enzymes are restricted to the lipid membranes of the endoplasmic reticulum making the highly water-soluble AGEs inaccessible to them. Phase 2 coupling reactions require side groups, which are not typical of most AGEs except for their acidic groups that may be esterified. Information about metabolic processes for change of dietary AGEs into metabolic products is scarce at this time (Poulsen et al., 2013).

Excretion has been measured by renal clearance of AGEs since LMW compounds, with relatively short half-lives, are rapidly absorbed, cleared through glomerular filtration and excreted in urine. Studies in rats indicate that there is a high excretion rate of free LMW AGEs and a relatively short time for interaction with other functional proteins. Tissue retention of AGEs was attributed to the higher affinity of peptide-bound AGEs for proteins (Poulsen et al., 2013). In human beings, renal absorption of AGEs was as much as 30% in healthy individuals

and as low as 5% in patients with renal compromise (Koschinsky et al., 1997). Urinary excretion was used to compare LMW pyrraline and pentosidine which, had a 50% and 60% recovery (percentage excreted of total ingested) rate respectively, while HMW pentosidine was associated with a 2% recovery (Förster, Kühne, & Henle, 2005; Poulsen et al., 2013).

2.1.3 Measurement

Quantification of AGEs in food is required to establish the amount consumed in a meal. Once this meal is ingested, AGEs can take two biological pathways through the body. The first is absorption in the small intestine into the circulation where they are distributed to organs or are excreted via the kidneys. The second route is taken by molecules that are too large for absorption which, are retained in the intestinal lumen until they reach the colon to be excreted in feces or used as substrates by colonic microbes. The effects of AGEs on the body as well as bioavailability and absorption are not fully understood. Reliable analytical methods for measurement of AGEs in food, biofluids and tissues are limited.

2.1.3.1 Measurement of AGEs in food

Dietary sources of AGEs have been estimated using one or, rarely, two markers which, is a major limitation since there is considerable variation in the content of individual AGEs in different foods. AGE compounds formed in foods are dependent on the type of food and method of heat treatment; however, only a few markers have been used for quantification. The two most commonly measured AGEs are carboxymethyllysine (CML) and methylglyoxal (MGO) derivatives (Uribarri et al., 2015). CML is formed through different pathways; thus, it is commonly used as a marker in determining exogenous levels of AGEs in food (M. U. Ahmed, Thorpe, & Baynes, 1986; Delgado-Andrade, 2016). Databases for the CML content of numerous human foods have been created and used for measurement of AGE concentrations in the body.

One such database has been created for 549 foods and can be found at http://www.adajournal.org (Uribarri et al., 2010). However, CML can be derived from lipid oxidation as well as glycation (Fu et al., 1996) resulting in an overestimation of AGEs in high fat foods (Krause, Knoll, & Henle, 2003; Uribarri et al., 2015). Furosine (ε-*N*-(furoyl-methyl)-L-lysine), formed during acid hydrolysis of the protein bound Amadori compound fructoselysine and produced by the reaction of ε-amino groups of lysine with glucose, is a specific marker of the Amadori compound generated in the early stage of the Maillard reaction and is used in AGE quantification (Erbersdobler & Somoza, 2007; Finot, Bricout, Viani, & Mauron, 1968; van Rooijen et al., 2013). Limitations to its use in estimation of glycation reactions are due to the inability to distinguish between different precursors (Henle, 2005) once it is obtained from the acid degradation process (Poulsen et al., 2013).

Methods for quantification are instrumental and immunochemical. Instrumental methods include ultra-high (UHPLC) and high-performance liquid chromatography (HPLC) with diode array detector (DAD), fluorescence detector and tandem mass spectrometry (MS/MS) and gas chromatography (GC) together with mass spectrometry. Enzyme-linked immunosorbent assay (ELISA) is the primary immunochemical method is used measure CML, MGO derivatives and some nonspecific AGEs (Poulsen et al., 2013). Advantages of ELISA for CML measurement include relatively high specificity, speed, simplicity of laboratory equipment and easy sample preparation before analysis. Demerits of this method may be the cost of the ELISA kit, decreased antibody specificity related to the kit used and difficulty of comparison with different analytical techniques because of the assigned units of measurement relative to CML-modified bovine serum albumin (BSA) and not direct evaluation of amount in the food specimen (Uribarri et al., 2015).

Despite ELISA being used to measure CML, method validation has not often been reported. The main challenge being incomplete characterization of antibody epitope recognition in quantification of the AGE content of the specimen (Kellow & Coughlan, 2015; Vlassara, Uribarri, Cai, & Striker, 2008). As a result, chromatographic methods such as gas chromatography (GC) and liquid chromatography (LC) combined with mass spectrometry methods, have been used to repeat and compare these measurements with results obtained by ELISA. Differences in results between ELISA and chromatographic methods are due to poor selectivity of ELISA in the presence of fat globules or advanced lipoxidation products found in food which may bind to the ELISA antibody (Uribarri et al., 2015).

Liquid chromatography – mass spectrometry (LC-MS/MS) may not require derivatization and has increased sensitivity when compared with UV and fluorescence. The preferred method for MS/MS detection is multiple reaction monitoring where a selected parent ion, fragments into specific daughter ions. Ideally, the mass transition and retention time should be relevant to a single compound so that quantification may be precise. Specific markers of the advanced stage of the Maillard reaction such as HMF and CML have often been analyzed by HPLC and sometimes combined with MS (Poulsen et al., 2013). While chromatographic methods require extensive sample preparation in addition to more elaborate materials and equipment, these techniques offer more accurate CML measurements.

Presently, there is not a fully validated method for determination of CML in food (Uribarri et al., 2015). CML also represents only one compound in this heterogeneous group suggesting that a method for detecting multiple AGEs is needed, which should be standardized with accompanying reference materials. Recently, LC-MS has been successfully applied to the measurement of CML, CEL and MG-H1 in common human foods resulting in the creation of

another database (Scheijen et al., 2016) but as yet, there has been no standardization of methodology. Establishing validated methods for AGE concentrations would provide accurate measurements in food as well as meaningful comparison of data.

2.1.3.2 Measurement of advanced glycation end products in pet food

Pet foods are subjected to processing that favors the Maillard reaction and formation of AGEs (Hullar, Fekete, & Szocs, 1998). Previous research has investigated the reduction in bioavailability of the essential amino acid lysine in pet foods when lysine participates in the Maillard reaction (Rutherford, Rutherford-Marwick, & Moughan, 2007; Williams, Hodgkinson, Rutherford, & Hendricks, 2006). Bioavailable lysine is defined as the reactive lysine that is digested, absorbed and utilized for metabolism. It is determined using an animal growth assay, which measures the ability of the animal to deposit protein or amino acid from a test diet or by the true ileal amino acid digestibility assay. Both methods are accurate but are costly and time-consuming (Moughan, 2003; Rutherfurd & Moughan, 2007; van Rooijen et al., 2013). In foods, the bioavailable fraction of lysine depends on the degree to which the fraction of lysine has undergone the Maillard reaction and the ileal digestibility of the reactive lysine (van Rooijen et al., 2013). Reactive lysine is undamaged lysine that has a reactive ε-amino group. There have been few studies on ileal reactive and total lysine digestibility in dogs leading to a scarcity of data related to lysine digestibility. Studies related to apparent ileal crude protein digestibility suggest that lysine digestibility is likely to be highly variable (Hendriks, van Baal, & Bosch, 2012; van Rooijen et al., 2013).

Lysine as the reactant during MR, results in formation of Amadori compounds, lysinoalanine (LAL) and fructoselysine (FL). As MR proceeds, Amadori compounds follow several pathways to yield AGEs. In a study examining different processed forms of dog and cat

foods, the AGEs FL, CML, hydroxymethylfurfural (HMF) and LAL as a crosslink were measured using UHPLC-MS (van Rooijen et al., 2014a). It was observed that there were differences in MRP content between and within types of processed foods. Based on dry matter analysis, it was found that canned pet foods contained the most FL (4534mg/kg), HMF (1417mg/kg) and CML (37mg/kg) followed in decreasing order by pelleted and extruded foods. Between the dry foods, mean contents of FL, CML and HMF were higher in the pelleted (844, 20 and 1161 mg/kg respectively) than the extruded form (646, 13 and 880 mg/kg respectively). Both wet and dry foods had similar levels of LAL. The overall conclusion was that commercial pet foods are a significant source of AGEs for dogs and cats with the awareness that processing significantly influences AGE levels. This study is the second of its kind to report MRPs in pet food. The first reported an FL content of 2840 mg/kg in one dry dog food (Chiang, 1983). There are no data to compare the other AGEs mentioned. It was also noted that in pelleting of pet foods, many of the ingredients are preprocessed indicating that pelleting may not be the source of the AGE levels determined in the study. Further work is required to determine AGE levels in the individual preprocessed ingredients to distinguish any differences (van Rooijen et al., 2014a).

Based on quantitation of AGEs in pet foods, daily intake was estimated. It was estimated that daily intake of canned and pelleted foods was higher than for extruded foods. When compared to human diets, dogs could ingest up to 122 times more HMF from an extruded food while for cats on this type of diet, ingestion of HMF is 38 times greater. Canned and pelleted diets provide higher amounts of HMF and CML than extruded diets. No data were available for LAL in human subjects so it could not be compared in this study. Since both CML and HMF can be absorbed in the small intestine and enter circulation, there is a need for further investigation

into the contribution to the AGE pool in the body as well as development of AGE-related diseases (van Rooijen et al., 2014a).

2.1.3.3 Measurement of advanced glycation end products in biological samples

In biological samples, fluorometric and spectrophotometric methods were the earliest techniques used to identify AGEs with natural fluorescence due to their crosslinked structure. These included pentosidine, pyrraline, glyoxal lysine dimer and methylglyoxal dimer among others. The simplicity of the techniques makes them applicable today but the need for increased selectivity of these approaches has attracted considerable attention. Samples used in these assays included saliva, urine and skin samples (Garay-Sevilla et al., 2005; Uribarri et al., 2015).

If LC is used with optical spectrometry for non-fluorescent AGEs such as CML, the CML must first be converted into a fluorescent species by use of derivatizing agents. The reactive dicarbonyl species may be measured as an indicator of oxidative and carbonyl stress. This proves particularly difficult because of the high reactivity and ubiquitous but low physiologic abundances of these compounds. In biological fluids, there is also risk of sample contamination by reagents, air and water of already highly chemically complex substances. More recently, MS has become increasingly useful for quantification as well as structural classification of known or new species which have enabled AGE analysis in biological samples (Uribarri et al., 2015). Data on urinary excretion of AGEs: FL, CML and LAL were obtained using ultra high-performance liquid chromatography – mass spectrometry (UHPLC-MS) in cats fed commercial moist and dry foods. Urinary excretion of FL and CML increased with intake for dry food while LAL showed no difference. Urinary recovery (percentage of AGEs excreted of total ingested) showed a negative relationship with daily intake for all three AGEs in the dry foods and for CML and LAL in the moist foods. Decreasing urinary recovery with increasing diet

suggests that digestion and absorption capability was surpassed, *in vivo* metabolism also increased or renal excretion was maximized. Despite variations concerning the type of diet or the AGE, this study gives evidence that AGEs were absorbed and subsequently excreted in urine in cats proving UHPLC-MS to be a valid analytical method for this investigation (van Rooijen et al., 2016). In human beings, plasma and urine AGE measurements were obtained and correlated with a food frequency questionnaire (FFQ) to determine the influence of dietary AGE intake on free and protein bound AGEs (Scheijen et al., 2018). In this study, both free plasma and urine AGEs correlated with diet but urine seemed to be a better indicator of AGE intake. These two studies show that in both cats and human beings, urine may be a reliable and accurate biofluid for dietary AGE studies.

2.1.4 The receptor for advanced glycation end products (RAGE)

The receptor for AGE (RAGE) is a cell surface, pattern-recognition receptor of the immunoglobulin superfamily that is expressed in a range of tissues. The term pattern-recognition is used not only because of its tendency to retain classes of molecules and individual ligands, but also because it shares ligands and signaling pathways with some members of the toll-like receptor family (TLR) thereby regulating immune and inflammatory reactions. It is considered a multi-ligand receptor with a key role in inflammatory processes (Kellow & Coughlan, 2015; Poulsen et al., 2013). During embryonic development, RAGE expression is high, while in mature tissues, levels of expression are low and widespread except for the lung, which remains persistently high for the lifespan of the organism (Sterenczak et al., 2011). Endothelial cells, smooth muscle tissue, neurons, lymphocytes, macrophages and dendritic cells express basal levels of RAGE but the expression is markedly increased when stimulated by increased presence of ligands associated with chronic disease states. Ligands known to interact with RAGE include

proinflammatory cytokine-like mediators such as calcium-binding S100 proteins (calgranulins), high-mobility group box 1 protein (HMGB1/amphoterin) a nuclear protein that is released upon cellular necrosis, phosphatidylserine, Mac-1 and β-amyloid protein (Kellow & Coughlan, 2015; Poulsen et al., 2013). Due to their interaction with these pattern-recognition receptors, RAGE ligands are classified as pathogen-associated molecular patterns (recognition of microbial products) or danger-associated molecular patterns (interaction with endogenous molecules called alarmins in the presence of tissue damage and inflammation) suggesting a role for RAGE in innate immunity (Kellow & Coughlan, 2015; Uribarri et al., 2015).

2.1.4.1 AGE-RAGE Interaction

Activation of RAGE causes oxidative stress and inflammation and regulates transcription factors and activators of transcription. Another role involves down-regulation of enzymes in the glyoxalase system thereby decreasing protection against protein glycation caused by carbonyl stress.

Generally, AGEs are ligands for RAGE but studies have reported conflicting results. Some suggestions for the discrepancy include insufficient distinction between binding and non-binding AGEs, contamination of AGE preparations by endotoxins and differences in the proportion of AGE modifications in the proteins used in the respective studies. Additionally, expression of RAGE in the gastrointestinal tract may also be low (Brett et al., 1993), making the AGE-RAGE interaction appear less significant. Molecular size of the AGEs is also thought to be a determinant of AGE-RAGE interaction where it was found that high molecular weight (HMW) CML interacts with RAGE. Studies related to dietary AGEs produced by cooking and RAGE activation have not considered intestinal degradation of HMW AGEs to LMW AGEs and their resultant absorption. A high AGE diet increases RAGE expression, which in turn increases

transcription of factors involved in oxidative stress and inflammation. While this has not been a consistent finding, the fact that RAGE expression may be increased in certain instances warrants further investigation (Poulsen et al., 2013).

AGEs are ligands for RAGE whose interaction results in increased reactive oxygen species production and establishment of a proinflammatory state (Leuner et al., 2012). The AGE-RAGE interaction converts prolonged proinflammatory signals into continual cellular damage, dysfunction and illness as illustrated in Figure 2.2 (Bengmark, 2007; Kellow & Coughlan, 2015). When AGEs engage with RAGE, NADPH oxidase activation increases intracellular reactive oxygen species (ROS) production. This leads to sustained activation of nuclear factor-κB (NF-κB), which moves from the cytoplasm to the nucleus of the cell to begin transcription of proinflammatory cytokines (interleukin 6, tumor necrosis factor-α), growth factors (vascular endothelial growth factor), adhesion molecules, chemokines, C-reactive protein and procoagulants such as thrombin. NF-κB maintains its own activation and enhances cell surface expression of RAGE to prolong and intensify the inflammatory state. The activity of NF-κB is complemented by RAGE activation of transcription factors STAT3, AP-1 and forkhead box O1 as well as various kinases all of which, ensure that AGE-RAGE signaling induces inflammation and cellular migration (Kellow & Coughlan, 2015).

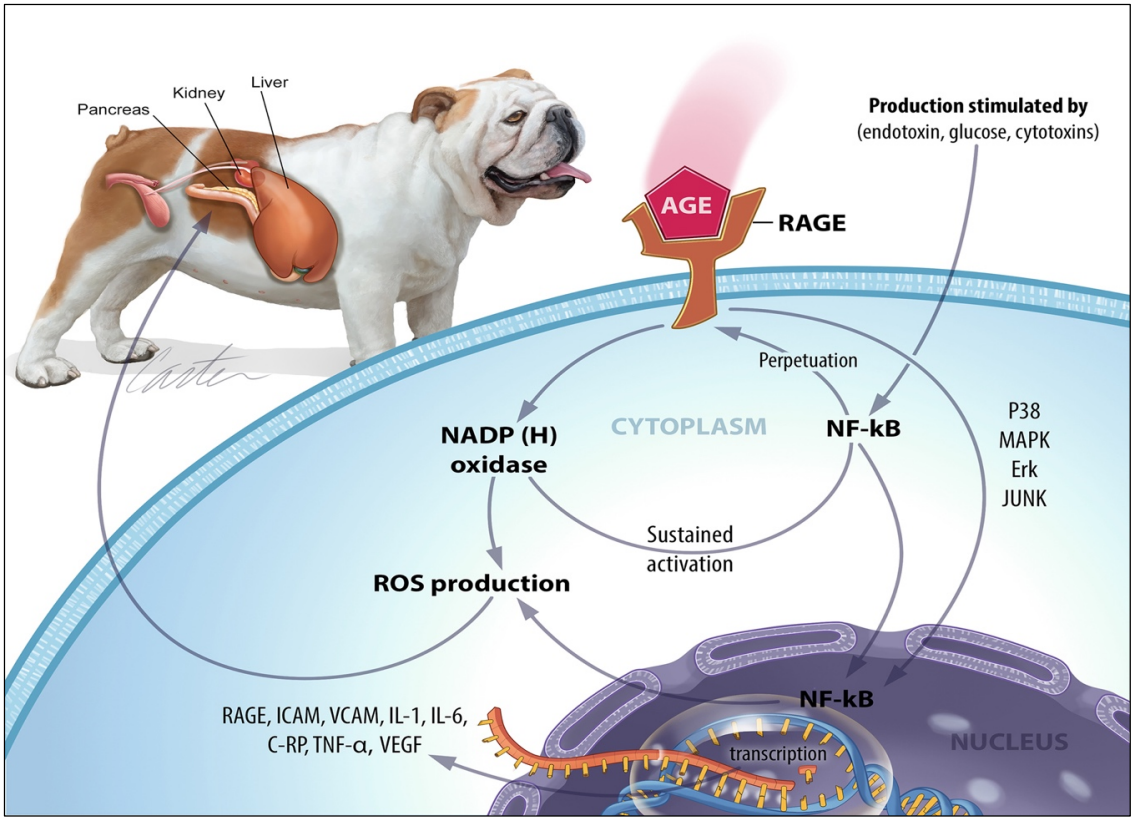

Fig. 2.2 Illustration showing the AGE-RAGE activation and cellular signaling pathways to initiate and perpetuate inflammation and oxidative stress.

Abbreviations: ROS – reactive oxygen species; NADP(H) – nicotinamide adenine dinucleotide phosphate hydrogen; RAGE – receptor for advanced glycation end products; ICAM – intercellular adhesion molecule; VCAM – vascular cell adhesion molecule; IL-1 – interleukin-1; IL-6 – interleukin-6; C-RP – C-reactive protein; TNF-α - tumor necrosis factor-α; VEGF – vascular endothelial growth factor; NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells; MAPK – mitogen-activated protein kinases; P38 – a specific subset of MAPKs; Erk – extracellular signal-regulated kinase; JUNK – Jun N-terminal kinase.

2.1.4.2 Variants of RAGE

A variant of RAGE known as soluble variant (sRAGE) has been identified, whose corresponding mRNA is a C-terminal truncated splice-variant of RAGE that does not encode the transmembrane and cytoplasmic domains leaving only the extracellular domain. This structure facilitates AGE clearance since it acts as a decoy receptor, sequestering ligands to prevent binding to cellular RAGE and propagation of signaling pathways (Heilmann et al., 2014; Poulsen et al., 2013; Uribarri et al., 2015). Upon secretion from the cell, it is referred to as endogenous secretory RAGE (esRAGE) while the native cellular RAGE can be cleaved to give soluble RAGE (sRAGE) (Hanford et al., 2004). Both isoforms are referred to as sRAGE (Hu et al., 2015) and cancel the effects of AGEs in cultured cells (Uribarri et al., 2015). A role for esRAGE has been suggested for cardiovascular disease (Falcone et al., 2013; Fujisawa et al., 2013; Lanati, Emanuele, Brondino, & Geroldi, 2010), complications of diabetes mellitus, atherosclerosis and metabolic syndrome (Katakami et al., 2009; Piarulli et al., 2013; Yang et al., 2013). While it is believed that esRAGE is involved in preventing RAGE signaling and offers protection from low-grade inflammation, the low serum levels may be more associated with its use a biomarker of risk (Kislinger et al., 1999) instead of its ability to reduce ligand concentration. Another potential function of esRAGE is preventing RAGE signaling via a feedback mechanism that blocks the harmful effects of RAGE activation. It should be noted that the endogenous sRAGE variant can act as a proinflammatory and chemotactic molecule suggesting a greater role than just a RAGE signaling decoy (Maillard-Lefebvre et al., 2009; Uribarri et al., 2015).

2.1.4.3 Measurement of sRAGE

sRAGE and esRAGE concentrations in the circulation have been determined by ELISA. If sRAGE is involved in the depression of the toxic effects of RAGE signaling then it is expected

that diets high in AGEs might alter plasma levels of sRAGE. One pilot study did not support this proposed response but should be investigated further since this study was limited to ten subjects (Uribarri et al., 2015). There is much more scope for investigation into the role of sRAGE in disease and pathophysiological processes. Soluble RAGE is recognized as a potential therapeutic target in human beings with chronic inflammatory conditions leading to work being done in dogs with inflammatory bowel disease (IBD). This study validated and used ELISA to measure sRAGE in dogs and to determine serum sRAGE and serum/fecal S100A12 levels related to the diagnosis and treatment of canine IBD. Measurement of canine sRAGE in serum via ELISA was proven to be adequately sensitive, accurate, linear and reproducible. While it is apparent that sRAGE concentrations are reduced in canine IBD patients compared to healthy dogs, as is the case with human subjects, it could not be determined from the data whether the decrease in sRAGE is related to consumption or decreased production of this variant receptor. Further work is needed to determine the role of sRAGE and RAGE in dogs for this disease (Heilmann et al., 2014).

2.1.4.4 The advanced glycation end product receptor 1 (AGER1)

AGEs are degraded enzymatically primarily by glyoxalase I and II and eliminated by the kidneys. The effects of endogenously formed AGEs are decreased by antioxidant systems or regulated by renal detoxification and urinary elimination (van Rooijen et al., 2013; Vlassara & Striker, 2011). The advanced glycation end product receptor 1 (AGER1) is an AGE receptor involved in the binding, suppression of signaling and degradation of AGEs (Cai, He, Zhu, & Vlassara, 2006; Uribarri et al., 2015).

Since AGEs interact with RAGE causing increased oxidative stress and inflammation via activation of nuclear factor κ-B, the AGE-RAGE axis has been proposed as having the following

different components: serum AGEs, sRAGE, RAGE and AGER1. In human diabetic subjects, significant associations were found between components of the axis and between sRAGE and noninvasive markers for risk of cardiovascular disease. This finding has implications for serum sRAGE as a potential surrogate marker of AGE-RAGE interaction in diabetic patients (Villegas-Rodriguez et al., 2016). Serum levels of CML were positively associated with RAGE and sRAGE but inversely associated with AGER1, while RAGE itself showed positive association with sRAGE but inverse association with AGER1 (Villegas-Rodriguez et al., 2016). The receptor expression and function of AGER1 is enhanced in mice fed low-glycoxidant diets compared with those fed a standard AGE content diet. The increase in function of this receptor may be due to negative regulation of pro-oxidant RAGE and $p66^{sch}$ proteins (an adaptor molecule associated with oxidant injury and lifespan). Low AGE diet fed mice had reduced levels of oxidative stress, less severe age-related organ changes and longer lifespan suggesting that decreased intake of AGEs contributes to preservation of innate defense mechanisms as well as organ function in mice (Cai et al., 2007). Restriction of AGE intake has also been investigated in human diabetic subjects related to insulin resistance (IR) in type 2 diabetes mellitus. These subjects showed an improvement in IR, upregulation of AGER1 to a normalized level as well as decline in RAGE and tumor necrosis factor-α (TNF-α) providing sufficient support for an AGE restricted diet as part of the medical management of patients diagnosed with type 2 diabetes mellitus (Uribarri et al., 2011).

2.1.5. Thermal Processing of Foods

Heat processing of food increases palatability, aroma and shelf-life while simultaneously reducing food-borne diseases (Poulsen et al., 2013). The final stage of MR which, occurs during heat processing of foods, produces melanoidins that gives cooked food the favorable brown

color. This coloration is due to heat pyrolysis of sugar and is not due to the amino acid component of reactants. Melanoidins are brown, nitrogen-containing HMW pigments that produce the color, desirable flavor and aroma of foods. During MR, the texture of the food can also be affected by protein crosslinking (ALjahdali & Carbonero, 2017). Factors related to heat processing that affect the rate of AGE formation include nutrient composition, temperature and duration of heat exposure, pH, humidity and presence of trace metals. The rate of the Maillard reaction doubles for every increase of 10^{o}C (Ledl & Schleicher, 1990) while alkaline pH in low moisture conditions increases the formation of MRPs (Poulsen et al., 2013). These differences in conditions suggests that different forms of heat processing can affect the AGE content of food without a concurrent change in nutrient composition. Dry heat promotes AGE formation by greater than 10 to 100-fold above the uncooked state in most foods (Uribarri et al., 2010). This is consistent with the general finding that animal food sources which, are AGE-rich because of fat and protein content, that were cooked at high temperature for an extended duration under low humidity conditions have had the highest AGE content (Uribarri et al., 2015; Uribarri et al., 2010). It follows that cooking in an oven for a longer time at low temperature in the presence of humidity and low pH, from ingredients like lemon juice, causes lower AGE levels in the cooked food (Poulsen et al., 2013).

The nutritional value of food declines in the presence of MR especially when proteins become non-digestible or not bioavailable. Lysine can be glycated becoming unavailable for assimilation while the protein efficiency ratio (PER) decreases when glycine and glucose react. Numerous elements have also been affected such as nitrogen which, is increased in the stool of young adult human subjects (Seiquer et al., 2006), iron (Garcia, Seiquer, Delgado-Andrade, Galdo, & Navarro, 2009); magnesium in rats (Delgado-Andrade, Seiquer, & Navarro, 2007), and

phosphorus in mice (Delgado-Andrade, Seiquer, Garcia, Galdo, & Navarro, 2011), have decreased bioavailability when high MRP foods are consumed (ALjahdali & Carbonero, 2017).

There has been a human dietary evolution towards increased AGE intake. After birth, infants often receive formula, a high MRP source, as the main form of nutrition either in conjunction with or as a substitution for breast-feeding. Many of the current generation of human beings either have no time to cook or are not familiar with traditional cooking techniques and since pre-cooked and preprocessed foods are readily available, then it is more often the preferred form of food. As a final consideration, adolescents often prefer fast food and snacks to their own detriment due to exposure to these dietary patterns as they mature. While these may be the most popular reasons for the switch to high MRP foods, in the current economic environment, processed and fast foods predominate dietary choices because it represents convenient and inexpensive options (Delgado-Andrade, 2014).

The "Western diet", also called the standard American diet, has been described as one that features higher intake of red meat, fast foods, fried and baked foods, high-fat dairy products, high-sugar drinks with concurrently reduced fiber and whole grains. People whose nutrition is based on this type of diet most likely also consume high concentrations of AGEs (Hull et al., 2012) as a result of the preparation methods: frying, roasting, and toasting. These methods of cooking increase the aroma, color and taste of the food but are also the reason for the high levels of MRPs in more than 200 staple items of this diet (ALjahdali & Carbonero, 2017).

2.1.5.1 Pet food processing

The first commercial dog food, a biscuit, was brought to the United States from England by its manufacturer, James Spratt, in the mid-nineteenth century. The evolution of dog food from that time seemed to follow the occurrence of the world wars leading up to its existence today. Pet

dogs were either fed scraps or meals designed by their owners while working dogs obtained food sources from their environment. In the post-First World War period, there was a glut of horse meat promoting the production of canned dog food. During the Second World War, a tin shortage made canning difficult so pelleting emerged as the new form of dog food processing with the production of dry kibble involving MR (Gentzel, 2013). The ingredients consisted primarily of rendered animal meat and bone meal representing a shift in the diet of dogs that parallels that of the human Western diet. This type of processing of these dog food ingredients, marked by similar conditions to the thermal processing of human foods, has been associated with an increase in chronic disease in both species (Egger & Dixon, 2010; Gentzel, 2013).

During pet food manufacturing, commercial pet foods undergo thermal treatments to improve safety and nutritive value. These treatments consist of pre-conditioning, extrusion cooking, retorting and pelleting which, improves the digestibility of protein and starch via denaturation and gelatinization respectively. Additional benefits of these thermal treatments include inactivation of anti-nutritional components in legumes and cereals, improvement in food safety and shelf-life as a result of elimination of bacterial cells and microbial spores. However, heat application to foods can also have deleterious effects on protein quality as a result of cross-linking, racemization, oxidation of sulfur-based amino acids and amino acids in the Maillard reaction (van Rooijen et al., 2013). Similar to cooking and heat processing of human food, MR is necessary for desirable flavor, color and aroma but results in reduction in bioavailability of essential amino acids. Approximately 62% of lysine in pet foods contain a bound ε-amino group that is available for participation in the Maillard reaction (van Rooijen et al., 2013). This form of the amino acid can be absorbed during digestion but cannot be utilized by the animal. Lysine is the first or second limiting essential amino acid in commercial pet food; therefore, a reduction in

utilization by the animal results in the food having lowered nutritive value. The significance of the nutritive value of pet food is related to commercial pet foods being fed often or for long periods of the pet's life so that if there is nutrient deficiency or imbalance, the animal may be at risk for associated diseases. Another consideration is the potential health implications due to formation and daily intake of MRP's in pet foods which, has been associated with age-related diseases in humans and dogs (Comazzi, Bertazzolo, Bonfanti, Spagnolo, & Sartorelli, 2008; Rivera-Velez, Hwang, Navas, & Villarino, 2019; Uribarri et al., 2015).

Thermal processing of pet foods and resultant dietary intake of CML, pentosidine, HMF and pyrraline are thought to contribute to the AGE pool in dogs. These AGEs are possibly partially digestible and absorbed into general circulation. In human studies comparing ingested MRPs and serum concentrations, it was found that about 10% of dietary AGEs were observed in serum (Koschinsky et al., 1997) and thus part of the AGE pool. The level of MRPs in pet foods may be derived in three ways: the pet food ingredients, the conditions of processing and lysine reactions during coating and storage of the food (van Rooijen et al., 2013).

2.1.5.2 Methods of pet food processing

Dry pet foods may be manufactured using extrusion or pelleting of ingredients. Moist foods are produced using heat sterilization and retorting. In pet foods, the extent of MR may be measured as a change in the total and reactive lysine content because of its ε-amino group. It should be noted that an underestimation of this change is possible if total lysine content decreases when MR converts lysine to AGEs. Each method of processing possesses specific conditions that determine the rate and extent of MR.

Extrusion (80 – 200^{o}C) allows the dehydration, expansion and shaping of kibble. It is a method of cooking under conditions of high temperature and short time to enhance the

digestibility of raw ingredients. The limited number of studies investigating the change in total and reactive lysine at different extrusion temperatures are not only scarce but also contradictory regarding effect of temperature on reactive lysine. Further studies are needed to determine the significance of different extrusion temperatures and the extent of MR.

Pelleting uses lower temperatures than extrusion (60 – 90^{o}C) and includes mash conditioning with subsequent drying or cooling. Even though this process employs reduced temperatures, MR occurs during pre-conditioning and possibly pelleting causing a decrease in reactive lysine. Research on pelleted piglet diets reveal increased furosine, HMF and furfural (Delgado-Andrade et al., 2010).

Retorting involves placement of food in airtight cans, containers or pouches before thermal processing. The treatment of the food is a sterilization process to preserve the high moisture food for a long duration. The moist food is heated and mixed before packaging the hot contents. This package is then retorted at a temperature-time combination determined by the need to destroy bacteria only or both bacteria and spores. Information is currently lacking on the effects of retort processing temperatures on total and reactive lysine content as few studies have addressed it.

Drying of dog foods requires high temperatures that can affect total and reactive lysine. This process makes the food less palatable necessitating the addition of palatability enhancers and digests produced using heat and enzymes that also affect lysine content while increasing flavor.

To the authors' knowledge, no studies have been performed on pet foods to determine the stability of lysine during storage conditions. One study reported doubling in the amount of furosine over 12 weeks as a result of the increase in storage temperature from 22.2^{o}C to 37.8^{o}C

(Chiang, 1983). There was also a negative correlation between furosine and lysine concentrations (Chiang, 1983; van Rooijen et al., 2013).

Work done on commercial pet foods so far has chosen lysine as an indicator of the extent of MR. The inconsistent findings of the effect of processing conditions on the difference in total and reactive lysine concentrations suggest the need for further research regarding this crucial amino acid. The evidence indicates that ingredients, pre-treatment of chosen ingredients and final conditions of processing and storage play a role in reactive lysine content, bioavailability of lysine and the extent of MR. Furthermore, the production of AGEs in pet foods via processing provides the foundation for further investigations into the AGE quantity in the processed pet foods, the bioavailability of nutrients and the overall fate of the AGEs in the canine body.

2.1.6 Advanced glycation end products and disease - mechanism of action

AGE-associated diseases develop via three mechanisms: (i) structural alteration or inter- and intramolecular crosslinking of proteins that change their molecular properties and function, or (ii) by activation of cellular signaling pathways through receptor binding, or (iii) mechanisms independent of receptor interaction, producing reactive oxygen species and an inflammatory response correlated with the AGE concentration (Delgado-Andrade, 2014; Uribarri et al., 2015).

Protein crosslinking depends on the sugar concentration and protein turnover as seen in increased endogenous AGE production occurring with diabetes mellitus. The turnover rate of proteins in the body determines the potential for tissue accumulation of AGEs such that long-lived proteins are more susceptible. Proteins with a slower turnover rate such as collagens are more sensitive to accumulation. Since the AGE-protein crosslinks are resistant to degradation, accumulation delays the turnover rate and impedes tissue repair. Collagen and low-density lipoprotein (LDL) are associated with crosslinking leading to arterial stiffness and reduction in

LDL uptake while in the kidneys crosslinking causes thickening of the basement membrane (Poulsen et al., 2013). Crosslinking associated with diabetes and age-related vascular dysfunction may also occur in elastin, tubulin, myelin and lens crystallins (Kellow & Coughlan, 2015). MGO is implicated in amyloidosis and Alzheimer's disease suggesting its potential role in pathogenic protein aggregation. This is an indication that several AGEs, possessing high protein affinity, may have the capacity to cause aggregation of specific protein structures leading to manifestation of AGE-related diseases and toxicity (Poulsen et al., 2013). In the intracellular environment, AGE-modified mitochondrial proteins alter electron transport to increase the production of reactive oxygen species (ROS) thereby compromising mitochondrial function while glycation of guanine nucleotides by reactive dicarbonyl species in high concentrations increases the frequency of mutations and reduces DNA replication (Kellow & Coughlan, 2015).

AGEs may also incite formation of ROS without receptor binding to produce oxidative stress and inflammation in tissues. Free radical formation occurs during MR via donation of electrons from the Schiff base to the dicarbonyl species and is pathogenic to cells with reduced antioxidant activity or detoxification enzymes. Since pancreatic β-cells are of this nature, high dietary AGE consumption in rodent animal models showed diminished insulin secretion and β-cell death in response to elevated ROS production (Kellow & Coughlan, 2015).

AGE receptors are expressed in several cell types as stated earlier. One of the main binding proteins is RAGE which, has been studied with respect to its CML-activation leading to a sustained inflammatory response. The other important receptor is the advanced glycation end product receptor - AGER1, thought to have a protective effect being involved in AGE degradation and removal instead of activation of inflammation.

2.1.6.1 Association of dietary advanced glycation end products with chronic diseases

The clinical significance of AGEs is tied to their association with oxidative stress and inflammation leading to chronic diseases such as obesity, cardiovascular disease, diabetes mellitus, chronic kidney disease, metabolic syndrome, and neurodegenerative diseases (Kellow & Coughlan, 2015; Uribarri et al., 2015). Chronic inflammation is characterized by persistent and uncontrolled cellular signals that cause continued cellular damage and stress. A low-grade form of this type of inflammation manifests as increased circulatory levels of inflammatory markers and activated immune cells, consistent with the aforementioned diseases. Information about the role of AGEs in human health and disease was lacking because of the assumption that they were poorly absorbed. Later, several studies provided evidence for absorption in human subjects using a single AGE-rich meal, and in rodents using labeled single protein-AGEs or specific AGE-enriched diets demonstrating that there was contribution to the AGE pool of the body (Uribarri et al., 2005). While AGE serum concentrations and the occurrence of diabetes mellitus and renal disease are thought to be positively correlated, it has been shown that high AGE concentrations are also associated with risk factors for disease in healthy human beings suggesting a role in the development of diabetes mellitus and cardiovascular conditions (Poulsen et al., 2013). Additional conditions associated with immune cell activation and low-grade chronic inflammation include asthma, allergy, nonalcoholic steatohepatitis, inflammatory bowel disease (IBD) and neoplastic diseases (Kellow & Coughlan, 2015).

High circulating levels of AGEs were first described in relation to diabetic complications and were thought to be a result of *in vivo* formation attributed to similarly high circulating blood glucose concentrations (Poulsen et al., 2013). It was later discovered that the Western diet,

consisting of highly processed foods as well as high levels of fat and sugars, led to increased exposure to AGEs formed during heat processing of foods.

In human beings, AGEs may be a component of the etiology of age-related diseases such as atherosclerosis, nephropathy, retinopathy, osteoarthritis and neurodegenerative diseases such as Alzheimer's disease (Bengmark, 2007; Uribarri et al., 2015), insulin resistance and diabetes mellitus (Uribarri et al., 2011). In dogs, age-related diseases with possible AGE involvement include diabetes mellitus, cardiovascular, renal and neurodegenerative diseases all of which, have demonstrated similarities to these medical conditions in people (van Rooijen et al., 2014a).

In rats, high AGE/high fat diets have been the most detrimental in causing insulin resistance (Uribarri et al., 2011). Dietary AGEs in rats were also associated with renal tissue degeneration, atherosclerotic plaques, age-related insulin resistance and reduced lifespan (Cai et al., 2007; Uribarri et al., 2011). One problem of using rodents as animal models for AGE research is that their normal diet does not consist of heat-treated foods like human beings so processing their diets via heat and feeding for long durations may produce other deleterious effects not associated with AGEs (Poulsen et al., 2013).

In studies involving human subjects, high AGE diets correlate with increased biomarkers for oxidative stress and inflammation. Subsequent studies confirm that a low AGE diet decreases these deleterious effects and reduces oxidative stress and inflammation (Cai et al., 2007; Uribarri et al., 2011). Low AGE diets also reduce biomarkers for oxidative stress and inflammation suggesting that lowering dietary intake of AGEs may delay onset of chronic disease and aging in human subjects (Uribarri et al., 2010). In mice, AGE restricted diets prevent vascular and kidney dysfunction and diabetes mellitus, improve insulin sensitivity, encourage wound healing and lengthen life span. Generally, reduction of dietary AGEs has led to a reduction in circulating

concentrations as well as markers of oxidative stress, inflammation, endothelial dysfunction and insulin resistance (Uribarri et al., 2015). These findings justify the need for investigation into the effect of dietary AGEs both in healthy and chronically ill dogs. The information obtained can be applied both to veterinary nutritional management and human AGE research especially if dogs are identified as being better dietary experimental models than rodents.

2.2 Metabolomics and metagenomics

Metabolomics is the systemic identification and quantitative measurement of (Idle & Gonzalez, 2007) biological small molecules (< 1500 Da) (Song, Wang, Yin, Deng, & Jiang, 2019) in cells and tissues of living organisms to detect the dynamic metabolic changes in response to genetic alterations or physiological stimulus (Xie, Zhang, Zheng, & Jia, 2013). These areas of study not only facilitate understanding of complex metabolic pathways in an organism but also may allow for diagnostic and prognostic information about gastrointestinal (GI) diseases. The metabolic fingerprint that is obtained from this information describes the host's dietary or disease status while monitoring the metabolic variations providing details of the regulatory process at the cellular level that maintains metabolic homeostasis (Xie et al., 2013).

The study of metabolomics has rapidly become the method for identifying small molecules in biological samples related to host-microbe interactions. In disease states, identification of metabolites can provide an overall functional overview of biochemical pathways involved during varied physiological and pathophysiological states (Minamoto et al., 2015). The metabolome, which describes the biochemical environment determined by the microbial metabolic activity, is just as important when considering the gastrointestinal ecosystem

(Honneffer, Steiner, Lidbury, & Suchodolski, 2017). More recently, it has been found that like the microbiome, the metabolome also varies with the segment of the gastrointestinal tract (Honneffer et al., 2017).

The genome of the microbiome adapts to its own community needs and that of the individual host whose environmental factors change in response to diet, disease and treatment. There is a constant exchange of chemical information between the microbial community and host metabolic pathways, which serves to maintain host physiological processes. This chemical communication occurs through signaling pathways via peptides and proteins, low molecular weight metabolic products as well as indirect relay from immune-mediated pathways. This exchange lays the foundation for the host-microbiome interaction in terms of microbial ecology and host metabolic regulation and control. It is believed that terminal microbial metabolites or host-microbial cometabolites are either excreted in feces or enter the systemic circulation via absorption in the gut to be excreted in urine and may be involved in propagation of this chemical information exchange between the eukaryotic cells of the mammalian host and the prokaryotic cells of the gut microbiota (Xie et al., 2013).

A study comparing the microbiome and metabolome using plasma to detect metabolites in small and large breed dogs confirmed differences despite them being in the same environment and fed the same diets. This proved variation not only among species but also within breeds of the same species, providing evidence for some of the common diseases to which some breeds and sizes of dog may be predisposed. In particular, it was observed that small dogs possessed metabolites and clinical parameters strongly associated with kidney function (Middleton et al., 2017). Metabolite fingerprinting can also be done using urine samples to detect differences within canine breeds related to dietary metabolism. Using evidence that inherited conditions

result in chronic metabolic defects and that breed-specific genetic disorders are associated with pure breeds of dog, mass spectrometry analysis revealed differences between Labrador Retrievers and Miniature Schnauzers related to dietary components (Beckmann et al., 2010). Urine is commonly studied in metabolomics since it contains numerous LMW compounds and provides information about previously unknown metabolic phenotype and pathways. This information is vital to understanding numerous aspects of physiology and nutrition as it relates to diseases (Riviera-Vélez & Villarino, 2017).

Metagenomics is the study of the collective genome of an environmental sample, which indicates the functional potential of an organism. Metabolomics complements metagenomics through the identification of the gastrointestinal ecosystem changes related to host and bacterial by-products. While there are few investigations combining both these "omics" in dogs, studies to date have revealed similarities in the functional core between humans and dogs. The functional core comprises host-microbial interactions, short-chain fatty acids (SCFAs), amino acids and vitamins (Guard & Suchodolski, 2016). Metagenomics goes beyond the study of the microbiota. It is the sequencing of the whole sample DNA whereas sequencing techniques of the 16S rRNA gene can only give information at the phylogenetic level. The first study to look at the use of metagenomics to appraise the phylogeny and functional capacity of the canine GI microbiome showed similarity between dogs with humans and mice. Functional categories were similar to other mammalian GI microbiomes demonstrating links with carbohydrates, protein including DNA and RNA metabolism, vitamin and cell wall component biosynthesis as well as virulence (Swanson et al., 2011). Whole metagenome shotgun (WMS) sequencing of DNA from the community provides short reads of the genomes that can be assembled and referenced against the microbiome and the metabolome. This assembly of sequences can be mapped to microbial genes

to give species-level identification as well as functional genes of the sample. The use of metagenomics has provided the most details regarding composition and functional capacity of the GI ecosystem although limitations are recognized. Bacterial organisms that are relatively less abundant makes genetic information less available for sequencing. Another limitation is presented when there are unmatched sequences to reference databases making assignment of function unlikely (Guard & Suchodolski, 2016).

The first metagenomics study done in dogs compared the effect of amounts of fiber on fecal microbiota of healthy dogs. Primary functional categories found included carbohydrate metabolism, protein metabolism, DNA metabolism, cofactors and vitamins, amino acid metabolism, cell wall and capsule, and virulence. Even though it was found that the pathways were not altered by high fiber diets used in the study, this work is the first holistic view of the functional capacity of the canine fecal microbiome (Guard & Suchodolski, 2016; Swanson et al., 2011).

The two aforementioned studies were able to show breed and size-specific differences in the metabolome, using plasma in the first case and urine in the second study, proving that there are multiple factors affecting each fingerprint. The information gathered by these studies can be used as the foundation for further studies related to commercial diet preparation. If it is known that certain breeds or sizes of dogs are predisposed to some metabolic condition or related disease, then dietary components can be altered to decrease the incidence of disease in the specified populations.

2.2.1 The metabolic profile

There are two approaches used in the establishment of a metabolic profile. Untargeted metabolomics uses multiple platforms for qualitative and semi-quantitative results to give an unbiased metabolic profile while targeted metabolomics compares unknown samples with pure standards for identification and quantification. Biological samples usually include serum and urine although feces can be used with some degree of difficulty due to significant amount of sample impurities and lack of gold standards for normalization of fecal metabolite concentrations (Guard & Suchodolski, 2016). A study looking at the variation of the microbiota and metabolome at four different sites in the GI tract of dogs (Honneffer et al., 2017) used fecal samples with an untargeted metabolomics approach and suggested that the host-microbiome interaction may indeed be determined by metabolites and location. It was established that while the main function of the intestine is absorption, this process is not a linear one since many nutrient substrates must be broken down into different metabolites. Each metabolite then takes its own unique path, either to react further or be absorbed, such that normal physiological processes seem to be dependent both on the metabolite and location along the tract (Honneffer et al., 2017).

Over the last twenty years, establishment of individual human susceptibility to health and disease was made possible by the systems biology approach which, provided descriptions of the host gut microbial-metabolic interactions. This approach looks at the interactions among genes, proteins, metabolites and additional cellular elements of the entire host or community of organisms to establish models for the host-microbiome relationships (Xie et al., 2013). The top-down systems biology approach to host-microbe metabolomics means that the metabolic phenotype is measured from urine, serum and whole blood, using nuclear magnetic resonance (NMR) spectroscopy. The interpretation of these data is used to establish links between the

microbes and host metabolism (Heinken & Thiele, 2015). NMR spectroscopy has been widely used in, but not limited to, other applications such as the discovery of biomarkers for disease. A few examples include Alzheimer's disease in mice models (Fukuhara et al., 2013), bladder cancer in dogs (J. Zhang et al., 2012), atherosclerosis in porcine models (Zabek et al., 2017) and in humans with IDB (Schicho et al., 2012) serving as a useful technique in early diagnosis of oxidative stress, inflammation and disease.

Generally, even though the most common analytical techniques are NMR, GC-MS or LC-MS (Emwas et al., 2019), it should be noted that no single method will derive the complete endogenous metabolic composition. Multiple complementary platforms should be used for global metabolic profiling (Xie et al., 2013). NMR has several advantages over mass spectrometry such as being nondestructive of samples; requiring little sample treatment or chemical derivatization and allowing easy quantitative analysis and identification of novel compounds. Its greatest deficiency is its lack of sensitivity being anywhere from 10 to 100 less sensitive than LC-MS or GC-MS (Emwas et al., 2019).

This microbe-metabolite dynamic in the gut enables a holistic approach to gaining knowledge of the gastrointestinal ecosystem to be able to link it to disease states. The microbiome and metabolome provide information on bacterial members and their metabolism as well as how their activities and/or numbers are related to, dependent on or affected by the host environmental factors. The host diet is a significant factor since the undigested portion becomes the microbial substrate resulting in the production of metabolites which, may be detrimental to the microbial population, the host or both. Given the evidence of microbial and metabolic changes in diseases such as obesity, diabetes mellitus, cardiovascular disease and metabolic syndrome (Martinez, Leone, & Chang, 2017) as well as inflammatory bowel disease (Guard &

Suchodolski, 2016), there is need for investigation into the changes that occur in response to diet as well as during the course of disease. Most of the information to date relates to the changes associated with the Western diet which, is believed to be high in AGEs but changes have not been specific to the effect of AGEs. Furthermore, in dogs, there have been no studies found that explore the effect of heat processed foods and AGEs on the canine microbiome and metabolome. The work done in this project attempts to uncover the relationship between these two "omics" to build comparatively on the information available for the Western diet as described for human subjects. Investigations into canine biofluid metabolomic profiles allow for identification of potential biomarkers for disease conditions associated with thermally processed foods and AGEs. Knowledge of biomarkers associated with dietary AGE intake will aid diagnosis and management of diseases in pets making invaluable contribution to veterinary nutrition and medicine.

2.3 Canine Gastrointestinal Microbiome

The mammalian gastrointestinal (GI) system comprises a complex and diverse microbiota comprising bacteria, archaea, fungi, protozoa and viruses (Suchodolski, 2011a, 2011b). The term 'microbiota' was referred to in the past as 'microflora' used to describe mucosal bacterial organisms, either without or within the GI lumen (Schmitz & Suchodolski, 2016). In any given mammalian organism, the number of GI microbes are about ten times that of host cells (Suchodolski, 2011b) and molecular studies using 16S rRNA gene analysis has identified several hundred to thousands of bacterial phylotypes in the GI system alone (Handl, Dowd, Garcia-Mazcorro, Steiner, & Suchodolski, 2011; Suchodolski, 2011a). The term 'microbiome', coined

by Joshua Lederberg, describes the ecological community of commensal, symbiotic and pathogenic microorganisms in a host organism and is recognized as a determinant of health and disease (Lederberg & McCray, 2001; Schmitz & Suchodolski, 2016). GI microbes demonstrate a symbiotic relationship with their host contributing to health or conversely playing a role in disease in the presence of imbalance (Handl et al., 2011; Hooda, Minamoto, Suchodolski, & Swanson, 2012). The establishment and continuance of the core intestinal microbiome depends on host factors such as genetics, breed and sex, as well as early environmental factors, and diet. The genome of the GI microorganisms makes up the intestinal microbiome and it is the host-microbiome interaction, which is crucial for the health status of both humans and animals (Handl et al., 2011; Minamoto et al., 2015; Schmitz & Suchodolski, 2016; Suchodolski, 2011b). Intestinal dysbiosis is the condition in which there is an alteration of the gastrointestinal microbiome usually associated with inflammatory disease (Minamoto et al., 2015).

2.3.1 Role the microbiome

The microbiome protects against invasion by pathogens by competing for space and nutrients, digesting dietary complex carbohydrates that host digestive enzymes cannot, and degrades sloughed epithelial cells and mucus creating a favorable environment for continued host epithelial cell proliferation. Microbes utilize substrates via nutrients from the host's dietary intake as well as endogenous secretions and in turn synthesizes products that can only become available to the host through bacterial metabolic activity (Honneffer et al., 2017; Minamoto et al., 2015). The GI tract contains the largest microbial load in the body with the colon as the primary site of fermentation, harboring the highest bacterial numbers most of which are anaerobes (Hooda et al., 2012). Fermentation is a key process in the GI tract that alters pH to provide a suitable environment for the microbial population, prevent diseases and maintain the

nutritional status of the host. While dogs may not depend on fermentation as an essential part of digestion, commercial dog foods affect bacterial activity, numbers and capacity to perform fermentation in response to ingredients (Hooda et al., 2012; Swanson et al., 2011). The increased microbial diversity of the colon is a result of decreased ingesta transit time allowing for longer availability of nutrients. The main functions of these microbes are to produce energy from nutrients and to prevent invasion by pathogenic bacteria through competitive inhibition. The relationship is symbiotic since the bacterial organisms metabolize complex nutrients for both host and microbial benefit. These metabolic products are modified by dietary changes in protein and fiber. Approximately 7% of the metabolic energy of dogs is produced by bacteria localized in the canine colon (Suchodolski, 2011a). Sloughed cells, mucus and undigested material as complex carbohydrates (starch and dietary fiber such as cellulose, pectin and fructans) from the small intestine are metabolized via fermentation to produce SCFAs that are used as an energy source by bacteria and promote epithelial cell growth (Suchodolski, 2011a).

Knowledge of the host microbiota is important in health and disease but should be accompanied by the functional aspects of these microbes. The metabolic products of the canine and feline microbiota include volatile fatty acids (VFA), lactate, ammonia and other end products. Gastrointestinal health relates to these microbes' capacity to ferment digested products into SCFAs of which acetate (60%), propionate (25%) and butyrate (10%) (Suchodolski, 2011a) are in the highest proportions of the VFA's and serve to maintain a low colonic pH (Gibson, McCartney, & Rastall, 2005). Beneficial effects of the SFCA's include some anti-inflammatory protection to the intestines (Correa-Oliveira, Fachi, Vieira, Sato, & Vinolo, 2016), butyrate as the main source of energy for colonocytes (Bourassa, Alim, Bultman, & Ratan, 2016) and creating a low pH environment that reduces growth of pathogenic bacteria (Herstad et al., 2017). The

products of fermentation of fiber in the colon have physiological benefits and serve to maintain the health of the gut and that of the host. Minor VFA's are branched-chain SFCA's such as 2-methylpropionate, 2-methylbutyrate and 3-methylbutyrate (Jackson & Jewell, 2019), formed by putrefaction of amino acids valine, isoleucine and leucine, respectively, when there is bacterial proteolysis in the colon instead of fermentation. Free amino acids, branched-chain SFCA's and polyamines in the feces are evidence of proteolytic break down by microbes and can be toxic and detrimental to the health of the host. Bacteria may be capable of saccharolytic fermentation or proteolytic putrefaction depending on the macronutrient composition of the diet. Fiber addition to the diet increases saccharolysis and decreases pH while reducing proteolysis suggesting that it is possible to manipulate the microbiome towards a healthy state (Jackson & Jewell, 2019). Bacterial metabolites, when beneficial to the host, demonstrate the core of symbiosis seen as the host-microbiome interaction (Honneffer et al., 2017).

2.3.2 Methods and techniques for determining the composition of the gastrointestinal microbiome

Previous methods of determining GI microbiota were restricted to cultivation but with the advent of molecular-based techniques and DNA sequencing techniques, there has been an upsurge of information concerning GI microbial identification and classification. Bacterial culture methods possess several limitations: insufficient information about optimal growth requirements for various organisms, most GI bacteria are anaerobic resulting in deleterious effects during sample handling, many bacteria are mutualistic with other host microbes so growth on culture media is not ideal, selective culture media lack sufficient specificity for the desired targets and may include other microbes, and biochemical identification tests often do not definitively classify GI microbes (Suchodolski, 2011b; Suchodolski, Camacho, & Steiner, 2008). These limitations have

prevented both complete identification and description of the GI microbiota as well as the accuracy of identification and classification of those organisms that have been successfully isolated by cultivation.

Molecular methods are considered the standard approach to determining the composition of microbial communities of the GI tract in addition to interactions between, and function of the various groups of organisms (Handl et al., 2011; Suchodolski, 2011a, 2011b; Suchodolski et al., 2008; Swanson et al., 2011). Such methods involve DNA or RNA extraction from gastrointestinal samples including feces, tissue biopsy or luminal contents. A specific gene is amplified using universal primers that target conserved regions flanking different areas within the gene so that sequencing facilitates phylogenetic identification and classification (Suchodolski, 2011b). Molecular fingerprinting, like phylogenetic identification, relies on the same concept. The universal primers generate a mixture of polymerase chain reaction (PCR) amplicons that are separated to give a characteristic fingerprint of the microbial community of the sample (Suchodolski, 2011b; Suchodolski et al., 2008). Separation and sequencing of the PCR amplicons is done by the creation of 16S rRNA gene clone libraries or automated high-throughput sequencing platforms such as 454-pyrosequencing that allows rapid analysis of several thousand sequences in a few hours (Suchodolski, 2011b). Following phylogenetic identification, quantification of bacterial groups is done using quantitative real-time PCR (qPCR) and fluorescent in-situ hybridization (FISH). The greater accuracy of FISH is attributed to this method allowing direct microscopic counting of fluorescence-labeled bacteria as well as visualization of the intracellular, adherent or invasive position of bacteria relative to epithelial cells (Suchodolski, 2011b). For bacterial and archaeal identification, 16S rRNA gene pyrosequencing is used to describe diverse microbiome in fecal samples of healthy dogs

(Beloshapka et al., 2013; Handl et al., 2011; Handl et al., 2013; Suchodolski, 2011b). High-throughput sequencing of the 16S rRNA gene is a DNA-based technique that allows for comprehensive evaluation of the GI microbiota and is superior to traditional culture methods (Beloshapka et al., 2013).

Although molecular methods surpass cultivation methods in terms of identification of organisms, several limitations still exist. Different DNA extraction methods and PCR primers will give varied results between samples and because of the wide bacterial diversity in the gut, groups of bacteria that are relatively fewer in number may not be identified by these methods. There is also bias in quantification via PCR since it depends on the various operon numbers within bacterial phylotypes (Suchodolski, 2011b). One study comparing FISH and 454-pyrosequencing found that different methods described varied abundances of predominant microorganisms in healthy dogs. The expected discrepancies between different methods further emphasize the need for use of multiple methods (Garcia-Mazcorro, Dowd, Poulsen, Steiner, & Suchodolski, 2012). As a result of these limitations, methods should be used complementarily and it should be recognized that there is no optimal method for absolute accuracy in description of all microbes of the GI tract (Suchodolski, 2011b). However, description of the normal canine GI microbiome, afforded by these methods is necessary so that a baseline is created, which allows investigation of changes related to diet, environmental factors or disease. In addition to knowing the members of the microbial community, it is also necessary to have information about the functional capacity of the GI microbiome. While the microbiota may differ between individual animals, the metabolome bears little variation. The similarity in microbial genes and metabolic pathways is often present despite differences in phylogenetics and indicates the functional stability of the gut microbiome (Suchodolski, 2011b).

DNA sequencing techniques focused on fecal samples have been applied to determine phylogenetics and functional capacity of the canine GI microbiota (Swanson et al., 2011). It should be noted that there is a difference in population between intestinal biopsy and fecal samples so that fecal samples may not provide true representation of the GI microbial population especially of the upper gastrointestinal regions (Hooda et al., 2012). In addition to differences in enteric tissue microbial community, luminal bacterial populations may also vary among each segment of the small and large intestine along the GI tract (Suchodolski et al., 2008).

2.3.3 The members of the canine microbiome

Previous work on fecal as well as luminal content samples has identified the major phyla present in the intestinal tract of dogs as *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria* and *Actinobacteria* (Handl et al., 2011; Honneffer et al., 2017; Suchodolski, 2011b; Suchodolski et al., 2008). It has been documented that *Firmicutes* was the most abundant phylum (40%) while *Bacteroidetes* and *Fusobacteria* were lower (30%) and *Proteobacteria* was lowest (1.4%) of the sequences generated from samples of canine colon luminal content (Suchodolski et al., 2008). The three most abundant bacterial phyla are *Firmicutes* (mainly *Clostridiales*), *Bacteroidetes* and *Fusobacteria* in the colon and feces of dogs (Suchodolski, 2011b). Differences between studies are noted in the relative abundances of *Firmicutes* compared with *Bacteroidetes* and *Fusobacteria* and remain as discrepancies possibly as a result of variation in methodology, which should be further investigated (Handl et al., 2011; Suchodolski, 2011b). These high-throughput methods produce results at the phylogenetic level but differences at lower taxonomic levels are superior for determining microbial interactions and presence in healthy and diseased states (Beloshapka et al., 2013).

2.3.4 Factors that influence changes in the microbiome

Molecular fingerprinting has shown that individual dogs have a stable microbiome. While higher level phylogeny may be similar, the microbiome differs significantly as the species or strain level with only a 5-20% overlap between individuals (Suchodolski, 2011b). Differences at lower taxonomic levels may be more significant with dietary changes giving rise to clinically relevant information regarding dietary influences. For example, a comparison of high animal-derived protein or carbohydrates compared with a dry commercial diet showed significant dietary effects (Hang et al., 2012) and warrants further investigation into dietary components as a factor in altering microbiota. A study comparing fecal microbiota in lean and obese pet dogs (Handl et al., 2013) showed that major differences between the two groups were not as clear as previous work in mice (Turnbaugh, Backhed, Fulton, & Gordon, 2008) and human beings (Ley, Turnbaugh, S., & Gordon, 2006; Turnbaugh et al., 2009), but it was noted that these differences may not be apparent at the phylum level of identification since significant compositional changes that affect only bacterial subgroups may go undetected. The predominant phyla identified were *Firmicutes*, *Fusobacteria* and *Actinobacteria* but changes were noted within *Actinobacteria* where the genus *Roseburia* was more abundant in obese pet dogs while at the order taxa level, *Clostridiales* was significantly more abundant in the research control dogs that were fed *ad libitum* (Handl et al., 2013). In healthy dogs, the most abundant bacterial class was *Clostridia* showing significant variation in species (Handl et al., 2011). Also in this study, gastrointestinal pathogens *Clostridium perfringens*, *Enterococcus* spp., *E. coli* and *Helicobacter* spp. were identified in these clinically healthy dogs suggesting that these pathogens are part of the normal GI microbiota and the pathophysiological processes that lead to disease manifestation require further investigation. Protein source may alter the microbial population in the GI tract since

individual bacterial populations were found to be different between beef and chicken diets (Beloshapka et al., 2013). Combinations of next-generation sequencing technology and molecular tools are recommended for further investigation into role of diet in determining the gastrointestinal microbial balance (Beloshapka et al., 2013; Hang et al., 2012). There has been limited use of high-throughput molecular techniques to determine changes for specific dietary components.

Fungal phyla described included *Ascomycota*, *Basidiomycota*, *Glomeromycota* and *Zygomycota* with 66 fungal genera (Handl et al., 2011). While the presence of other microbial organisms such as fungi, archaea and viruses have been confirmed by molecular methods, the medical significance of these microbes is unknown (Suchodolski, 2011b). Studies of this nature prove that genera and species information are of clear significance if we are to detect subtle differences in changes in the microbiome between individual dogs but also with individual dietary changes or disease processes.

In vitro experiments performed using human fecal suspensions for incubation with furosine, CML, pyrraline and maltosine revealed that colonic microbiota can derive energy by degradation of dietary glycated compounds (Hellwig et al., 2015). Of these four AGEs, furosine degradation was greatest while CML break down was variable by individual and observed to be to a lesser extent than furosine. Pyrraline degradation was noted but less than CML and maltosine was not degraded under these conditions. *In vivo* studies in human beings have investigated the effects of dietary AGEs on the fecal microbiome of male adolescents (Seiquer, Rubio, Peinado, Delgado-Andrade, & Navarro, 2014) and peritoneal dialysis patients (Yacoub et al., 2017). Further research into the effect of dietary AGEs is needed to determine alterations of the colonic microbiome. I*n vivo* animal experiments, while greater in number, have also

provided contradictory information regarding changes in the fecal microbiota in response to AGEs. High AGE levels in bread crust fed to rats (Delgado-Andrade et al., 2017; Helou et al., 2017), heat treated fish fed to mice and catfish (Z. Zhang & Li, 2018), heat treated, high fat diets fed to mice (Marungruang, Fak, & Tareke, 2016) and glycated fish protein fed to rats (Han et al., 2018) are some studies designed to investigate the effects of AGEs on the microbiome resulting in conflicting findings. Research into the effects of high AGE diets needs to continue both in human and animal subjects in the hope of obtaining consistent and clinically useful findings. Furthermore, in dogs and cats, direct effect of AGEs on the gastrointestinal microbiota has not been examined despite their diets being often comprised of various thermally processed commercial foods. A study done in adult female cats by van Rooijen et al., 2016, measured CML, fructoselysine (FL) and lysinoalanine (LAL) in urine. Urinary recovery is defined as the percentage in urine of the dietary amount and was found to have an inverse relationship with increased dietary intake suggesting that digestion, absorption, metabolism as well as urinary excretion may be limiting factors and requires further investigation. In this study, the members or role of the microbiota was not established but since MRP's may pass the small intestine and enter the large intestine to become substrates for intestinal microbes, the possibility of microbial degradation exists but was not part of that investigation (van Rooijen et al., 2016).

To date there are no studies on dietary AGE levels directly affecting GI microbiota of dogs; however, since thermally treated diets can affect the microbiome and AGE levels are associated with these diets, then there may be links worth exploring. Another consideration relates to the finding that not all AGEs are absorbed in the colon, making them a potential substrate for microbial breakdown and a potential factor in microbial alterations related to the

process of digestion in the host organism. This leads to the possibility of AGEs in commercial pet foods having a direct effect on the microbiome and is a key area for investigation.

2.3.5 Diseases associated with alterations in the microbiome

In human beings, the GI microbiome has been associated with colon cancer, IBD, obesity and diabetes mellitus all of which are also associated with increased AGE intake (Hooda et al., 2012). The establishment of the Western diet is believed to be associated with these diseases and has been linked to gut microbiota through gut microbial transplant experiments showing that microbes from lean individuals can improve insulin sensitivity in a host with metabolic syndrome (Martinez et al., 2017). Experimental evidence has shown that dysbiosis is linked to obesity and its metabolic complications but yet no solid causal relationships have been defined (Moran-Ramos, Lopez-Contreras, & Canizales-Quinteros, 2017). Based on the knowledge gained in studies in human subjects consuming Western diets and exhibiting clinical chronic diseases, it is reasonable to assume that dogs fed similarly processed foods should be comparably affected.

There are several gastrointestinal diseases of dogs that are associated with non-specific alterations in the microbiome. These include small intestinal bacterial overgrowth (SIBO) or antibiotic-responsive diarrhea, exocrine pancreatic insufficiency, and any disease associated with acute or chronic diarrhea. Studies have shown differences in the canine GI microbiome in acute versus chronic diarrhea (Schmitz & Suchodolski, 2016). Dogs with acute diarrhea had reduced numbers of normal colonic microbes (*Faecalibacterium*, *Ruminococcaceae* and *Blautia* spp.) with corresponding increases in *Lactobacillus*, *Enterococcus*, *E. coli* and *C. perfringens*. *Bacteroides* sp. was detected, using fluorescent in situ hybridization analysis, in higher abundance in patients with chronic diarrhea. Compared with healthy dogs, reduction in counts of

Fusobacteria, *Ruminococcacaea*, *Blautia* spp. and *Faecalibacterium* spp. were identified using qPCR with significant increases in abundances of *Bifidobacterium* spp., *Lactobacillus* spp. and *E. coli*.

Widespread research into GI microbial alterations has been conducted in human patients with IBD and since this disease is also present in dogs and cats, studies have also been focused on detecting alterations in canine patients. The use of pre- and probiotics to treat conditions related to intestinal dysbiosis has recently become a significant area of research but entails knowing the composition of the canine gastrointestinal microbiome in disease states in order to apply the findings (Schmitz & Suchodolski, 2016). A study done by Minamoto *et al*., 2015, evaluating the microbiome and its metabolic activities, showed that dogs with IBD were affected by oxidative stress in the GI tract due to inflammation. The subsequent alteration in the microbiome, as a result of this oxidative stress, perpetuated the disease (Minamoto et al., 2015). It has also been found that members of the *Clostridium* clusters XIVa and IV are often depleted in intestinal inflammation suggesting patterns of alteration in inflammatory conditions (Suchodolski, 2011a). These findings bear similarity to microbiome changes in human beings with IBD but are debatable, in both species, as to how these results relate to the immune reactions in the gut in this condition. More recently, it is believed that the microbial changes correspond to the metabolic changes which, aggravate the degree of inflammation in the organism (Schmitz & Suchodolski, 2016).

In mice models, obesity has been associated with significant changes in the cecal microbiome seen as a decrease in bacterial phylum *Bacteroidetes* with a distinct increase in *Firmicutes*. This corresponded to increased cecal concentrations of acetate and butyrate of the obese mice that were reversible through diet-induced weight loss (Handl et al., 2013; Ley et al.,

2005; Turnbaugh et al., 2008; Turnbaugh et al., 2006). Similar findings were noted in human beings where obese subjects possessed specific bacterial groups involved in fermentation of indigestible carbohydrates. Studies comparing the microbiome of obese with non-obese human subjects revealed characteristics associated with excess weight and metabolic syndrome manifesting as an increased *Firmicutes:Bacteroidetes* ratio (Ley et al., 2005). Some species appear to be protective against the development of obesity, such as *Bifidobacteria* and *Bacteroides* spp. while generally those of the phylum *Firmicutes* are associated with increased calorie release from colonic substrates and greater adipose tissue deposition in the host (Icaza-Chávez, 2013; Turnbaugh et al., 2006). It is suggested that the GI microbiome influences obesity via fermentation of carbohydrates (Handl et al., 2013). Several studies of the canine microbiome based on fecal samples has shown that, as in human beings, the predominant bacterial phyla are *Firmicutes* and *Bacteroidetes (Garcia-Mazcorro et al., 2012; Handl et al., 2011; Swanson et al., 2011). Fusobacteria* in dogs, whereas not abundant in human beings, may be either predominant or co-dominant with the aforementioned two phyla (Barko, McMichael, Swanson, & Williams, 2018; Hand, Wallis, Colyer, & Penn, 2013; Middelbos et al., 2010; Panasevich et al., 2015). The similarities between the microbiome between human beings and dogs suggest that changes in response to diet or as a result of diseases should be similar.

Imbalances in the microbial ecosystem are linked to diseases such as IBD, obesity and diabetes mellitus in human and animal models (Guard & Suchodolski, 2016; Handl et al., 2013). Intestinal dysbiosis, as defined earlier, is used to describe this condition where there are changes to composition or numbers of the bacterial species in the GI of an organism as a result of inflammatory disease. No direct cause and effect relationship has been clarified but it is considered to be a risk factor for development of disease in susceptible subjects (Suchodolski,

2016). It is believed that there is a correlation between the Western diet and the host-microbial interaction (Martinez et al., 2017). Studies have mainly looked at nutrient influences on gut microbiota in human beings and mice but not the specific effects of MRPs. It has been shown that germ-free mice fed high fat and sugar diets are not susceptible to diet-induced obesity (DIO) (Leone et al., 2015; Rabot et al., 2010) but transplant of microbes from mice that have been induced by Western diet or that are obese results in higher fat mass of the recipients (Turnbaugh et al., 2006; Turnbaugh et al., 2009). A similar but reverse situation occurred in human beings when microbial transplant from lean subjects to those diagnosed with metabolic syndrome caused improvement in insulin sensitivity (Vrieze et al., 2012). These studies indicate that the Western diet affects gut microbial structure and function. It follows that since the Western diet is also high in AGEs, it is possible that AGEs similarly affect GI microbes and this may even be extended further to suggest that AGEs play a role in the pathophysiological processes that lead to diseases such as obesity, metabolic syndrome, diabetes mellitus and their multi-systemic complications.

A dysbiosis index has been described such that the degree of dysbiosis is quantified using a numerical value. Negative values indicate normobiosis while increasing positive values indicate a greater degree of dysbiosis. An index value of 0, based on the mathematical model, indicates that the test sample is equidistant from both states and has 74% sensitivity and 95% specificity for identification of healthy dogs. The use of the dysbiosis index has been described as the tool with the highest discriminatory power for distinction between healthy dogs and patients with enteropathies. The dysbiosis index is a useful tool in clinical work that serves as a guide during patient treatment to return to normobiosis (AlShawaqfeh et al., 2017).

This potential link between GI microbiota, fermentation of indigestible carbohydrates, obesity and AGE intake justifies further investigation into dietary AGEs as it relates to the microbiome. There is an existing theory that in human beings, there has been an increasing loss of bacterial diversity caused by the shift to the Western diet over the last few decades. A study using a mouse model harboring human GI microbiota demonstrated that the Western diet characterized by high fats, simple sugars and low fiber caused a reversible loss of diversity after one generation which, could not be restored by alterations in diet after several generations. It is suggested that this sudden dietary change has compromised the human gut microbiome leading to additional fat storage and obesity (Sonnenburg et al., 2016). It is possible that addition of probiotics to the diet and fecal microbiota transplant (FMT) to restore the GI microbiome (Martinez et al., 2017), may decrease predisposition or even reverse obesity and associated diseases. Whether this theory also applies to dogs being fed commercial thermally processed food remains undetermined. Further investigation into the microbiome of dogs under similar conditions as those for the human subject trials would be needed to draw comparisons. If it is discovered that the canine gut microbiome responds to thermally processed diets in the same way that the human colonic microbiota responds to the Western diet, then other options for pet food processing will need to be explored to reduce the risk of obesity and its comorbidities. Raw and lightly cooked diets may be superior options but more research into differently processed diets, dietary AGE quantities and effects on the gut microbiome is necessary before alterations to commercial pet food processing can be recommended.

2.4 Purpose of study

Even though dietary AGEs were discovered more than 100 years ago, information about metabolic fate in the body remains largely unexplored. As a possible etiologic agent in chronic inflammation and diseases in human subjects, AGEs have only been studied over the past few decades. Animal models for human diseases have mostly been restricted to mice and/or rats so while much of the data relate to these species, the information cannot be applied confidently to human beings because the dietary components are vastly different between these two organisms.

Dogs offer a superior model for human disease for two reasons: firstly, many of the diseases present in human beings are also common to dogs and secondly, dogs eat commercial food processed under similar conditions to human processed foods allowing the effects of that diet to be investigated reliably. The same type of processed dog food is often fed to dogs for long periods during their lives making it simpler to establish associations with pathological processes related to diet unlike humans who may consume a wide variety of processed foods, making analysis of the data difficult. In addition to similarity in diseases and diet, the lifespan of a dog is significantly less than that of a human being making it easier to perform prospective studies with respect to development of age-related and chronic inflammatory disease.

The current study is a crossover study involving eight dogs being fed four differently processed diets. AGE levels of the ingredients, diets, blood and urine of the dogs after consumption of each diet were obtained. Data on AGE quantities of commercial pet foods as well as the fate of AGEs in the canine body are scarce. To the authors' knowledge, this is the first study to follow AGE levels from food to dog, obtaining measurements from the diet and from the subject to determine absorption and excretion.

Another aspect of this study is the consideration of sRAGE levels in the serum of these dogs. Studies described suggest that sRAGE levels decrease as AGE levels in the diet and circulation increase but data so far have been inconsistent. Data in dogs are sparse so in this work, a commercial ELISA kit will be used to measure the concentrations of sRAGE in serum samples to establish patterns related to dietary intake in dogs.

With current advances in studies of the canine microbiome, metabolomics and metagenomics in addition to the lack of existing information about the fate of HMW AGEs which are not absorbed into the circulation, this study will go one step further to include the effect of AGEs in the colon. The gastrointestinal microbiome of the dogs will be determined from fecal samples corresponding to diet and will be correlated with the analysis of the diets. These findings will be compared with NMR analysis of serum and urine metabolome to determine the fate of the microbial metabolites as well as biochemical pathways that may be altered by consuming differently processed diets.

The association of dietary AGEs and resultant RAGE activation, to produce persistent and continued oxidative stress and inflammation leading to the development of chronic disease, forms the basis of this research. This project not only establishes baseline AGE levels for the diets, influence on plasma concentration with concurrent evaluation of serum concentration of sRAGE but also affords a chance to investigate any changes in the microbiota or metabolome that could be associated with excessive dietary AGE intake-related diseases. The literature reveals a seemingly interdigitating network of all these elements that may lead to chronic disease development in several species. The aim of this work is to determine the effect of dietary AGE levels on plasma AGE concentrations, corresponding serum sRAGE concentrations while simultaneously examining the fecal microbiome and biofluid metabolome to elucidate any

changes. If changes are detected then new questions arise as to the cause and meaning of these changes, whether they are reversible and the role they play in disease predisposition or development. These discoveries set the foundation for further research in this area to improve upon the knowledge of how dietary AGEs may contribute to development of chronic inflammation and diseases.

CHAPTER 3

THE INFLUENCE OF DIETARY ADVANCED GLYCATION END PRODUCTS ON PLASMA ADVANCED GLYCATION END PRODUCTS (AGES) AND SERUM RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS (RAGE) IN DOGS[1]

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Contributors

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Abstract

Advanced glycation end products (AGEs), formed via the Maillard reaction (MR) during high-heat processing of foods, have been implicated in inflammatory and degenerative diseases in human beings. Cellular damage is primarily caused by AGE binding with the receptor for AGEs (RAGE) on cell membranes. An isoform of RAGE, soluble RAGE (sRAGE), acts as a decoy receptor binding circulating AGEs preventing cellular activation. Pet food processing methods, such as retorting and extrusion, employ high heat that may expose dogs to dietary AGEs. We hypothesized that diet and plasma AGEs as well as serum sRAGE concentrations would differ between differently processed diets of similar nutrient composition. This study examines total amount of the following AGEs: carboxymethyllysine (CML), carboxyethyllysine (CEL) and methylglyoxal hydroimidazolone-1 (MG-H1) in four differently processed diets - high heat processed: canned wet (WF) and dry kibble (DF) compared with low heat processed: air-dried (ADF) and mildly cooked/raw (RF), their influence on total plasma levels of AGEs: CML, CEL,

MG-H1, glyoxal hydroimidazolone-1 (GH-1) and argpyrimidine (AP) in addition to changes in serum sRAGE concentration. Ultra-high-performance liquid chromatography – tandem mass spectrometry (LC-MS/MS) was used to measure AGEs. sRAGE concentrations were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit. Statistically significant differences ($p<0.05$) indicated by the superscript letters were found between diets and plasma. Total dietary AGEs (mg/100kcal as fed) were highest in $WF^{b}>ADF^{a}>DF^{a}>RF^{a}$. In plasma, total AGEs (nM/50µL) were highest $WF^{a}>DF^{b}>ADF^{b,c}>RF^{b,d}$. No significant differences were found in serum sRAGE concentrations between diets. In conclusion, different methods of processing of pet foods are associated with varied quantities of AGEs influencing total plasma AGE concentrations in dogs. In this study, serum sRAGE levels, as an indicator of inflammation, did not vary but since dietary AGEs may play a pathological role in diseases, further investigation is warranted.

3.1 INTRODUCTION

The Maillard reaction was first described by the French chemist, Louis-Camille Maillard in 1912, as a series of spontaneous, non-enzymatic reactions that occur in heat-treated foods between a carbonyl group of a reducing sugar and the amino group of an amino acid to produce the dark pigmented melanoidins (ALjahdali & Carbonero, 2017). This "browning" (Maillard, 1912) reaction gives cooked food its characteristic color, aroma and flavor while increasing palatability and desirable appearance (Friedman, 1996). Melanoidins are the final products of the Maillard reaction but there are numerous compounds formed during the reaction. These compounds have beneficial effects but also have the potential to be deleterious to human and

animal health. Thermal processing of foods increases food safety by reducing bacterial numbers as well as increasing the shelf-life or storage times of the food (Poulsen et al., 2013). Thus, while heat processing conveys several positive attributes to food as a result of the Maillard reaction, it can also predispose to development of diseases (Delgado-Andrade, 2014). The reaction is divided into initial, intermediate and late stages, with the formation of several compounds at each stage, before the final production of melanoidins. One group of these compounds is known as advanced glycation end products (AGEs) which, as the name suggests, are produced in the late stage of the Maillard reaction. This group consists of a large number of heterogeneous compounds that are known to be formed not only between sugars and amino acids, proteins, lipids or nucleic acids via the Maillard reaction (Uribarri et al., 2015; Y. Zhu, Snooks, & Sang, 2018) but also by several other reactions including but not limited to oxidation of sugars, amino acids and lipids that bind to proteins through the production of reactive aldehydes (Uribarri et al., 2015). The most commonly measured AGEs in foods, biofluids and tissues are N^{ε}-carboxymethyllysine (CML), N^{ε}-carboxyethyllysine (CEL), N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine more commonly referred to as methylglyoxal hydroimidazolone-1 (MG-H1), pentosidine, glucosepane and pyrraline (Kellow & Coughlan, 2015).

In human beings, AGEs are associated with the "Western diet" and related lifestyle diseases such as obesity, type 2 diabetes mellitus, cardiovascular disease, atherosclerosis and stroke (Nowotny, Schroter, Schreiner, & Grune, 2018; Sebekova & Sebekova, 2019). The typical Western diet comprises red meat, dairy, fast and heat processed foods, and drinks high in refined sugars and fats with minimal amounts of fiber (ALjahdali & Carbonero, 2017; Bettiga et al., 2019; Delgado-Andrade, 2016). Due to the high palatability of these foods, individuals ingest more calories than needed for maintenance energy expenditure leading to diseases such as

obesity and diabetes mellitus. The foods included in the Western diet are thermally processed, relatively low cost, convenient to serve, and readily available to the consumer (Delgado-Andrade, 2014). The implication is that high AGE quantities exist in these commonly consumed food items suggesting a potential role in the pathogenesis of these diseases (Chaudhuri et al., 2018).

The endogenous production of AGEs occurs as a part of normal physiological processes in the body. First recognized in type 2 diabetes mellitus due to hyperglycemia, a glucose-derived hemoglobin $A1_c$ ($HbA1_c$) adduct was observed as the formation of fructosyllysine in hemoglobin, and use of this marker has significantly improved the diagnosis and monitoring of diabetic patients. The glycation of proteins can proceed via the Maillard reaction, independently of hyperglycemia (Poulsen et al., 2013), in all fluids and tissues containing glucose, fructose or dicarbonyls (Sell, Lapolla, Odetti, Fogarty, & Monnier, 1992). Methylglyoxal (MGO), glyoxal (GO) and 3-deoxyglucosone (3DG) are highly reactive α-dicarbonyls, produced as by-products of anaerobic glycolysis and less so by lipid peroxidation. They readily react with proteins, lipids and nucleic acids to form endogenous AGEs. MGO is significantly more reactive than glucose and is largely responsible for intracellular glycation to form AGEs related to a range of inflammatory and degenerative diseases (Chaudhuri et al., 2018). Exogenous sources in food contribute to the circulating AGE pool thereby increasing the potential for a state of inflammation and oxidative stress. These two sources are indistinguishable from each other in structure and function, and gradually accumulate in tissues or circulate in the body exerting their effects (Uribarri et al., 2015).

There are three broad categories of pathophysiological effects of high amounts of circulating AGEs whether endogenous, exogenous or as additive effects of both sources (Kellow

& Coughlan, 2015): 1) AGE modification of proteins leading to structural and functional changes most importantly seen as protein crosslinking causing vascular dysfunction related to aging and diabetes mellitus; 2) the formation of reactive oxygen species (ROS) via AGE catalytic activity at sites of their accumulation and, 3) binding and activation of a range of receptors, especially the receptor for advanced glycation end products (RAGE), that initiates a cascade of inflammatory reactions and release of proinflammatory mediators which, sustain inflammation and oxidative stress.

The most significant mechanism by which AGE-induced inflammation is initiated and sustained is via AGE-RAGE binding (Ramasamy et al., 2012). The receptor is a member of the immunoglobulin G (IgG) superfamily (Gupta et al., 2018) and is a multi-ligand cell surface pattern recognition receptor (PRR) with toll-like receptor (TLR) activity (Kellow & Coughlan, 2015). In addition to AGEs, its ligands include amyloid-β-protein, phosphatidylserine, S-100/calgranulins, high mobility group box 1 (HMGB1/amphoterin) and macrophage antigen-1 (integerin Mac-1) (Kellow & Coughlan, 2015; Ramasamy et al., 2012). The structure of RAGE consists of one "V"-type region responsible for ligand binding and two "C"-type domains, a short transmembranous portion and a cytoplasmic tail that is essential for intracellular signaling (Bierhaus et al., 2005). Two functionally equivalent isoforms of RAGE have been identified as decoy receptors that attenuate the effect of cellular AGE-RAGE interaction by binding to the AGEs in circulation. Two mechanisms have been proposed for the formation of these alternate forms of the receptor. Cleavage of RAGE at the "V" domain can be executed by matrix metalloproteinase-9 (MMP9) and sheddase A Disintegrin and Metalloprotease-10 (ADAM10) to produce a soluble form called cleaved RAGE (cRAGE) (Maillard-Lefebvre et al., 2009; L. Zhang et al., 2008). The other known mechanism is alternative splicing of pre-mRNA during

transcription to produce a truncated form with an absent transmembranous region and cytoplasmic tail known as endogenous secretory RAGE (esRAGE). Both of these forms are known as soluble RAGE (sRAGE) and the terms are often used interchangeably in the literature (Ciccocioppo et al., 2015; Maillard-Lefebvre et al., 2009). Investigations in human beings, dogs and rodents have evaluated the concentration of sRAGE in blood of healthy controls as well as in diseased individuals. Studies have shown decreased concentrations of this form of the receptor in chronic inflammatory conditions (Bierhaus et al., 2005) such as inflammatory bowel disease (Heilmann et al., 2014; Meijer et al., 2014), ulcerative colitis and Crohn's Disease (Ciccocioppo et al., 2015). Research related to cardiovascular disease has produced inconsistent results showing both positive and negative correlations of sRAGE. It still needs to be ascertained whether high dietary AGE intake induces changes in blood sRAGE concentration to clarify their role as decoy receptors mounting defense against RAGE cellular signaling (Uribarri et al., 2015).

Commercial pet food processing is similar to the Western diet in terms of the use of high heat preparation. While human foods are prepared using methods such as grilling, roasting and deep frying (ALjahdali & Carbonero, 2017), pet food companies use processes such as extrusion, retorting and pelleting to create their final product. These methods involve the use of high heat with varying amounts of moisture to give pet food its characteristic shape, color, texture and appearance (van Rooijen et al., 2013). The Maillard reaction in the pet foods ensues, not only allowing for the formation of AGEs but also for a reduction in the bioavailability of amino acids, such as lysine, that are utilized as reactants (van Rooijen et al., 2013). Studies have shown that there is a significant reduction or alteration of nutrients in food that undergoes thermal processing for animals and human beings (Anese, Nicoli, et al., 1999; Hendricks, Moughan, Boer, & van der Poel, 1994; Moughan & Rutherford, 2008; Rutherford et al., 2007).

Increased public awareness about the harmful effects of highly processed foods has influenced pet owners' response to pet food products subjected to similar conditions (Algya et al., 2018). There has been a movement toward air-dried or freeze-dried meat as well as raw meat (Algya et al., 2018; J. Kim, An, Kim, Lee, & Cho, 2017) whose characteristics include decreased heat processing, more natural appearance and decreased artificial preservatives or additives (Nieto-Lozano, Reguera-Useros, del C. Peláez-Martinez, & Hardisson de la Torre, 2006). Air-dried or freeze-dried foods depend on dehydration by room temperature air currents or sublimation, respectively, thereby removing water from the food that would otherwise promote bacterial growth (Schafer, 2018). Raw diets consist of meat and selected bones alone or in combination with vegetables, starches and fiber (Fredriksson-Ahomaa et al., 2017; Strohmeyer et al., 2006). They have the appeal of being similar to the natural canine diet with no processing therefore perceived to be superior. The main disadvantage is the possibility of increased bacterial content or bacterial proliferation during storage (Fredriksson-Ahomaa et al., 2017). One option to reduce this risk is to lightly cook the food such as boiling or heating at low temperature for a short period (J. Kim et al., 2017). Another option eliminates heat by use of the bacterial fermentation product, bacteriocin, formed by the species *Pediococcus acidilactici,* and mixed with the emulsified raw food as a natural preservative (Nieto-Lozano et al., 2006). As investigation into AGEs linked to the potential negative consequences of thermal processing of commercial pet foods develops, owners may want to avoid the possible risks associated with these high heat methods by seeking alternative food preparation options (van Rooijen et al., 2014a). As pet owner awareness increases, the commercial pet food industry may reconsider their approach to pet food processing in order to meet consumer demands, and create additional dietary options for pets.

There is limited information on absorption, digestion, metabolism and absorption of AGEs. Initially, it was thought that dietary sources of AGEs were not absorbed so their contribution to the AGE pool was largely ignored. More recent studies have examined serum or plasma concentrations as well as urinary excretion of AGEs as a means of determining any correlation with dietary intake of AGEs. A comparison of dietary intake of pyrraline and pentosidine showed that AGEs differ in their behavior (Nowotny et al., 2018). Dietary pyrraline is almost completely excreted in urine (Föerster & Henle, 2003) whereas renal excretion of pentosidine is affected by its source (Förster et al., 2005). This was attributed to whether pentosidine was in the free state or bound to proteins in the foods. This is also true for AGEs CML, CEL and MG-H1. When the free forms of these AGEs were measured in plasma and urine using ultra-high-performance liquid chromatography – tandem mass spectrometry (UPLC-MS/MS), there were significantly higher concentrations associated with increased intake. Conversely, when the protein bound AGEs were evaluated in the diet compared with the urine and plasma, no differences were observed (Scheijen et al., 2018). Once absorbed and not excreted in urine, dietary AGEs are known to accumulate in blood and tissues possibly contributing to inflammation and oxidative stress (Poulsen et al., 2013). This is noteworthy especially in renal failure patients with diminished capability to remove toxins from the blood resulting in accumulation of glycotoxins and other harmful metabolites in the body (Rabbani & Thornalley, 2018).

The fate of dietary protein bound AGEs seems to be restricted to the digestive tract either to be excreted in feces or be degraded by gut microbial organisms (ALjahdali & Carbonero, 2017; Kellow & Coughlan, 2015). In addition to host mechanisms of digestion and absorption of AGEs, colonic microbiota also can degrade glycated compounds (Hellwig et al., 2015) using

them as substrates for metabolic processes (Helou et al., 2014) and releasing products that may either be beneficial or detrimental to the host. The use of AGE substrates by the colonic microbiome still needs to be explored. Much of the work in progress concerns changes in microbiota associated with the development of obesity and insulin resistance in human beings consuming Western-style diets (Mills, Stanton, Lane, Smith, & Ross, 2019).

This study aims to determine the AGE concentrations in plasma after feeding four commercial dog foods for which CML, CEL and MG-H1 quantities are known. The four diets have similar macronutrient composition but differ by processing method. The diets are canned wet food (WF) and extruded kibble (DF) that were high heat processed as well as an air-dried (ADF) and a mildly cooked/raw diet (RF) that were low heat processed and contained a *Pediococcus acidilactici* fermentation product as a natural bactericidal preservative. Serum concentrations of sRAGE using a commercial ELISA kit for canine RAGE were also determined after feeding each diet. Our hypothesis states that diets high in AGEs will correlate with higher plasma AGE concentrations in the dogs while serum sRAGE concentrations will have an inverse relationship with dietary AGE quantity.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Eight purpose-bred laboratory colony Beagle dogs[a], 4 males and 4 females, reproductively intact, ranging from 3-7 years old were used. Their health status was confirmed through physical examination, complete blood cell counts, serum biochemical analyses and urinalyses (Appendix A-D). Dogs were housed individually in cages under a 12-hour light-dark cycle with temperature

range maintained within 50^{o} to 85^{o}F. Cages and bowls were cleaned and sanitized daily. Fresh tap water fit for human consumption was available *ad libitum* for the duration of the study period.

3.2.2 Study foods

Four diets of similar nutrient composition consisting of kangaroo meat and sweet potato that were differently processed were evaluated: 1 – a high heat, retorted canned wet food (WF); 2 – a high heat, extruded dry kibble (DF); 3 – a low heat, air-dried food (ADF); and 4 – a low heat, mildly cooked/raw meat diet (RF). A *Pediococcus acidilactici* fermentation product was added to both low heat processed diets as a biological preservative with bactericidal activity. Nutritional composition of each diet in the study is seen in Table 3.1. These diets met the requirements for complete and nutritionally balanced dog foods as established by the Association of American Feed Control Officials (AAFCO). Further details about ingredients and nutrient analyses are provided in Appendix E. Additional processing and the feeding regimen are provided in Appendix F.

In this experiment, high heat processing of diets was performed at temperatures greater than 200^{o}F while low heat methods employed temperatures under 150^{o}F as seen in Table 3.2.

Table 3.1. Macronutrient composition of the four differently processed diets based on 100% dry matter (DM).

Macronutrient Composition	**DIET (100% DM amount)**			
	Canned Wet	**Dry Kibble**	**Air-dried**	**Mildly Cooked/Raw**
Protein	39.48	33.94	45.77	45.66
Carbohydrates	45.82	49.32	34.65	34.62
Fat	8.61	8.40	10.97	10.96
Crude Fiber	1.11	2.25	1.65	1.65
Ash	5.82	6.09	8.27	8.42
ME (kcal/kg)	3717.29	3,628.08	3746.83	3741.33

Abbreviations – ME – metabolizable energy; DM – dry matter

Table 3.2 Method of processing and corresponding maximum temperatures employed during manufacture of the four diets.

Diet	Process	Maximum temperature (^{o}F)	Comments
Canned Wet (WF)	Retorting	254	Exposed to high temperatures for 60 – 90 minutes
Dry Kibble (DF)	Extrusion	265	After extrusion, food is dried to <10% moisture
Air-dried (ADF)	Dehydration	140	Dried for 12 hours until <12% moisture
Mildly Cooked/Raw (RF)	Mild and slow cooking	105	Cooked for 10 hours then frozen

3.2.3 Study Design

A Latin-square design (Appendix G) allowed for minimization of differences enabling comparison among diets consumed by the same dog. Four diet sequences were created for feeding to avoid differences because of the order of consumption of the diets. Pairs of dogs were assigned to one of four diet group sequences based on a table of random numbers. Each pair received the first diet in their sequence for four weeks, samples collected then dogs were crossed over to the next diet until all dogs completed each diet in the predetermined sequence. Samples were collected before beginning the sequence of feeding to obtain a record of "baseline" parameter values of these healthy dogs. Feeding quantities were based on daily caloric requirement determined by body weight that was measured weekly and the feeding amount adjusted to maintain body weight within 5% of baseline (see Appendix H for body weights). The

canned wet and mildly cooked/raw diets were offered twice daily for a minimum of one hour. The dry kibble and air-dried diets were offered once daily for a minimum of one hour.

3.2.4 Sample collection

The sample collection schedule for this experiment is presented in Appendix I.

Food Samples

A single batch of food of each type was manufactured for the entire period of the study. A sample of each of the diets was collected at the beginning of each four-week feeding period, stored at -80^{o}C, shipped overnight on dry ice then stored at -80^{o}C until analysis. For analysis of protein bound AGEs in the food samples, composite samples were made for each of the diets for a total of four samples of each of the diets – WF, DF, ADF and RF.

Plasma and serum samples

Blood was collected prior to the start of feeding (baseline) and once weekly at the end of the seven-day period prior to the morning feeding via jugular venepuncture in sterile syringes. Samples were split into red top serum separator tubes and green top heparinized tubes (3mls each). Each sample was centrifuged at 4^{o}C for 15 minutes at 3000RPM after clotting (red) and mixing (green) so that serum and plasma could be obtained via pipette and stored in cryovials identified by dog identification number (ID), diet, date and specimen type. All samples were stored at -70^{o}C, shipped on dry ice, stored at -80^{o}C at the laboratory until analysis. Plasma AGEs were measured using liquid-chromatography mass spectrometry and serum samples were used for quantitative analysis of the canine sRAGE using a commercial ELISA kit.

3.2.5 Analysis of AGEs in food and plasma

3.2.5.1 Food samples – CML, CEL and MG-H1 assay

Materials

Boric acid, sodium borohydride, trifluoroacetic acid (TFA), 1-butanol, methanol, sodium hydroxide, hydrochloric acid (HCl), water and acetonitrile were obtained from Sigma Aldrich. N^{ε}-(1-carboxymethyl)-L-lysine (CML) and N^{ε}-(1-carboxyethyl)-L-lysine (CEL) were obtained from Cayman Chemicals. All other reagents and solvents were of analytical or ultra-high-performance liquid chromatography – mass spectrometry (UPLC/MS) grade.

Sample preparation

The sample preparation method was adapted and modified to suit the dog food samples from work done on human foods investigating protein-bound AGEs (Scheijen et al., 2016). Approximately 6g of each food sample was soaked in about 40mls of water for 72 hours at 4^{o}C to aid in homogenization. These samples were then homogenized using a commercial food processor and lyophilized to dryness. For analysis, ≈ 50mg of each dried food sample for each of the four-week feeding periods was used. All diets were <20% fat eliminating the need for a defatting step. All samples were mixed and deproteinized with 1000µL cold (4^{o}C) TFA, centrifuged (4300g, 4^{o}C, 20 minutes) and the supernatant carefully removed with a Pasteur pipette. Subsequent hydrolysis was performed by addition of 1000µL 6N HCl and incubation at 110^{o}C for 24 hours. After hydrolysis, 80µL of hydrolysate was evaporated to dryness under nitrogen gas at 70^{o}C. After drying, samples were derivatized with 100µL 1-butanol:HCl (3:1 v/v) for 90 minutes at 70^{o}C. Samples were then evaporated to dryness under nitrogen, redissolved in

300μL water, vortexed and centrifuged at 15000g for 20 minutes. For UPLC-MS analysis, 100μL of sample was transferred to a sample vial for analysis.

Instrumentation

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed on an Orbitrap Elite Mass Spectrometer (Thermo Fisher Scientific, Massachusetts, USA) coupled to an RSLC liquid chromatography system (Thermo Fisher Scientific, Massachusetts, USA), and equipped with an electrospray ion source. Prepared samples were injected (volume 20μL) onto the separation column (Waters Acquity UPLC BEH C18 – 2.1x150mm with 1.7μm particle size). The separation was performed under a linear gradient from 5% to 100% B (solvent A – 100% water with 0.1% formic acid; solvent B – 80% acetonitrile with 0.1% formic acid) at a flow rate of 0.3μL/min. Electrospray was carried out in positive ion mode, and programs monitoring the expected ion masses (SIM) as well as fragmentation of those masses (SRM – CID) were carried out using the Thermo Fisher Excalibur software. The chromatograms for the CML internal standard (Fig. 3.1) and CEL (not shown) were used to construct a calibration curve, as seen in Fig. 3.2, ranging from 1 to 100μM concentrations of CML and CEL to calculate the quantity of all three AGEs in the diets: absolute CML and CEL concentrations and relative concentrations of MG-H1 to the CML internal standard, and to confirm identification by retention time.

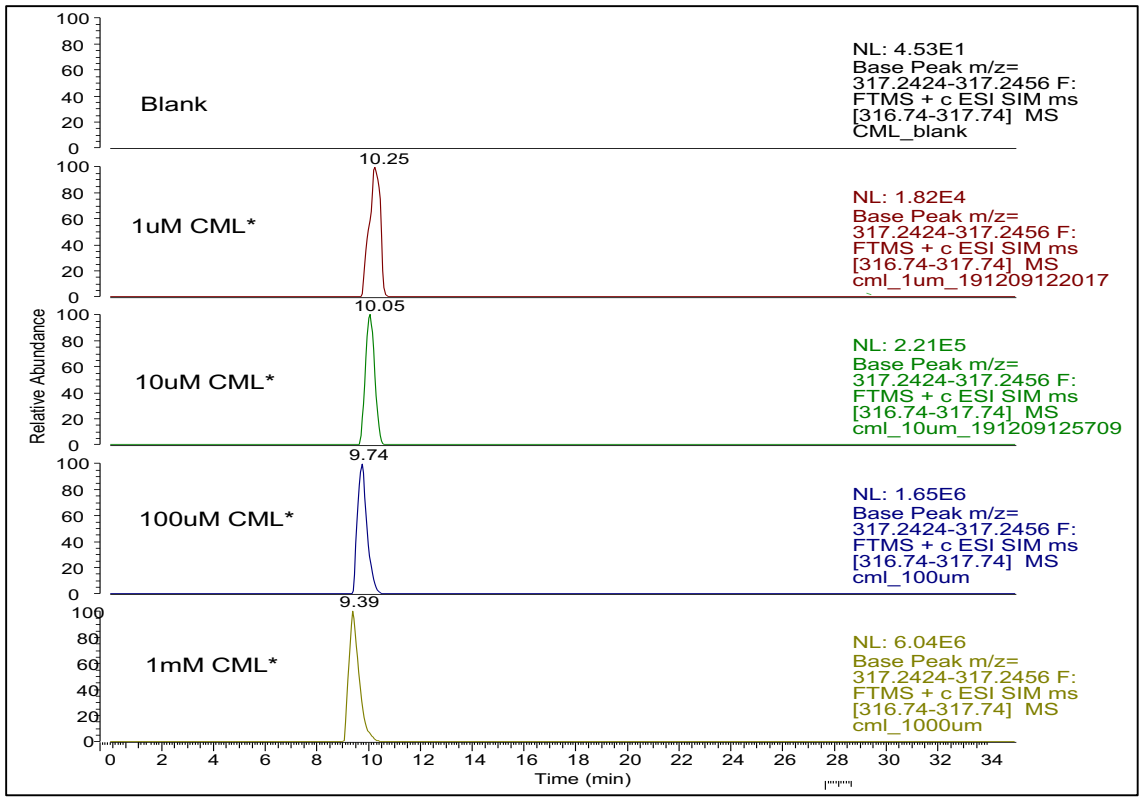

Figure 3.1. Chromatogram of carboxymethyllysine (CML) internal standard used to construct the calibration curve for calculation of amount of CML in diets. * indicates butanol/hydrochloric acid derivatized CML.

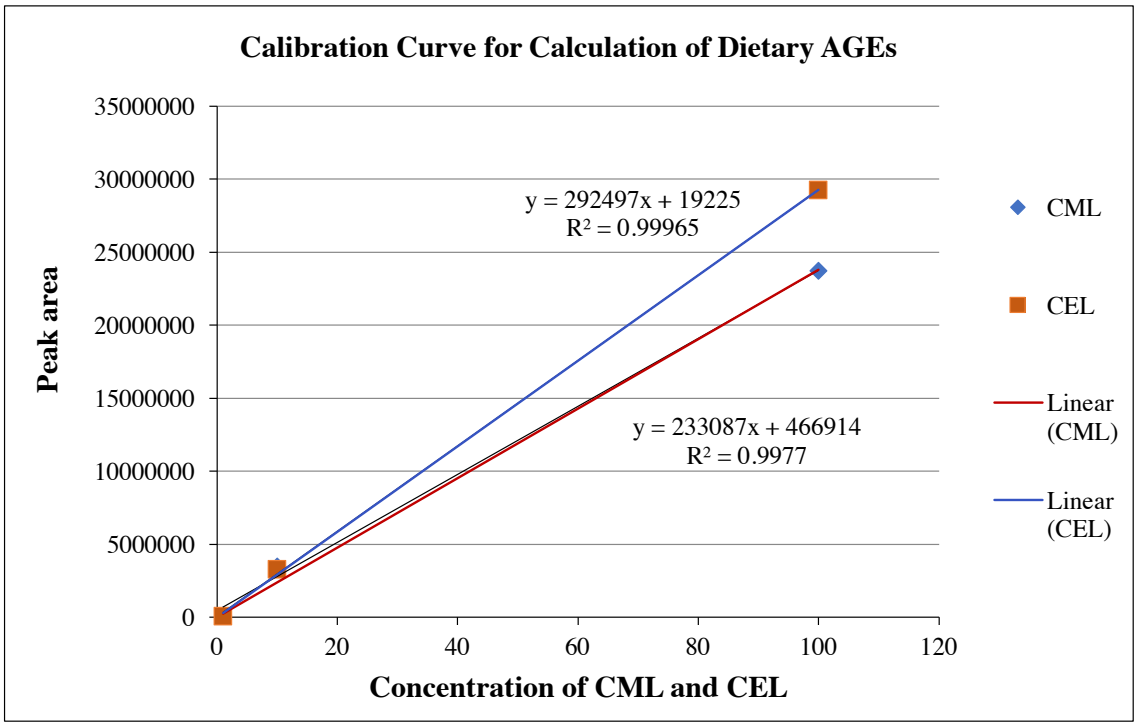

Figure 3.2 Calibration curve for N^{ε}-(carboxymethyl)lysine (CML) and N^{ε}-(carboxyethyl)lysine (CEL) internal standards used for calculation of absolute concentrations of CML and CEL and relative concentrations of methylgloxal hydroimidazolone-1 (MG-H1) in diet samples.

3.2.5.2 Plasma samples – CML, CEL, MG-H1, glyoxal hydroimidazolone-1 (GH-1) and argpyrimidine (AP) assay

Materials

The internal standard (IS) used was N^{ε}-(1-carboxymethyl)-L-lysine-d3 (CML-d3) and was purchased from Cayman Chemical. A solution of picrylsulfonic acid (5% w/v) was purchased from Sigma Aldrich. Distilled water, methanol (MeOH) and formic acid (FA) were all of LC-MS grade. All other chemicals were of laboratory analytical reagent grade.

Sample preparation

A volume of 50µL of plasma was pipetted into a 10KDa MWCO centrifugal filter (Pall Life Sciences, California, USA) followed by the addition of a 150µL of 10nM IS and 1.0% sodium chloride (NaCl) solution containing 0.1% FA. Centrifugal filtration was done at 14000g at 4^{o}C for 30 minutes, and the flow through was evaporated to dryness. Once the sample was dried, 2,4,6-trinitrobenzene sulfonate (TNBS) derivatization was performed by addition of a 0.1M 50µL sodium borate and 50µL of a 150mM TNBS solution (pH 8.5) at 30^{o}C for 30 minutes. Following the derivatization step, 100µL of 0.2% FA was added to the solution to stop the reaction, and 100µL was placed into sample vials for injection. This method was applied successfully to determine free AGEs in rat plasma (Hashimoto et al., 2013) and, to the authors' knowledge, is the first time it was applied to determine free AGE concentrations in canine plasma.

Instrumentation

LC-MS/MS was performed on an Orbitrap QExactive Mass Spectrometer (Thermo Fisher Scientific, Massachusetts, USA) coupled to a Vanquish UPLC (Thermo Fisher Scientific, Massachusetts, USA), and equipped with an electrospray ion source. Prepared samples were injected (volume 20μL) to the separation column (Agilent Zorbax Eclipse XDB-18 – 2.1x150 mm, 1.8um). The separation was performed under a linear gradient from 0% to 100% B (solvent A – 100% water with 0.1% formic acid; solvent B – 100% MeOH with 0.1% formic acid) at a flow rate of 200μL/min. Electrospray was carried out in positive ion mode and programs monitoring the expected ion masses (SIM) as well as fragmentation of those masses (PRM – HCD) were carried out using the Thermo Fisher Excalibur software. The chromatogram for one

sample in Fig. 3.3 shows the peaks for all five AGEs and the CML internal standard used to calculate concentrations of AGEs in the sample vials as a peak area ratio of the unlabeled peak to the CML internal standard peak area.

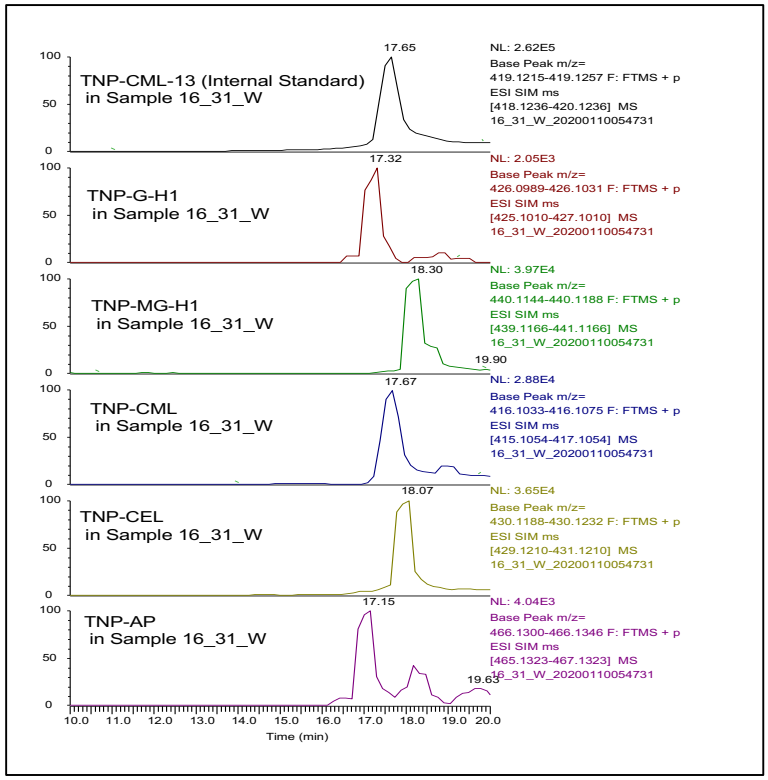

Figure 3.3 Chromatogram showing the derivatized trinitrophenyl-carboxymethyllysine (TNP-CML) internal standard (top) and all five TNP-AGEs in the sample: GH-1, MG-H1, CML, CEL and AP. The concentrations of AGEs in the sample were calculated as a ratio of the unlabeled peak area to the CML internal standard peak area.

Abbreviations: TNP-AGEs – trinitrophenyl-advanced glycation end products; GH-1 – glyoxal hydroimidazolone-1; MG-H1 – methylglyoxal hydroimidazolone-1; CML – N^{ε}-(carboxymethyl)lysine; CEL – N^{ε}-(carboxyethyl)lysine; AP - argpyrimidine

3.2.6 Analysis of canine receptor for advanced glycation end products (RAGE) in serum

Serum samples were used to measure soluble RAGE (sRAGE) using the commercial RayBiotech Inc.® Canine RAGE ELISA kit (Georgia, USA). Samples were analyzed according to the manufacturer's instructions and plates were read using a BioTek Synergy™ HT microplate reader with Gen5™ Microplate Reader and Imager software (Vermont, USA). Standard curves were constructed and used to calculate sRAGE concentrations based on optical density at 450nm.

3.2.7 Statistical Analyses

Data were analyzed using Analyse-It (v5.11.1) and IBM SPSS® (Statistical Package for the Social Sciences, version 23) software platforms. The Shapiro-Wilk test revealed that data were not normally distributed so statistical analyses were performed using non-parametric tests. AGE concentrations in the diets were tested for significant differences using the Kruskal-Wallis test. Global differences in plasma AGEs and sRAGE concentrations across all diets were determined using the Friedman's test for repeated measures. Statistically significant differences were determined using Wilcoxon-Mann-Whitney test for individual comparisons. Differences were considered to be statistically significant at $p < 0.05$.

3.3 RESULTS

3.3.1 General characteristics of the laboratory colony study population.

Clinical parameters were obtained and recorded for each dog during this study (Appendices A-D). Body weights were recorded each week for each diet and the % change determined at the end of the four-week feeding (Appendix H). Daily food consumption for each diet was recorded and averages were tallied per week and over four weeks (Appendix J). Blood and urine samples were collected weekly for hematology, serum chemistry and urinalysis. All parameters were within normal limits for the duration of the study. Statistical analyses revealed significant differences for a few parameters between diets but since all values were within normal reference ranges, there was no clinical significance of these findings (Appendix K).

3.3.2 Advanced glycation end products in the commercial diets.

Four replicates of each diet were analyzed for the AGEs: CML, CEL and MG-H1 [mg/100kcal AF (as-fed)] corresponding to each four-week feeding period of the crossover design (Appendix G). A Kruskal-Wallis H test showed that there was no statistically significant difference in the effect of time $\chi^2(3) = 5.01, p = 0.1713$, on the AGE quantities in the food samples over the 16 weeks of feeding. This finding suggests that storage conditions did not significantly alter the AGE quantity in diets and that the sequence of feeding the four diets did not influence the results.

The canned wet diet contained the highest amount of CML followed by the air-dried food then dry kibble. The diet with the lowest CML was the mildly cooked/raw meat diet (Table 3.3). Significant differences for CML quantity between diets were observed between all diets except the canned wet and air-dried diets (Table 3.4a).

In decreasing order, CEL median value was highest in the canned diet followed by the mildly cooked/raw, dry kibble and lowest in the air-dried diet (Table 3.3) with the canned wet being significantly different from all other diets (Table 3.4a).

Relative quantities were obtained for MG-H1 based on CML concentrations (IS) (Table 3.3). The highest median value of MG-H1 was seen in the mildly cooked/raw diet then in decreasing order, the dry kibble, the canned wet and lowest in the air-dried diet. No significant differences were observed between diets (Table 3.4a).

Table 3.3 Average amount (mg/100kcal) as fed of individual and total dietary advanced glycation end products (AGEs) in the four differently processed diets: canned wet, dry kibble, air-dried and mildly cooked/raw. Values are medians with interquartile ranges. Statistically significant differences indicated by superscript letters – a, b and c as determined by Kruskal-Wallis tests and Wilcoxon Mann Whitney post-hoc individual comparisons.

Advanced glycation end product (AGEs)	Diet			
	Canned Wet	Dry Kibble	Air-Dried	Mildly Cooked/ Raw
CML	0.97 (0.78 - 1.14)^{b}	0.50 (0.40 - 0.58)^{a}	0.70 (0.64 - 0.94)^{b}	0.21 (0.18 - 0.26)^{c}
CEL	1.18 (0.92 - 1.35)^{b}	0.32 (0.24 - 0.36)^{a}	0.23 (0.16 - 0.28)^{a}	0.35 (0.21 - 0.44)^{a}
MGH1	0.40 (0.26 - 0.86)^{a}	0.45 (0.32 - 0.47)^{a}	0.36 (0.19 - 0.52)^{a}	0.68 (0.51 - 1.06)^{a}
CML + CEL	2.16 (1.72 - 2.47)^{b}	0.82 (0.65 - 0.93)^{ac}	0.92 (0.82 - 1.20)^{a}	0.58 (0.43 - 0.66)^{c}
CML + CEL + MGH1	2.55 (1.98 - 3.33)^{b}	1.29 (0.98 - 1.39)^{a}	1.37 (1.10 - 1.61)^{a}	1.25 (0.97 - 1.68)^{a}

Abbreviations: CML - N^{ε}-(carboxymethyl)lysine ; CEL - N^{ε}-(carboxyethyl)lysine ; MG-H1 – methylglyoxal hydroimidazolone-1

Total AGEs can be described as the sum of the absolute quantities of CML and CEL as well as the sum of all three measured in the food recalling that in this study, MG-H1 concentrations were calculated relative to the CML internal standard. The sum of CML and CEL median values matched quantities as for CML alone: canned wet > air-dried > dry kibble > mildly cooked/raw (Table 3.3). The canned wet diet contained significantly higher amounts of CML and CEL compared to the other three diets and for the sum of these two AGEs, the air-dried diet was significantly higher than the mildly cooked/raw diet (Table 3.4b). Total AGEs (sum of all 3) produced similar results described by median values (Table 3.3): canned wet > air-dried > dry kibble > mildly cooked/raw. Overall, the canned wet diet contained a statistically significant greater amount of these three AGEs compared to the other three diets (Table 3.4b).

Table 3.4. Comparisons of individual (a) and total (b) advanced glycation end products (AGEs) in the four diets. Wilcoxon Mann Whitney post-hoc analyses were performed after global Kruskal Wallis tests with significance set at $p < 0.05$. Values shown for each AGE comparison between diets are 95% confidence intervals (C.I) and p-values.

(a)

Diet Contrast	**CML**		**CEL**		**MG-H1**	
	95% C.I.	p-value	95% C.I.	p-value	95% C.I.	p-value
WF - DF	0.22, 0.75	0.0209	0.41, 1.15	0.0209	-0.20, 0.64	0.5637
WF - ADF	-0.22, 0.52	0.1489	0.50, 1.24	0.0209	-0.37, 0.73	0.7728
WF - RF	0.50, 0.98	0.0209	0.35, 1.14	0.0209	-0.50, 1.00	0.2482
DF - ADF	0.03, 0.59	0.0209	-0.05, 0.20	0.1489	-0.19, 0.39	0.5637
DF - RF	0.11, 0.43	0.0209	-0.19, 0.20	0.1489	-0.01, 0.81	0.1489
ADF - RF	0.38, 0.89	0.0209	-0.11, 0.27	0.1489	-0.05, 0.91	0.0833

(b)

Diet Contrast	**CML + CEL**		**CML + CEL + MG-H1**	
	95% C.I.	p-value	95% C.I.	p-value
WF - DF	0.65, 1.80	0.0209	0.45, 2.44	0.0209
WF - ADF	0.52, 1.74	0.0209	0.32, 2.41	0.0209
WF - RF	0.88, 2.06	0.0209	0.28. 2.68	0.0433
DF - ADF	-0.13. 0.56	0.3865	-0.34, 0.65	0.3865
DF - RF	-0.06, 0.49	0.1489	-0.43, 0.70	0.5637
ADF - RF	0.18, 0.81	0.0209	-0.52, 0.70	1.000

Abbreviations: WF – canned wet; DF – dry kibble; ADF – air-dried; RF – mildly cooked/raw; CML – N^{ε}-(carboxymethyl)lysine; CEL – N^{ε}-(carboxyethyl)lysine; MG-H1 – methylgloxal hydroimidazolone -1

3.3.3 Advanced glycation end products in plasma.

The AGEs measured in the plasma were absolute quantities of CML, for which an internal standard (IS) was used, in addition to CEL, GH-1 and MG-H1 and AP all relative to the concentration of CML in the samples.

The median values for plasma CML (Table 3.5a) show that concentrations were highest when the air-dried diet was consumed with lower amounts for the dry kibble and canned wet foods while the mildly cooked/raw meat diet was lowest. The comparison between diets (Table 3.6a) revealed significant differences between the mildly cooked/raw and all the other diets. Median values for CEL (Table 3.5a) showed the canned wet diet associated with the highest plasma concentration followed in decreasing order by the dry kibble, mildly cooked/raw and air-dried diets. Significant differences (Table 3.6a) were found between canned wet and the three other diets. The dicarbonyl GH-1 was highest in plasma (Table 3.5a) with the air-dried diet, lower with the canned wet food and the dry kibble diet and lowest with the mildly cooked/raw meal. There were significant differences (Table 3.6a) between the air-dried and mildly cooked/raw in addition to canned wet and mildly cooked/raw diets. The median values for MG-H1 (Table 3.5a) were higher than those observed for GH-1 being highest with the canned wet diet, lower with the dry kibble and air-dried food and lowest with the mildly cooked/raw meal. The comparison between diets (Table 3.6a) for MG-H1 in plasma showed significant differences between the canned wet and all other diets as well as between the air-dried and mildly cooked/raw diets. The median values for AP (Table 3.5a) showed this AGE to be highest in plasma when the mildly cooked/raw diet was fed followed in decreasing order by the canned wet, dry kibble and air-dried diets. Significant differences observed via diet comparison (Table 3.6a) were between the mildly cooked/raw diet and all other diets.

Pairs, a group and total AGE quantities in plasma were compared among the diets. For the purposes of this study, CML and CEL (lysine adducts) and GH-1 and MG-H1 (dicarbonyls derivatives) were considered as pairs then altogether as a group of four of these commonly measured AGEs and finally, a measure of total AGEs including AP (Table 3.5b). The median for the combination of CML and CEL was highest with the canned wet diet (Table 3.5b) with significant differences between the mildly cooked/raw (lowest CML + CEL) and each of the three other diets (Table 3.6b). For the dicarbonyl pair of AGEs, the high MG-H1 median for the canned wet food (Table 3.5a) influenced the results of the sum of both compounds such that the median for the pair was also the highest in the canned wet diet (Table 3.5b). This influence of the relatively high amounts of MG-H1 compared with GH-1 caused significant differences between diets in this pair to match those of MG-H1 alone. For this dicarbonyl pair, the plasma concentrations with the canned wet diet was different from all other diets and the air-dried diet was different from the mildly cooked/raw diet (Table 3.6b). When the plasma measurements of the CML, CEL, GH-1 and MG-H1 group are compared with the total AGE group the results are similar (Table 3.5b). The highest median values for the sum of the lysine adducts and dicarbonyls alone and total AGEs were observed when the canned wet food was consumed (Table 3.5b). About half of this amount was observed with the dry kibble and air-dried diets for both of these groups (Table 3.5b). Overall, the mildly cooked/raw diet had the lowest median values (Table 3.5b). Significant differences between diets were similar (Table 3.6b) when comparing the sum of four AGEs with the total of all five AGEs. Significant differences were observed for plasma concentrations of total AGEs with the canned wet diet and all other diets as well as between the air-dried diet and mildly cooked/raw diet.

Table 3.5(a) Average plasma concentrations (nM/50μL) of individual advanced glycation end products (AGEs) at the end of feeding (four weeks) the four differently processed diets: canned wet, dry kibble, air-dried and mildly cooked/raw. Values are medians with interquartile ranges. Statistically significant differences indicated by superscript letters – a, b, c and d as determined by Friedman's tests and Wilcoxon Mann Whitney post-hoc individual comparisons.

Advanced glycation end product	**Diet**			
	Canned Wet	**Dry Kibble**	**Air Dried**	**Mildly Cooked/Raw**
CML	1.50 (1.24 - 1.85)^{a}	1.78 (1.12 - 2.51)^{a}	2.34 (1.54, 3.91)^{a}	0.77 (0.61 - 0.91)^{b}
CEL	2.45 (1.67 - 3.23)^{a}	1.54 (0.85 - 1.72)^{b}	0.64 (0.51 - 1.03)^{b}	0.77 (0.61 - 1.07)^{b}
GH-1	0.08 (0.07 - 0.09)^{a}	0.07 (0.06 - 0.07)^{ab}	0.09 (0.07 - 0.12)^{a}	0.05 (0.04 - 0.05)^{b}
MG-H1	5.41 (3.39 - 7.40)^{a}	1.53 (0.61 - 3.26)^{b}	1.01 (0.61 - 2.12)^{bc}	0.37 (0.18 - 0.57)^{bd}
AP	0.46 (0.32 - 0.56)^{a}	0.35 (0.32 - 0.45)^{a}	0.34 (0.29 - 0.38)^{a}	0.62 (0.52 - 0.69)^{b}

Abbreviations: CML - N^{ε}-(carboxymethyl)lysine; CEL - N^{ε}-(carboxyethyl)lysine; GH-1 – glyoxal hydroimidazolone-1; MG-H1 – methylglyoxal hydroimidazolone-1; AP – argpyrimidine

Table 3.5(b) Average plasma concentrations (nM/50μL) of grouped and total advanced glycation end products (AGEs) at the end of feeding (four weeks) the four differently processed diets: canned wet, dry kibble, air-dried and mildly cooked/raw. Values are medians with interquartile ranges. Statistically significant differences indicated by superscript letters – a, b, c and d as determined by Friedman's tests and Wilcoxon Mann Whitney post-hoc multiple comparisons.

AGEs grouped	**Diet**			
	Canned Wet	**Dry Kibble**	**Air-Dried**	**Mildly cooked/ Raw**
CML + CEL	4.04 (2.94 - 5.04)^{a}	3.44 (1.98, 4.09)^{a}	3.12 (2.05, 4.82)^{a}	1.46 (1.36, 1.92)^{b}
GH-1 + MG-H1	5.49 (3.49 - 7.46)^{a}	1.59 (0.68 - 3.32)^{b}	1.13 (0.68 - 2.24)^{bc}	0.42 (0.22 - 0.64)^{bd}
CML + CEL + GH-1 + MG-H1	9.57 (6.43 - 12.51)^{a}	5.67 (2.65 - 6.81)^{b}	4.21 (2.77 – 7.00)^{bc}	1.89 (1.64 - 2.48)^{bd}
Total (CML + CEL + GH-1 + MG-H1 + AP)	9.99 (6.74 - 12.95)^{a}	6.01 (3.10 - 7.14)^{b}	4.53 (3.12 - 7.42)^{bc}	2.50 (2.33 - 3.14)^{bd}

Abbreviations: CML - N^{ε}-(carboxymethyl)lysine; CEL - N^{ε}-(carboxyethyl)lysine; GH-1 – glyoxal hydroimidazolone-1; MG-H1 – methylglyoxal hydroimidazolone-1; AP – argpyrimidine

Table 3.6(a). Significant differences between diets for individual plasma advanced glycation end products (AGEs). Statistically differences determined by Friedman's tests and Wilcoxon Mann Whitney post-hoc individual comparisons ($p < 0.05$).

AGE	Diet Contrast	Hodges-Lehmann Location Shift	95% Confidence Interval (C.I.)	p - value
CML	Air-dried - Raw	1.30	0.65 – 3.62	0.0046
	Air-dried - Canned Wet	0.75	-0.20 – 2.73	0.1152
	Air-dried - Dry Kibble	0.65	-0.69 – 2.56	0.3446
	Dry Kibble - Raw	0.97	0.09 – 1.77	0.0357
	Canned Wet - Raw	0.68	0.40 – 1.09	0.0046
	Dry Kibble - Canned Wet	0.29	-0.48 – 1.15	0.4008
CEL	Canned Wet - Air-dried	1.55	0.86 - 2.62	0.0016
	Canned Wet - Raw	1.41	0.77 - 2.52	0.0016
	Canned Wet - Dry Kibble	1.21	0.02 - 1.85	0.0460
	Dry Kibble - Air-dried	0.64	-0.05 - 1.21	0.0742
	Dry Kibble - Raw	0.61	-0.13 - 1.06	0.0929
	Raw - Air-dried	0.11	-0.29 - 0.51	0.5995
GH-1	Air-dried - Raw	0.04	0.20 - 0.07	0.0033
	Canned Wet - Raw	0.03	0.01 - 0.05	0.0274
	Air-dried - Dry Kibble	0.03	-0.01 - 0.06	0.1415
	Dry Kibble - Raw	0.02	-0.01 - 0.03	0.0587
	Air-dried - Canned Wet	0.02	-0.01 - 0.05	0.3446
	Canned Wet - Dry Kibble	0.01	-0.01 - 0.03	0.1152
MG-H1	Canned Wet - Raw	4.96	2.18 - 7.19	0.0016
	Canned Wet - Air-dried	4.26	1.77 - 6.57	0.0033
	Canned Wet - Dry Kibble	3.84	1.44 - 6.38	0.0063
	Dry Kibble - Raw	0.97	-0.03 - 2.92	0.0587
	Air-dried - Raw	0.64	0.01 - 2.01	0.0460
	Dry Kibble - Air-dried	0.46	-0.79 - 2.25	0.4622
AP	Raw - Air-dried	0.28	0.17 - 0.38	0.0011
	Raw - Dry Kibble	0.25	0.12 - 0.35	0.0033
	Raw - Canned Wet	0.17	0.04 - 0.33	0.0117
	Canned Wet - Air-dried	0.09	-0.03 - 0.23	0.1722
	Canned Wet - Dry Kibble	0.03	-0.06 - 0.22	0.2076
	Dry Kibble - Air-dried	0.02	-0.04 - 0.14	0.4622

Abbreviations: CML - N^{ε}-(carboxymethyl)lysine; CEL - N^{ε}-(carboxyethyl)lysine; GH-1 – glyoxal hydroimidazolone-1; MG-H1 – methylglyoxal hydroimidazolone-1; AP – argpyrimidine

Table 3.6(b). Significant differences between diets for grouped and total plasma advanced glycation end products (AGEs). Statistically differences determined by Friedman's tests and Wilcoxon Mann Whitney post-hoc individual comparisons ($p < 0.05$).

AGE	Diet Contrast	Hodges-Lehmann Location Shift	95% Confidence Interval (C.I.)	p-value
CML + CEL	Canned Wet - Raw	1.87	1.33 - 3.58	0.0033
	Dry Kibble - Raw	1.73	0.12 - 2.68	0.0460
	Air-dried - Raw	1.18	0.24 - 3.97	0.0357
	Canned Wet - Dry Kibble	0.95	-0.96 - 2.37	0.4008
	Canned Wet - Air-dried	0.88	-0.96 - 2.52	0.4008
	Air-dried - Dry Kibble	0.01	-1.81 - 2.13	1.0000
GH-1 + MG-H1	Canned Wet - Raw	4.97	2.22 - 7.22	0.0016
	Canned Wet - Air-dried	4.25	1.76 - 6.53	0.0033
	Canned Wet - Dry Kibble	3.82	1.35 - 6.39	0.0063
	Dry Kibble - Raw	0.99	-0.05 - 2.95	0.0742
	Air-dried - Raw	0.68	0.04 - 2.03	0.0460
	Dry Kibble - Air-dried	0.43	-0.87 - 2.18	0.4622
CML + CEL + GH-1 + MG-H1	Canned Wet - Raw	6.74	3.56 - 10.51	0.0016
	Canned Wet - Air-dried	4.70	1.08 - 9.14	0.0157
	Canned Wet - Dry Kibble	4.68	0.70 - 8.68	0.0274
	Dry Kibble - Raw	3.53	-0.06 - 4.98	0.0587
	Air-dried - Raw	1.92	0.38 - 5.15	0.0357
	Dry Kibble - Air-dried	0.14	-3.13 - 3.52	1.0000
Total (CML + CEL + GH-1 + MG-H1 + AP)	Canned Wet - Raw	6.59	3.28 - 10.55	0.0016
	Canned Wet - Air-dried	4.89	0.97 - 9.16	0.0157
	Canned Wet - Dry Kibble	4.53	0.71 - 8.53	0.0460
	Dry Kibble - Raw	3.24	-0.24 - 4.72	0.0929
	Air-dried - Raw	1.70	0.02 - 4.95	0.0460
	Dry Kibble - Air-dried	0.09	-3.30 - 3.49	1.0000

Abbreviations: CML - N^{ε}-(carboxymethyl)lysine; CEL - N^{ε}-(carboxyethyl)lysine; GH-1 – glyoxal hydroimidazolone-1; MG-H1 – methylglyoxal hydroimidazolone-1; AP – argpyrimidine

3.3.4 Serum sRAGE concentrations

Friedman's test revealed no statistically significant differences in serum concentrations of sRAGE concentrations between diets, $\chi^2(3) = 7.35$ ($p = 0.062$) although the mildly cooked/raw diet corresponded to the highest concentration of the receptor in serum (Table 3.7). Samples taken at the end of feeding of each diet were selected for statistical analysis. Details of all sRAGE measurements and statistical output are available in Appendices L and M.

Table 3.7. sRAGE concentration medians, interquartile ranges and mean rank values for serum at the end of feeding each diet.

Diet	Median	Interquartile Range	Mean Rank (Friedman's test)
Canned Wet	603.42	280.38 - 3006.02	2.50
Dry Kibble	460.11	198.42 - 2908.24	1.63
Air Dried	651.42	286.09 - 2829.24	2.50
Mildly cooked/Raw	690.35	317.01 - 3881.52	3.38

3.4 DISCUSSION

3.4.1 Clinical parameters of the canine colony

All dogs were clinically normal as confirmed by physical examination, hematological, biochemical and urinary laboratory data at the start and during the study as stated previously. Even though statistical analysis revealed significant differences of a few parameter means between the diets, there is no clinical significance of these differences since all parameters were

within normal reference ranges. This point is noteworthy in this investigation since data on dietary intake of AGEs in physiologically normal dogs is sparse and no clear role of AGEs in the pathogenesis of diseases has been described. It is yet to be determined if dietary AGEs are definitive etiological factors for development of various diseases and whether increased intake is a risk factor in normal dogs.

3.4.2 Dietary advanced glycation end products

Dietary sources of AGEs in human beings have gained the interest of researchers over the last few decades because of their association with the Western diet and various inflammatory and degenerative diseases (ALjahdali & Carbonero, 2017; Delgado-Andrade, 2016; Sharma, Kaur, Thind, Singh, & Raina, 2015). In commercial pet foods, exploration into these glycoxidant compounds is emerging due to the variant methods of thermal processing used in conventional pet food manufacturing (van Rooijen et al., 2013), a feature that bears many similarities to the method of food processing commonly associated with the Western diet (Sebekova & Sebekova, 2019). To the authors' knowledge, the first studies to determine AGE quantities in commercial pet food provided evidence not only of the extent of the Maillard reaction in thermally processed dog foods (van Rooijen et al., 2014a) but also to determine possible decrease in lysine bioavailability after this amino acid is consumed by participation in glycation reactions (van Rooijen et al., 2014b). These findings suggest that dietary AGEs contribute to the AGE pool in dogs and quantification of AGEs in canine biofluids will aid in investigation into the pathologic consequences of eating thermally processed foods for the long periods during their lifetime.

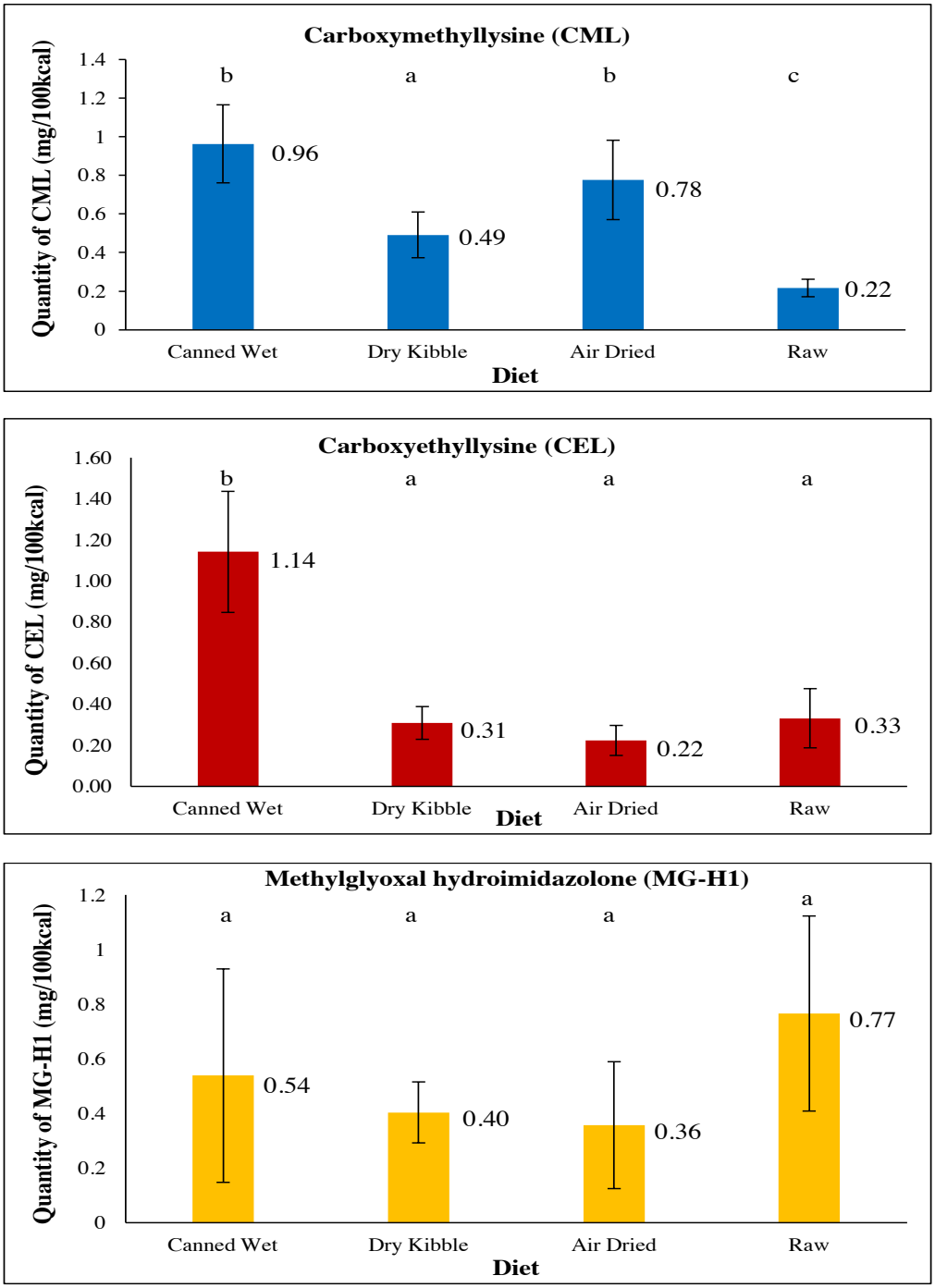

Figure 3.4 Mean quantities with standard deviations and statistically significant differences ($p < 0.05$) indicated by a, b and c, of individual dietary advanced glycation end products (AGEs) for all four diets of the feeding trial. AGEs measured in the diets were: carboxymethyllysine (top); carboxyethyllysine (middle) and methylglyoxal hydroimidazolone-1 (bottom).

The dietary AGEs evaluated in this study were absolute concentrations of CML and CEL as well as relative concentrations of MG-H1 to the CML internal standard. Lysine reactions with the α-dicarbonyls GO (glyoxal) and MGO (methylglyoxal) result in the formation of CML and CEL respectively, while the guanidino group of arginine reacts with MGO to give rise to MG-H1 (Hellwig, Gensberger-Reigl, Henle, & Pischetsrieder, 2018). The most commonly measured AGE is CML probably because it was the first AGE to be recognized (M. U. Ahmed et al., 1986; Delgado-Andrade, 2016) as the product of several pathways and, has been quantified in at least three databases of common human foods (Goldberg et al., 2004; Hull et al., 2012; Scheijen et al., 2016; Uribarri et al., 2010). No such database exists for commercial pet foods although information about AGE concentrations in mildly cooked/raw meat and other components used in dog food processing may be obtained from these sources.

The diets in this study comprised two high heat processed commercial diets[b]: a canned wet and dry kibble produced by extrusion and retorting respectively, and two similarly formulated low-heat processed diets: air-dried and mildly cooked/raw meat. One of the complications of previous AGE studies involved the difficulty in predicting the rate of AGE formation as well as the diversity of AGEs in the food item. The formation of AGEs is determined by numerous factors such as the components of the food, the presence of moisture, precursors, transition metals, antioxidants as well as the actual food preparation method including type and duration of heat exposure, maximum temperature, availability of water, pH, and type of food processing (Poulsen et al., 2013). In order to circumvent these obstacles, differences in components and nutrient composition were minimized so that only differences in processing related to AGE formation could be ascertained. The protein source for all diets was irradiated kangaroo meat combined with sweet potato as the carbohydrate component providing

a highly digestible, low-fat, novel protein diet designed for dogs with a range of dermatologic and digestive inflammatory disorders[b]. Maximum temperatures used for processing of the diets were: 254°F for retorting (WF); 265°F for extrusion; 140°F for air drying and 105°F for the mildly cooked/raw meat. The mean individual dietary AGEs, the sum of CML and CEL as well as the sum of all three dietary AGEs varied for each of these processing methods as shown in Figures 3.4 and 3.5. The canned wet diet contained the highest total amount of CML and CEL while MG-H1 was observed to be highest in the mildly cooked/raw diet but was not significantly different from the other diets (Fig. 3.4). Recall that MG-H1 values obtained via UPLC-MS were relative to CML leaving the possibility of over- or underestimation. While there were significant differences between diets for each of the measured AGEs individually, when considering dietary AGE contribution to the body's AGE pool, it is the total AGE burden of the diet that may be of pathological significance (Liang, Chen, Li, Li, & Yang, 2019; Nowotny et al., 2018; Šebeková & Somoza, 2007).

The comparison of the two lysine adducts and total amounts of AGEs in the diets resulted in the canned wet diet being set apart from the other diets by virtue of its highest AGE content. As seen in Fig. 3.5, the canned wet diet processed by retorting, had the highest mean CML and CEL (2.10mg/kcal as fed) and CML, CEL and MG-H1 (2.64mg/100kcal as fed) totals that were statistically different from the other three diets. Of the protein bound AGEs measured in this experiment, CEL was the only one that was statistically different between the canned wet and air-dried diets (Table 3.4a). This result is unexpected since there is a difference of almost 100°F between retorting and air-drying suggesting that the diet processed under the higher heat condition should contain a substantially higher amount of all AGEs. The air-dried diet, subjected

to a lower temperature during drying, contained the second highest total AGEs for both the pair of lysine adducts and all three AGEs (Table 3.3) that could be attributed to the addition of dextrose to this diet as a substrate for *Pediococcus acidilactici* fermentation. It is possible that processing conditions did not favor use of dextrose by the organisms, leaving this reducing sugar available for glycation reactions during low-heat processing. Significant differences between diets were noted for CML (air-dried vs. dry kibble and mildly cooked/raw), CEL (air-dried vs. canned wet) (Fig.3.4), total CML and CEL (air-dried vs. canned wet and mildly cooked/raw) while for the sum of all three AGEs, the only diet that showed significant difference from the air-dried diet was the canned wet diet (Fig. 3.5). Further investigation into processing of air-dried diets as well as the effect of addition of bacterial fermentation products to low-heat processed diets related to AGE formation is needed to determine the cause of the higher AGE content of the air-dried diet.

The canned wet diet contained the highest amount of total AGEs and was significantly different from all other diets (Fig. 3.5). In this study, even though the extruded dry kibble was also a high heat processed diet, the total AGEs was statistically different from that of the canned wet diet. This finding may be related to canned food processing done by retorting, under conditions of high temperature for a longer period of time than extrusion thereby favoring AGE formation. Furthermore, the total quantity of AGEs in the dry kibble was not statistically different from the low heat processed diets. The addition of dextrose to the low heat processed diets was mentioned earlier as a contributor to glycation reactions in these diets causing an increase in total AGEs that was similar to the quantity in the dry kibble. Existing literature reports lower protein bound AGES in raw meats (Sun et al., 2016) than after high heat exposure so additional studies are recommended to investigate AGE quantity in differently processed pet

foods and to make further comparisons between high and low heat methods with bacterial fermentation product additives.

Overall, comparison of the diets with the total amounts of AGEs measured in this investigation matches the findings of other studies that examined the AGE content of foods (Scheijen et al., 2016; van Rooijen et al., 2014a). It is expected that high heat processing increases the AGE quantity in the food that can be decreased via reduction of temperature in food preparation. Although information regarding pet foods is sparse, similar findings have been demonstrated in research from heat application to other meats such as pork (Yu et al., 2016), beef (Sun et al., 2015), different types of fish (Niu et al., 2017) and other commercial meats (Sun et al., 2016). Raw meat had significantly lower AGE levels that the corresponding cooked or processed product (Z. Zhu, Huang, Cheng, Khan, & Huang, 2020) as seen with the pet food formulations used in this case.

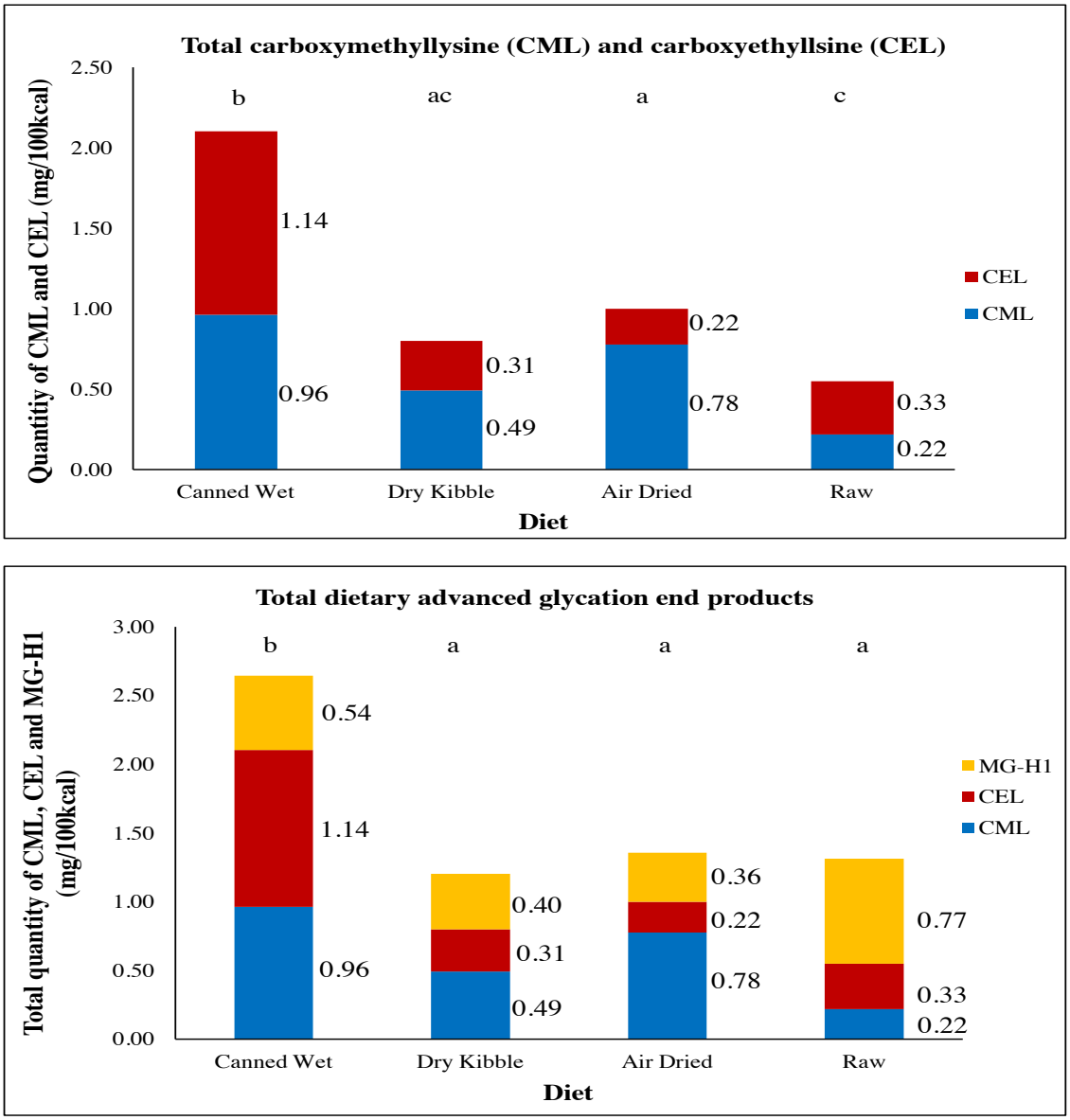

Figure 3.5. Stacked bar graphs showing the sum of CML and CEL only, and the total advanced glycation end products (AGEs) for each of the four diets: canned wet, dry kibble, air dried and mildly cooked/raw.

Abbreviations: CML – carboxymethyllysine; CEL – carboxyethyllysine; MG-H1 -methylglyoxal hydroimidazolone–1

a, b and c – superscripts indicate statistically significant differences between diets ($p < 0.05$)

3.4.3 Advanced glycation end products in plasma

Digestion, absorption, metabolism and elimination of AGEs in the body still need to be explored. It was previously assumed that dietary AGEs were not absorbed and didn't contribute to the body's AGE pool so until recently, its role in the pathogenesis of disease was largely ignored (Liang et al., 2019). As investigations continue into the metabolic fate of dietary AGEs, there is more guidance for researchers as to the biofluids and tissues that serve as useful samples for assessment of AGE metabolism and elimination (Scheijen et al., 2018).

Dietary AGEs, just as endogenous forms in the body, may be protein bound or free. Those that are protein bound are regarded as high molecular weight (HMW) AGEs that are not readily absorbed by the gastrointestinal tract and need to be degraded by host or microbial enzymes (Delgado-Andrade et al., 2011; Garcia et al., 2009; Poulsen et al., 2013; Seiquer et al., 2006) while smaller peptide-bound or free forms are termed low molecular weight (LMW) AGEs that can be absorbed by diffusion or peptide transporters (Hellwig et al., 2011). This existence of both HMW and LMW dietary AGEs suggest that after ingestion, the digestive fate of LMW AGEs is to be absorbed into the blood, undergo distribution to tissues and organs then be eliminated via the kidneys in urine. In contrast, HMW AGEs may be degraded by host enzymes and follow the same path as the LMW compounds or can by-pass host digestion and enter the colon where they either serve as substrates for gut microbial fermentation or to be excreted in feces (ALjahdali & Carbonero, 2017; Poulsen et al., 2013). There is evidence that foods high in protein bound dietary AGEs are positively associated with high concentrations of free AGEs in plasma and urine but not protein bound AGEs in plasma (Scheijen et al., 2018).

These associations in human beings serve as a foundation for this investigation into protein bound CML, CEL and MG-H1 in the canine diets and corresponding plasma

measurements of free CML, CEL, MG-H1, GH-1 and AP. These five AGEs have been successfully measured by UPLC-MS in rat plasma (Hashimoto et al., 2013) and to the authors' knowledge, this study is the first and valid application of the trinitrobenzene sulfonate (TNBS) derivatization method to quantify free AGEs in canine plasma. Mean individual measurements of the five AGEs are seen in Figure 3.6 and total plasma AGE measurements are depicted as stacked columns in Figure 3.7. for each of the diets showing that the highest sum of AGEs (measured in nM/50μL) corresponded to the canned wet diet and was attributed to the highest amount of CEL and MG-H1 compared to the values for these AGEs in any other diet. A comparison of pairs of all diets revealed that the total plasma AGEs associated with the canned wet diet was significantly higher than the other three diets. There was also a significant difference between the air-dried diet and the mildly cooked/raw diet where the higher quantity of total plasma AGEs associated with the air-dried diet was mostly due to differences in CML and MG-H1 quantities when compared to the mildly cooked/raw diet (Table 3.5a). When compared with the food analysis, the canned wet diet contained the highest amount of protein bound CML, CEL and MG-H1 that correlated with the highest plasma AGE concentrations when this diet was consumed. This finding is significant since it supports our hypothesis that diets higher in AGE concentrations will be associated with higher plasma AGE concentrations. This preliminary work serves as the foundation for further investigation into the influence of dietary AGEs in dogs.

As with the findings in human foods, total plasma AGE concentration associated with the mildly cooked/raw diet was the lowest of the four diets providing evidence to support previous findings that low AGE diets result in lower AGE plasma levels (Scheijen et al., 2018). The consistent finding in these comparisons; however, is that high heat processing allows for the formation of quantifiable AGEs in food and that consumption of high-heat processed foods allow

for dietary AGEs to accumulate as part of the AGE pool. It follows that the reverse happens when raw or mildly cooked/heated foods are consumed thereby reducing exogenous sources of AGEs (Uribarri et al., 2015). If the role of AGEs in the pathogenesis of inflammatory diseases can be elucidated then dietary modification for both human beings and animals can be a useful therapeutic approach.

Glyoxal (GO) and methylglyoxal (MGO) belong to the group of highly reactive α-dicarbonyls produced at the intermediate stage of the Maillard reaction. They are among many precursors for the formation of CML and CEL, and serve as the reactants at the intermediate stage of the Maillard reaction to produce the imidazolone AGEs: GH-1 and MG-H1. From Fig. 3.8, the canned wet diet is associated with the highest quantities of both groups of lysine and imidazolone adducts suggesting that digestion favored higher plasma AGE levels. GH-1 measurement revealed far smaller quantities than MG-H1 causing statistically significant differences to be primarily determined by the MG-H1 concentration. The difference in total concentration of the lysine adducts was only significant for the mildly cooked/raw diet compared with the other diets in contrast with that of the α-dicarbonyl products which, was significantly different between the canned wet and all other diets as well as between the air-dried and mildly cooked/raw diets (Table 3.6b).

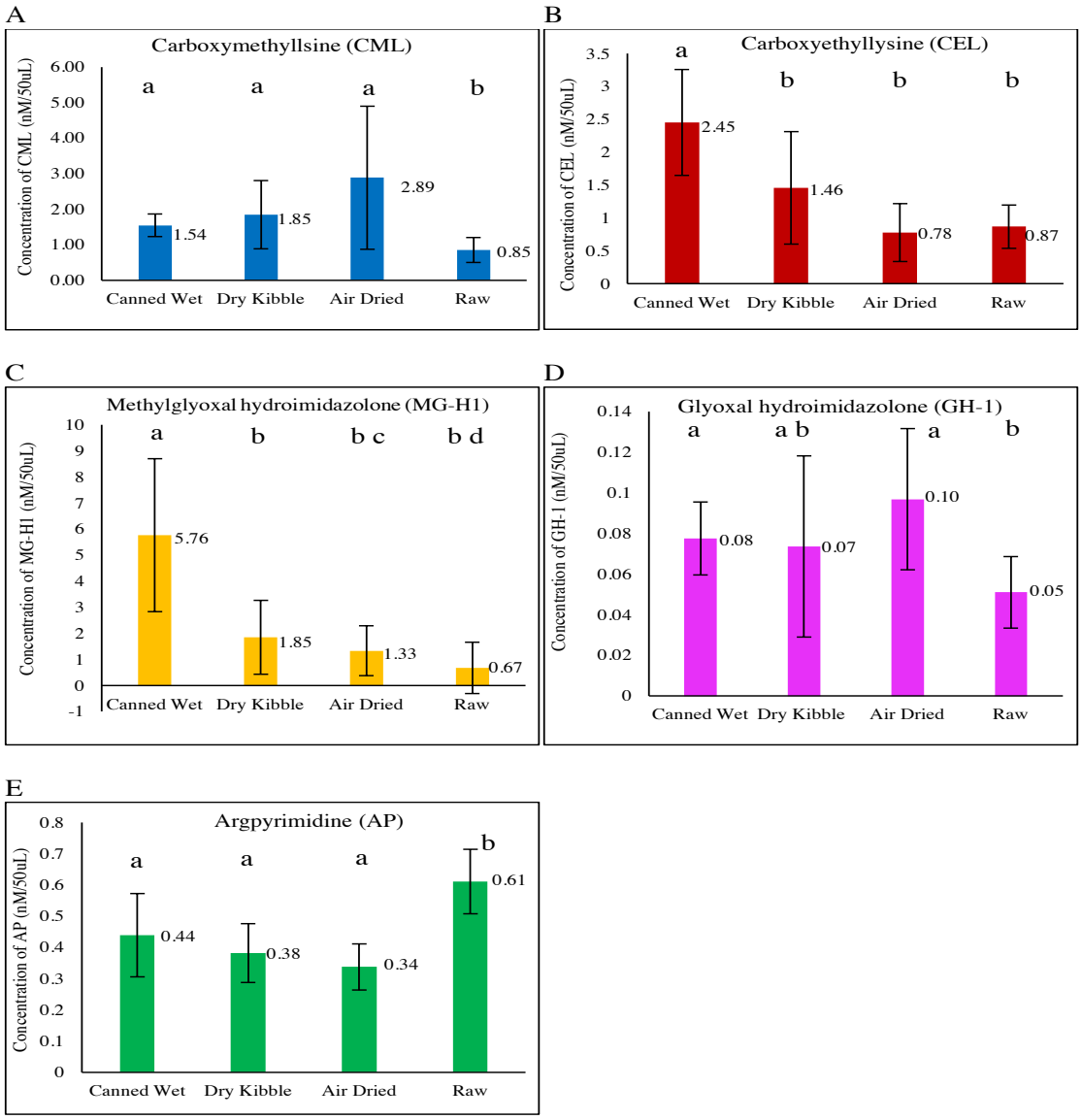

Figure 3.6 Mean concentrations (nM/50µL) with standard deviations of the five (A-E) individual plasma advanced glycation end products (AGEs).

a, b and c, d – superscripts indicate statistically significant differences ($p < 0.05$) between diets

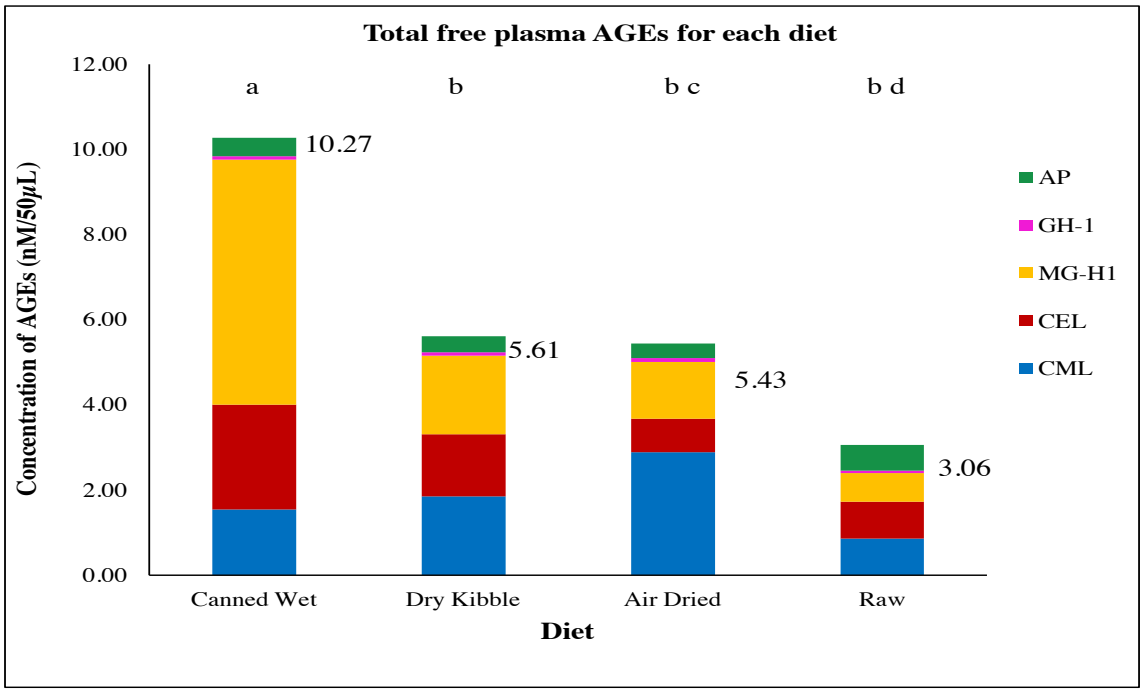

Figure 3.7. Stacked bar graph showing total mean advanced glycation end product (AGE) concentrations of the five AGEs measured in plasma.

Abbreviations: CML – carboxymethyllysine; CEL – carboxyethyllysine; MG-H1 methylglyoxal hydroimidazolone–1; GH-1 - glyoxal hydroimidizalone–1; AP – argpyrimidine

a, b and c, d – superscripts indicate statistically significant differences ($p < 0.05$) between diets

Another observation is that for all diets except the canned wet diet, the sum of CML and CEL exceeded the sum of the other two AGEs (Fig. 3.8). Whether this is of clinical significance is unknown and beyond the scope of this work but given that dicarbonyl stress in the body can lead to the development of diseases in human beings, and that there was a substantial amount of dicarbonyl derivatives in plasma associated with canned wet diets, it may be worthwhile to further investigate the existence of dicarbonyl stress in dogs on differently processed diets.

The dicarbonyls are very reactive and unstable compounds making measurement difficult. In this study, downstream products of MGO that were quantified included CEL, MG-H1 and AP (Wilker, Chellan, Arnold, & Nagaraj, 2001). While specific quantification of MGO is difficult because of its instability, the formation of these three products of its reaction is an estimate of its quantity in the food samples and in the plasma where both exogenous (dietary) and endogenous forms contribute to the calculated concentration. It should be noted that AP levels were relatively low in plasma compared to the other MGO products with significant differences between the mildly cooked/raw diet and all other diets (Table 3.6a). It is unknown why AP concentration would be greatest when the dogs were fed the mildly cooked/raw diet (Table 3.5a) but since this AGE was not assessed in these canine diets, it could be considered in future experiments to determine if this is a consistent finding in raw diets and if there is any clinical significance of dietary intake in dogs. As a result of these small amounts of AP, the statistically significant differences in the total amount of plasma AGEs between the diets, with or without AP values were the same.

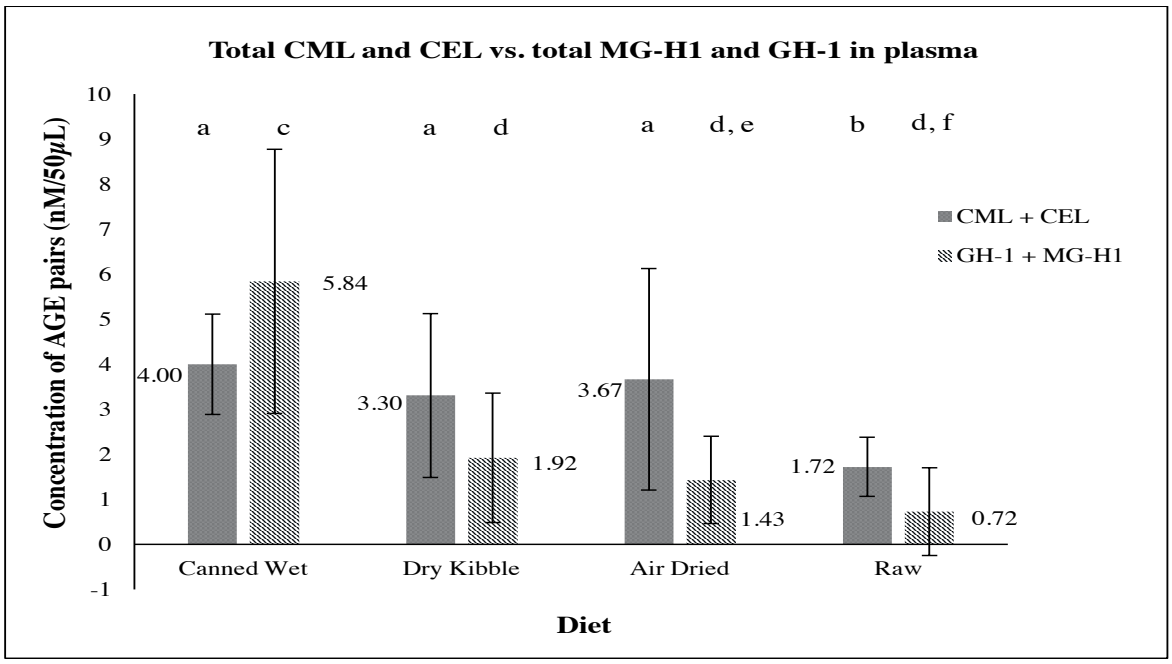

Figure 3.8. Clustered bar graph showing the total concentrations of lysine adducts CML and CEL compared with total concentrations of the α-dicarbonyl derived GH-1 and MG-H1 in plasma for each of the four diets.

Abbreviations: AGE – advanced glycation end product; CML – carboxymethyllysine; CEL – carboxyethyllysine; MG-H1 – methylglyoxal hydroimidazolone–1; GH-1- glyoxal hydroimidizalone–1

a, b – superscripts indicate significant differences in CML + CEL between diets

c, d, e and f – superscripts indicate significant differences in GH-1 and MG-H1 between diets.

3.4.4 Soluble Receptor for advanced glycation end products (sRAGE) in serum

In these dogs, there were no significant differences in sRAGE concentration between diets (Appendix M) after four weeks of feeding. It is important to note that these dogs were clinically normal, the diets were adult canine diets and the feeding period for each diet was limited to four weeks. Changes in RAGE activity and sRAGE concentrations in serum may depend on persistently high AGE intake and accumulation in the body. Levels of sRAGE in normal human beings have rarely been investigated and when these studies were performed, no relationship between dietary AGEs and sRAGE were found (Van Puyvelde, Mets, Njemini, Beyer, & Bautmans, 2014). More often, sRAGE concentrations have been determined during the course of diseases (Ciccocioppo et al., 2015; Fujisawa et al., 2013; Heilmann et al., 2014; Prasad, 2019; Wautier, Guillausseau, & Wautier, 2017) but to the authors' knowledge, there have not been any investigations into the changes in circulating sRAGE concentration in healthy dogs in response to dietary AGEs. Further research into this area in normal dogs is needed to be able to answer some of these questions about sRAGE activity and concentrations in response to dietary AGEs in this species.

In conclusion, we were able to identify and quantify AGEs in dog foods and determine their influence on plasma AGE concentrations using LC-MS as a state-of-the-art method. It was observed that AGE measurement is valid in pet foods and canine plasma and these findings serve as the foundation for further exploration in this area of pet nutrition. Most significantly, total dietary AGEs in the four differently processed pet foods influenced the total plasma AGE concentration suggesting that AGE burden may be of significance in dogs as an etiologic factor in the development of diseases related to inflammation and oxidative stress. Further long-term feeding trials and measurement of AGEs in both plasma and urine is recommended to continue

to build our knowledge of the role and effects of AGEs in companion animals as part of this comparative research.

Footnotes:

Animal Care and Use Approval:
This protocol with amendments was reviewed and approved by the Summit Ridge Farms' Institutional Animal Care and Use Committee (IACUC) in compliance with the Animal Welfare Act in addition to the IACUC at the University of Georgia.

a – Summit Ridge Farms, Susquehanna, PA

b - Rayne Clinical Nutrition™ diets (https://static1.squarespace.com/static/587795d586e6c05d5699c147/t/5cd9fc65a4222fedf3aabce2/1557789798091/051319_Rayne_DietPage_CanineKangaroo_DIAG_DietPage_VC401D_VC001D-6.pdf)

CHAPTER 4

CHANGES IN METABOLITE PROFILES IN CANINE SERUM AND URINE IN RESPONSE TO FOUR DIFFERENTLY PROCESSED DIETS

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[1] Siobhan Bridglalsingh, Xunan Shen, Joseph W. Bartges, Arthur S. Edison, Parastoo Azadi, Claire B. de La Serre, Rebecca L. Remillard. To be submitted to the *Journal of Animal Physiology and Animal Nutrition*

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Arthur S. Edison	– data analyses and editing
Parastoo Azadi	– editing
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Abstract

Dietary advanced glycation end products (AGEs) quantities in high heat processed commercial dog foods are similar to thermally processed foods characteristic of the human Western diet that is associated with a range of inflammatory and degenerative diseases. We hypothesized that serum and urine metabolite profiles in dogs, measured using nuclear magnetic resonance (NMR) spectroscopy, would change in response to four differently processed diets. These diets were two high heat processed diets - canned wet (WF) and dry kibble (DF); compared with two low heat processed diets - air-dried (ADF) and mildly cooked/raw (RF). Eight laboratory colony dogs were fed each diet over four weeks, as pairs in random sequence via a Latin square experimental design so that, after 16 weeks all dogs had consumed each diet. Serum and urine samples were collected weekly beginning at the start of feeding. 1D-NOESY PR spectra were collected for metabolite quantification and analysis and 2D HSQC and HSQC-TOCSY spectra were used for metabolite identification. In serum, ten out of 53 features were found to be statistically

significant and were identified as valine, glycine, acetic acid, creatine, acetylcarnitine, betaine and two unknowns. Similarly, six out of 79 features were identified in urine as the discriminatory metabolites acetic acid, creatinine, carnitine, dimethylamine, dimethyl-sulfone and methylnicotinamide. Overall, findings showed that the dry kibble diet was associated with lower metabolite concentrations for both serum and urine, and metabolite concentrations fluctuated minimally over four weeks indicating response to dietary change within seven days. A partial least squares regression model was able to predict one AGE (carboxymethyllysine) in the diets. This study serves as preliminary work into the effects of heat processing of pet foods on the canine metabolome and further investigations into effects of dietary AGEs is recommended.

Abbreviations

1D-NOESY PR – one-dimensional nuclear Overhauser enhancement spectroscopy

2D-HSQC – two-dimensional heteronuclear single quantum correlation spectroscopy

TOCSY – total correlation spectroscopy

4.1 INTRODUCTION

The Western diet is characterized by heat processed, calorie dense foods that contain advanced glycation end products (AGEs) formed during the Maillard reaction (Martinez et al., 2017; Qu et al., 2018). The Maillard reaction or "browning" reaction is a non-enzymatic reaction between a carbonyl group of a reducing sugar and the amino group of an amino acid, peptide or protein (Maillard, 1912). While this reaction is necessary for the desirable flavor, aroma, texture and

longer storage shelf-life of the foods (Poulsen et al., 2013), it results in production of this large group of heterogeneous compounds that have been implicated in inflammatory and degenerative conditions (Vlassara, 2005) for example, obesity, diabetes mellitus (Hu et al., 2015), atherosclerosis (Zabek et al., 2017) and cardiac disease, nephropathy (Rabbani & Thornalley, 2018), neurodegeneration (Uribarri et al., 2015), allergies (Gupta et al., 2018), and aging (Chaudhuri et al., 2018).

In veterinary medicine, commercial pet foods are subjected to high heat processing similar to foods consumed in the Western-style diet (van Rooijen et al., 2014a). The Maillard reaction proceeds in these heat-treated products, such as extruded kibble and canned foods, exposing dogs to dietary AGEs. Investigations into the direct effect of dietary AGEs on health in dogs are sparse and to the authors' knowledge, this study is the first to observe correlations between AGE quantities in diets and plasma with concurrent metabolic changes in serum and urine in dogs. While studies in both human beings and dogs have not determined causation between AGEs and disease, a significant amount of research has been done on obesity, the Western diet and gut microbial changes leading to metabolite changes (Delgado-Andrade et al., 2017; Martinez et al., 2017; Moran-Ramos et al., 2017; Sen et al., 2017; Snelson & Coughlan, 2019). The obesity epidemic is common both to dogs and human beings potentially because of the similarity in diet and lifestyle experienced by dogs as companion animals (Handl et al., 2013). This disease occurs as a result of over-consumption of highly processed foods with resultant changes in gut microbiota promoting low grade inflammation known as "metabolic endotoxemia" (Klingbeil & de La Serre, 2018), decrease in glucose tolerance, decrease in satiety and production of harmful metabolites. It seems reasonable to suggest that if a diet consisting solely of processed foods, that also have high quantities of AGEs due to the Maillard reaction,

leads to obesity and insulin resistance then there should also be a relationship between the excessive consumption of AGEs and these same diseases. There is existing evidence of the effect of dietary AGEs on the gastrointestinal microbiome and fecal metabolome of mice (Qu et al., 2018). This study demonstrated reduction in bacterial diversity and alteration in fecal metabolites after long-term AGE exposure alluding to mechanisms by which chronic AGE exposure may have negative impacts on gut microbes and host health.

Studies attempting to establish causation or association between dietary factors and health in human beings have relied on subjective methods such as diet records/history, food frequency questionnaires (FFQs) or 24-hour recalls (Uribarri et al., 2015). These methods are deficient in reliability, accuracy and objectivity often failing to measure actual dietary intake. When measuring dietary AGEs, accuracy is further complicated by details of the type of food processing, method of cooking, correct portion intake as well as the ensuing digestion and metabolism of both nutrients and AGEs in the food consumed. The challenge faced in this type of nutritional research is to develop a reliable method for determining dietary exposure to AGEs not only via quantification in the food but also by measurement of food-derived metabolites and dietary biomarkers that correlate with complex diet matrices (Radjursoga et al., 2019).

Experimental animal models using rodents to investigate the effects of dietary AGE intake on human beings poses some challenges when compared with the canine model. Rodent diets, lifespan, genetics and physiology are unlike dogs and humans while the latter two, since the domestication of the dog, bear significant similarities in microbiota, diet and lifestyle (Coelho et al., 2018; Handl et al., 2013). The lifespan of a dog as well as the possible long-term or repeated exposure to high heat processed diets, makes this species a suitable model for studying the effects of chronic dietary AGE exposure in human beings.

The field of metabolomics concerns the study of biological small molecules (<1500 Da) associated with the response to genetic variation or external environmental stimuli in fluids, cells and tissues of any living organism (Idle & Gonzalez, 2007; Song et al., 2019). Metabolomics complements genomics, transcriptomics and proteomics by identification of small molecules completely different from nucleic acid or proteins as well as offering a method to observe changes in metabolites between and within biological samples (Song et al., 2019). The metabolic profile of an individual organism is indicative of all the biological processes within that metabolic system that may be affected by, but certainly not limited to, diet and gastrointestinal microbiome degradation of any undigested material.

Nutritional metabolomics has been described as: "The study of endogenous and gut microbiota metabolic response to food (general diet or intervention) and the identification of metabolites that originate from food and could be used as biomarkers of exposure to these foods" (Fave, Beckmann, Draper, & Mathers, 2009), and offers an objective and more reliable method of obtaining information about and measuring dietary exposure to specific dietary components (Radjursoga et al., 2017; Radjursoga et al., 2019). The two most common high-throughput methods employed in metabolomics studies are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy (A. Zhang, Sun, & Wang, 2013). Of these two, MS is more frequently used due to its high sensitivity, ability to identify metabolites in low concentrations, accuracy in identification of molecular structure and quantification of metabolites when combined with separation methods: gas chromatography (GC) or liquid chromatography (LC) (Pan & Raftery, 2007). However, NMR spectroscopy has aided the understanding of metabolites in biological processes for almost fifty years with initial NMR-based metabolomics studies beginning in the 1980s with the analysis of human urine and serum using 1D ^{1}H NMR

spectroscopy (Wishart, 2019). When compared with MS, NMR spectroscopy has the advantage of minimal sample preparation, having no derivatization, separation or ionization steps, is non-destructive and non-biased, while producing accurate reproducible measurements, being highly quantitative and facilitating identification of complex unknown metabolites (Emwas et al., 2019; Markley et al., 2017; A. Zhang et al., 2013). Uses of these techniques have grown from initial analysis of metabolites in human biological samples to investigation into biomarkers of disease (Wishart, 2019), diagnoses, treatments (Song et al., 2019) and prognostic indicators, progression of disease (Fukuhara et al., 2013) and more recently, exploration of changes in metabolic profiles in human beings and rodents at risk for developing lifestyle diseases like obesity (S. H. Kim et al., 2009; A. Zhang et al., 2013). NMR-based metabolomics in serum and urine analyses have been applied to canine samples not only in disease conditions (J. Zhang et al., 2012) but also in diet-related trials in healthy animals (Richards et al., 2013; Soder et al., 2019).

Serum contains metabolites from the host digestion and absorption of nutrients from food as well as any microbial metabolites absorbed in the colon derived from bacterial degradation of available substrates. Urine metabolic profiling is restricted to water-soluble molecules excreted by the kidneys. Another significant source of metabolites is the fecal metabolic components but was not included in this study and remains an area to be investigated in future studies. Characterization of metabolites by NMR spectroscopy in biological samples in combination with unsupervised and supervised statistical methods can be applied to crossover dietary intervention studies to determine differences in the metabolome between and within treatments (Radjursoga et al., 2017; Radjursoga et al., 2019).

The aim of this study was to use NMR spectroscopy to determine the metabolic response in serum and urine to varied concentrations of dietary AGEs and whether these changes allow

discrimination among the four types of diet. We hypothesized that the serum and urine metabolic profiles of dogs feeding on high vs. low heat processed diets are different allowing distinction between the two types of foods. No direct pathological role has been determined for AGEs but since high heat processed foods are associated with high AGE quantities, it is possible that metabolic alterations can be associated with AGE concentration in the heat processed diets.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Eight purpose-bred laboratory colony Beagle dogs[a], 4 males and 4 females, all intact, ranging from 3 -7 years old were used. Their health status was confirmed through physical examination, complete blood cell counts, serum biochemical analyses and urinalyses (Appendices A – D). The study employed a crossover design for 16 weeks. Dogs were housed individually in cages under a 12-hour light-dark cycle with temperature range maintained within 50^{o} to 85^{o}F. Cages and bowls were cleaned and sanitized daily. Fresh tap water fit for human consumption was available *ad libitum* for the duration of the study period.

4.2.2 Study diets

Four diets of similar nutrient composition (Table 4.1) consisting of kangaroo meat and sweet potato but differing in AGE quantities were evaluated (See Appendix E for ingredients and nutrient analyses). The diets used in this study were subjected to different methods of processing to determine the effects on the serum and urine metabolome after feeding the dogs over four weeks. The canned wet and dry kibble diets were processed by retorting and extrusion respectively, serving as the two high-heat treated diets while the other two diets, air-dried and

mildly cooked/raw, were the low-heat processed diets. A *Pediococcus acidilactici* fermentation product (bacteriocin/pediocin) was added to both low heat processed diets as a bactericidal biological preservative. Table 4.2 shows the four diets, processing methods and maximum temperature employed. For this investigation, diets manufactured at temperatures greater than 200^{o}F were considered to be high heat processed while low heat processing referred to maximum temperatures less than 150^{o}F.

Table 4.1. Macronutrient composition of the four differently processed diets on 100% dry matter (DM) basis.

Macronutrient Composition	**DIET (100% DM amount)**			
	Canned Wet	**Dry Kibble**	**Air-Dried**	**Mildly Cooked/Raw**
Protein	39.48	33.94	45.77	45.66
Carbohydrates	45.82	49.32	34.65	34.62
Fat	8.61	8.40	10.97	10.96
Crude Fiber	1.11	2.25	1.65	1.65
Ash	5.82	6.09	8.27	8.42
ME (kcal/kg)	3717.29	3,628.08	3746.83	3741.33

Abbreviations: ME – metabolizable energy; DM – dry matter

Table 4.2. Maximum temperatures used for processing methods for each of the four diets in the feeding trial.

Diet	Process	Maximum temperature (^{o}F)	Additional details
Canned Wet (WF)	Retorting	254	Exposed to high temperatures for 60 – 90 minutes
Dry Kibble (DF)	Extrusion	265	After extrusion, food is dried to <10% moisture
Air-dried (ADF)	Dehydration	140	Dried for 12 hours until <12% moisture
Mildly cooked/Raw (RF)	Light and slow cooking	105	Lightly cooked for 10 hours then frozen until feeding

Three AGEs were measured in all diets: carboxymethyllysine (CML), carboxyethyllysine (CEL) and methylglyoxal hydroimidazolone-1 (MG-H1) but in considering their effects, it is the total amount of dietary AGEs and possible contribution to the body's AGE pool that is the determinant of the glycoxidant burden associated with risk of diseases (Delgado-Andrade & Fogliano, 2018). There was variation in the total amounts of AGEs (mg/100kcal of diet as fed) across the differently processed diets. The canned wet diet contained the highest dietary AGEs (2.55 mg/100kcal) as fed and was processed using a high maximum temperature of 254^{o}F for about 60 to 90 minutes followed in descending order by the air-dried (1.37mg/100kcal), dry kibble (1.29 mg/100kcal) and least in the mildly cooked/raw (1.25 mg/100kcal) diet. Statistical comparisons of AGE content in the diets revealed that the total AGEs in the canned wet diet was significantly higher than the other three diets.

4.2.3 Study Design

A Latin-square design allowed for minimization of differences enabling comparison among diets consumed by the same dog (Appendix G). Four diet sequences were created for feeding to avoid differences due to the order of consumption of the diets. Pairs of dogs were assigned to one of four diet group sequences based on a table of random numbers. Each pair received the first diet in their sequence for four weeks and samples were collected weekly, then dogs were crossed over to the next diet in their sequence until all dogs completed all four diets. A sample was collected prior to beginning the sequence of feeding to obtain a "baseline" record of the health status of the dogs. Feeding quantities were based on daily caloric requirement determined by body weight which, was measured weekly and feeding amount adjusted accordingly (see Appendix H for body weights) to maintain body weight within 5% of baseline. The canned wet and mildly cooked/raw diets were offered twice daily for a minimum of one hour. The dry kibble and air-dried diets were offered once daily for a minimum of one hour.

4.2.4 Sample collection

The sample collection schedule for this experiment is recorded in Appendix I.

Serum

Blood was collected at baseline and once weekly prior to the morning feeding via jugular venepuncture. Three milliliters (mls) of blood was placed into red-top separator tubes, allowed to clot then spun in a refrigerated centrifuge for 15 minutes at 3000rpm. Serum was removed using a pipette then placed in a cryovial. Each sample was labelled by dog identification number (ID), diet, date and specimen type. All samples were stored at -70^{o}C, shipped on dry ice then stored at -80^{o}C at the laboratory until analysis was performed.

Urine

Urine collection was done at baseline and once weekly. After the morning feeding, dogs were placed in metabolism cages with urine jars attached for overnight collection. Urine samples were collected the following morning prior to feeding. Each dog was palpated to determine if the bladder was distended with urine. If the bladder could be palpated, cystocentesis was performed to obtain a first morning urine sample. If no bladder could be palpated, the urine collected overnight in the urine jars was collected as the sample. Four to six milliliters (mls) of urine was placed in tubes labelled with the dog ID, diet, date and specimen type.

All samples were collected using sterile syringes then stored at -70^{o}C until shipped and stored at -80^{o}F until analysis was performed.

4.2.5 Sample processing – serum

4.2.5.1 Chemicals and Reagents

Monobasic (NaH_2PO_4), and dibasic (Na_2HPO_4) sodium phosphate were obtained from Fisher Scientific (Waltham, MA). Deuterium oxide (D_2O), DLM-4-100, 99.9%D and sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS-D6) were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). Pooled normal Human Red Cross plasma was used as quality control (QC) samples.

4.2.5.2 Preparation of Phosphate Buffer

Buffer solution was prepared by dissolving 928.6 mg of anhydrous NaH_2PO_4 and 320.9 mg of Na_2HPO_4 in 80 ml D_2O in a volumetric flask. This was made to a 1/3 mM concentration by adding 333.3 µl of 1.0 M DSS-D6 stock solution. The pH was adjusted to 7.4 and was brought to

a volume of 100 ml with D_2O and mixed well. The pH was rechecked and the buffer stored at 4^oC until use.

4.2.5.3 NMR sample preparation

The serum and QC samples were mixed with 100% cold methanol 1:2 ratio (v/v) in Eppendorf tubes on ice. The mixture was vortexed and incubated at -20^oC for 20 minutes then centrifuged at 14 000rpm for 30 minutes. The supernatant was transferred to a new 1.5ml Eppendorf tube and dried using a speed-vac concentrator. The pellet was again suspended in 600µl of phosphate buffer and each sample tube vortexed to ensure complete dissolution. Transfer of 590µl into a 5mm NMR tube was performed after centrifugation of the solution for 10 seconds. All samples were refrigerated at 4^oC until transfer to the NMR instrument.

4.2.6 Sample processing – urine

4.2.6.1 Chemicals and Reagents

Potassium dihydrogen phosphate (KH_2PO_4), potassium hydroxide (KOH) and hydrochloric acid (HCL) were obtained from Fisher Scientific (Waltham, MA). Sodium azide (NaN3 was obtained from United States Biochemical (Cleveland, OH). Deuterium oxide (D_2O), 99.9 atom % D and sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS-D6), 98 atom % D were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). Pooled quality control (QC) urine (ethanol, drug and nicotine free) samples were obtained from Golden West Biologicals, Inc. (Temecula, CA).

4.2.6.2 Preparation of Phosphate Buffer (1.5 M KH_2PO_4 buffer)

Buffer solution was prepared by dissolving 20.4g of the KH_2PO_4 80ml of D_2O and mixing with a solution of 133mg of DSS-D6 and 13mg of NaN3 in 10ml of D_2O via sonication. The pH was adjusted to 7.4 and the solution brought to 100ml in a volumetric flask by adding D_2O. It was mixed thoroughly, the pH rechecked and stored at 4^oC.

4.2.6.3 NMR sample preparation

Samples were thawed at 4^oC then for each one, 650 – 750µl of urine was centrifuged at 12000g for 5 minutes at this same temperature. 60µl of buffer was added to 540µl aliquot of study samples and QC samples each in 1.5ml Eppendorf tubes then vortexed for 2 minutes. 590µl of supernatant was transferred into 5mm NMR tubes and samples keep refrigerated at 4^oC until transfer to the NMR bay.

4.2.7 NMR data acquisition and processing

Serum and urine samples were run on an Avance III HD 600 MHz Bruker NMR spectrometer equipped with a 5-mm cryoprobe and Bruker SampleJet cooled to 5.6°C. One dimensional nuclear Overhauser enhancement spectroscopy (1D-NOESY PR) with water suppression (McKay, 2011) was collected on both serum and urine samples for data quantification and analyses. Two dimensional ^1H-^{13}C Heteronuclear Single Quantum Coherence (HSQC) and ^1H-^{13}C HSQC-1H-1H Total Correlated Spectroscopy (HSQC-TOCSY) (Bodenhausen & Ruben, 1980) spectra on internal pooled samples were collected on both serum and urine samples for metabolite identification. The spectra were processed using NMRPipe (Delaglio et al., 1995)

software or Bruker Topspin 4.0.7 software, and in-house MATLAB scripts (https://github.com/artedison/Edison_Lab_Shared_Metabolomics_UGA).

4.2.8 NMR statistics

4.2.8.1 One-way analysis of variance (ANOVA) for repeated measures with Tukey post hoc analysis

Samples from each week were selected to perform analyses and statistics. One dimensional proton nuclear magnetic resonance (1D ^{1}H-NMR) data were normalized using probabilistic quotient normalization (PQN). Non-overlapping peaks were selected from full resolution spectra (Fig. 4.1) to perform spectral binning using in-house MATLAB scripts (https://github.com/artedison/Edison_Lab_Shared_Metabolomics_UGA). A total of 53 features for serum and 79 features for urine were selected from the respective full resolution spectra, and the area under curve of selected features were integrated as relative intensities for each feature followed by univariate statistics. Initially, the Levene's test was performed on each feature to confirm the homoscedasticity. Features that passed the Levene's test were then used to detect differences between different diet types using one-way ANOVA for repeated measures, with p-value set at < 0.05. Tukey HSD post-hoc multiple comparisons tests were performed on significant features with the family-wise error rate (FWER) p-value adjusted to 0.1. Metabolites were identified using COLMARm (Bingol, Li, Zhang, & Bruschweiler, 2016). The metabolites were assigned a confidence level ranging from 1 to 5 according to criteria (Walejko, Chelliah, Keller-Wood, Gregg, & Edison, 2018).

The differences between relative intensities of metabolites during weeks one to four of feeding were determined using one way ANOVA for repeated measures with Tukey post-hoc

analysis with FWER < 0.1 to identify fluctuations in metabolite concentrations when dogs were consuming the same diet.

4.2.8.2 Boxplot analysis

Samples from each week with each food type were selected to generate boxplots. The area under the curve for selected features were integrated to calculate relative intensities as previous described. Samples were then grouped by its food type and week.

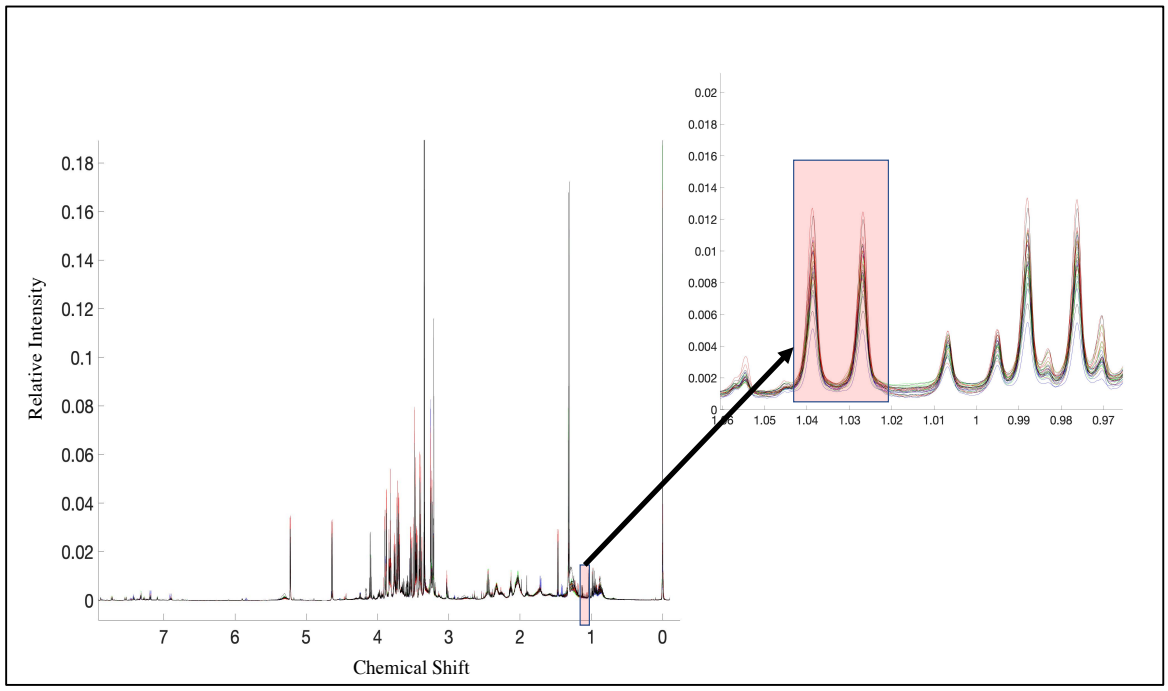


Figure 4.1 Full resolution 1D ^{1}H NMR spectra showing magnified image (on the right) of spectral binning of non-overlapping peaks selected as individual features for serum and urine.

Abbreviation: 1D ^{1}H NMR – one dimensional proton nuclear magnetic resonance

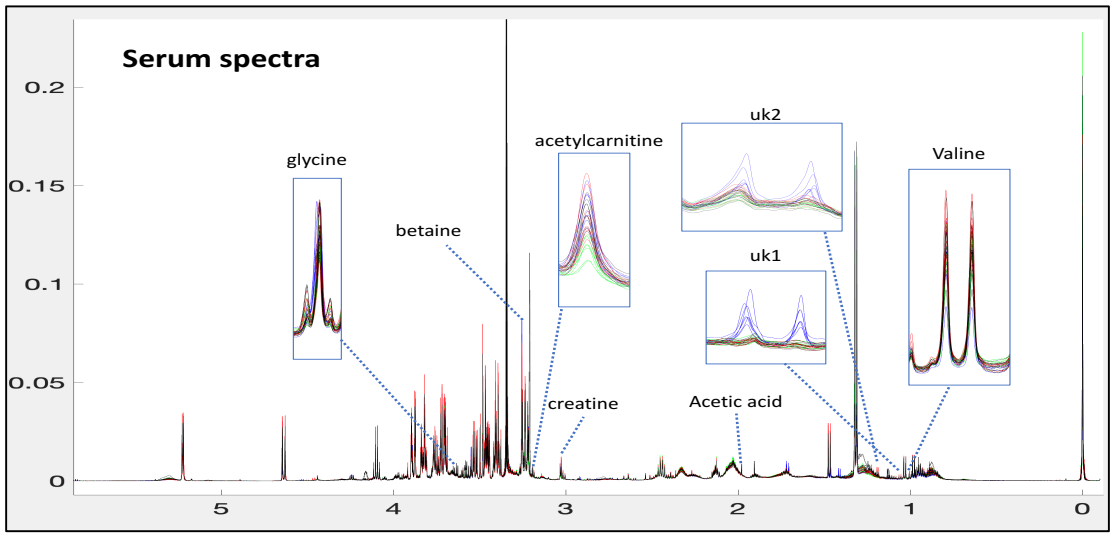
4.2.8.3 Partial least squares regression analysis

Significant metabolites selected by one-way ANOVA for repeated measures in both serum and urine were identified and assigned a confidence score as described above. Sample mean values for each week were used to predict CML values in dog food. Partial least squares regression was used to train the data with leave-one-out cross-validation method and calibrated $R^2 = 0.72$. Important metabolites were selected by variable importance in projection score (VIP score). All metabolites with VIP score ≥ 1.0 were selected to re-train the model. The calibrated R^2 increased to 0.77 after model re-training.

4.3 RESULTS

Spectral binning of non-overlapping peaks of full resolution spectra (Fig. 4.1) resulted in selection of 53 features for serum samples and 79 features for urine samples. Global ANOVA for repeated measures identified ten features for serum and six features for urine that were significantly different between diets for each week of feeding. Metabolites were identified using the COLMARm database (Bingol et al., 2016) as eight serum metabolites (Fig. 4.2a) and six urine metabolites (Fig. 4.2b).

a



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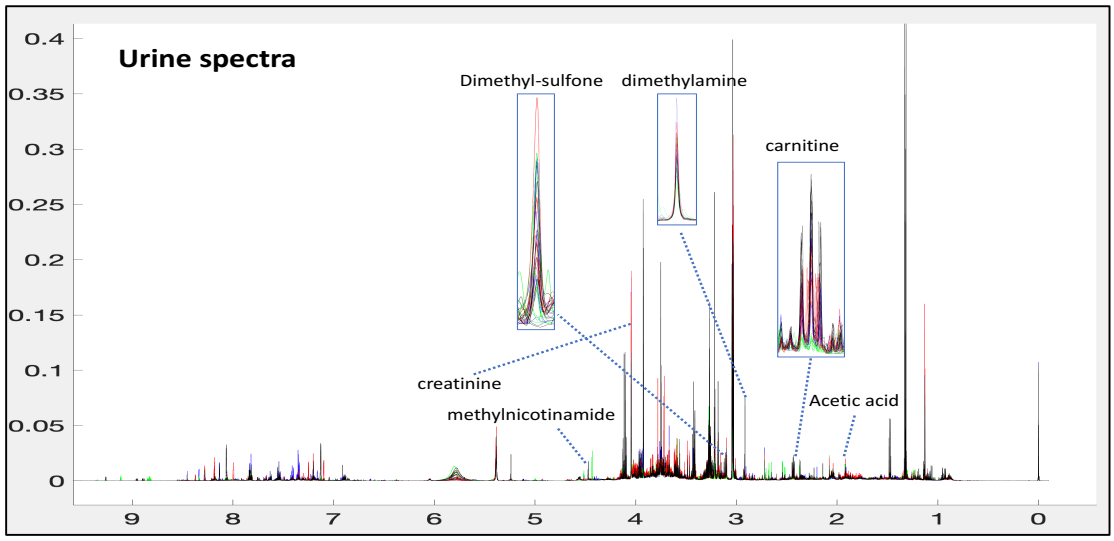


Figure 4.2. Full resolution 1D ^{1}H NMR spectra of serum samples (a) showing peaks selected as features that were identified as eight serum metabolites: glycine, betaine, acetylcarnitine, creatine, acetic acid, valine and unknown metabolites 1 (uk1) and 2 (uk2); and urine samples (b) showing peaks selected as features that were identified as six urine metabolites: creatinine, dimethyl-sulfone, methylnicotinamide, dimethylamine, carnitine and acetic acid.

Abbreviation: 1D NMR – one dimensional nuclear magnetic resonance

4.3.1 Serum metabolic response to the four diets

Changes in mean relative intensities for individual serum metabolites for the same diet over each week of feeding (Appendix N) were minimal with few statistically significant differences between weeks one to four of feeding observed only for valine, acetic acid, creatine and the unknown metabolites as seen in Table 4.3.

Table 4.3 Serum metabolite concentrations that were significantly different ($p < 0.05$) during the four weeks of feeding.

Metabolite	Diet	Weeks	Mean Difference	p-value	95% Confidence Interval	
					Lower	Upper
Valine	ADF	2 vs. 4	0.07	0.0567	0.03	0.13
Acetic acid	ADF	1 vs. 3	-0.03	0.0953	-0.05	0.00
		1 vs. 4	-0.04	0.0080	-0.07	-0.01
Creatine	RF	1 vs. 3	-0.03	0.0356	-0.05	0.00
Unknown 1	WF	1 vs. 3	0.05	0.0170	0.01	0.09
		1 vs. 4	0.04	0.0456	0.01	0.08
Unknown 2	WF	1 vs. 3	0.11	0.0195	0.03	0.19
		1 vs. 4	0.09	0.0842	0.00	0.18

Abbreviations: ADF – air-dried; RF – mildly cooked/raw; WF – canned wet

Statistical differences between diets for the eight serum discriminatory metabolites over the four week feeding trial are provided in Appendix O. Table 4.4 gives the mean values for the eight serum metabolites end of the feeding each diet and statistical differences between diets are listed in Table 4.5. Boxplots show these changes in metabolites for each week of feeding (Fig. 4.3) and after four weeks of feeding (Fig. 4.4) all four diets.

Table 4.4. Means and standard deviations (SD) of the relative intensities of each metabolite observed to be significantly different in serum between diets at the end of the four-week feeding trial.

Metabolite	**Diets**			
	Canned Wet	**Dry Kibble**	**Air Dried**	**Mildly cooked/Raw**
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Valine	$0.47^{a} \pm 0.07$	$0.51^{a,b} \pm 0.05$	$0.56^{b} \pm 0.05$	$0.52^{a,b} \pm 0.06$
Glycine	$0.50^{a} \pm 0.07$	$0.41^{b} \pm 0.05$	$0.44^{a,b} \pm 0.07$	$0.42^{b} \pm 0.04$
Acetic acid	$0.25^{a} \pm 0.01$	$0.25^{a} \pm 0.01$	$0.24^{a} \pm 0.01$	$0.31^{b} \pm 0.02$
Creatine	$0.14^{a,b} \pm 0.03$	$0.12^{a} \pm 0.04$	$0.18^{b} \pm 0.04$	$0.14^{a,b} \pm 0.02$
Acetylcarnitine	$0.17^{b} \pm 0.02$	$0.13^{a} \pm 0.01$	$0.16^{b} \pm 0.02$	$0.17^{b} \pm 0.02$
Betaine	$1.19^{b} \pm 0.38$	$0.99^{a,b} \pm 0.19$	$1.22^{b} \pm 0.25$	$0.82^{a} \pm 0.18$
Unknown 1	$0.33^{a} \pm 0.03$	$0.23^{b} \pm 0.02$	$0.23^{b} \pm 0.02$	$0.23^{b} \pm 0.02$
Unknown 2	$0.89^{a} \pm 0.11$	$0.77^{b} \pm 0.05$	$0.82^{a,b} \pm 0.06$	$0.78^{b} \pm 0.07$

a, b - superscript letters within rows indicate significant differences between diets at $p < 0.05$.

Table 4.5. Significant differences (FWER at 0.1) between diets for serum metabolites at week four after feeding.

Metabolite	ppm	Diet 1	Diet 2	Mean Difference (2-1)	p-adj	95% Confidence Interval	
						lower	upper
Valine	1.0387	WF	ADF	0.09	0.0266	0.01	0.18
Glycine	3.5495	WF	DF	-0.10	0.0265	-0.18	-0.01
		WF	RF	-0.08	0.0704	-0.16	0.00
Acetic Acid	1.9798	WF	RF	0.06	0.001	0.04	0.08
		DF	RF	0.06	0.001	0.03	0.08
		ADF	RF	0.07	0.001	0.05	0.09
Creatine	3.918	DF	ADF	0.05	0.0124	0.01	0.10
Acetylcarnitine	3.1852	WF	DF	-0.04	0.0014	-0.06	-0.01
		DF	ADF	0.03	0.0084	0.01	0.05
		DF	RF	0.03	0.0044	0.01	0.06
Betaine	3.2562	WF	RF	-0.37	0.0483	-0.74	0.00
		ADF	RF	-0.40	0.0249	-0.75	-0.04
Unknown1	1.4168	WF	DF	-0.10	0.001	-0.14	-0.07
		WF	ADF	-0.11	0.001	-0.14	-0.07
		WF	RF	-0.11	0.001	-0.14	-0.07
Unknown2	1.7189	WF	DF	-0.11	0.0378	-0.22	-0.01
		WF	RF	-0.11	0.0498	-0.21	0.00

Abbreviations: WF – canned wet food; DF – dry kibble; ADF – air-dried; RF – mildly cooked/raw, FWER – family-wise error rate

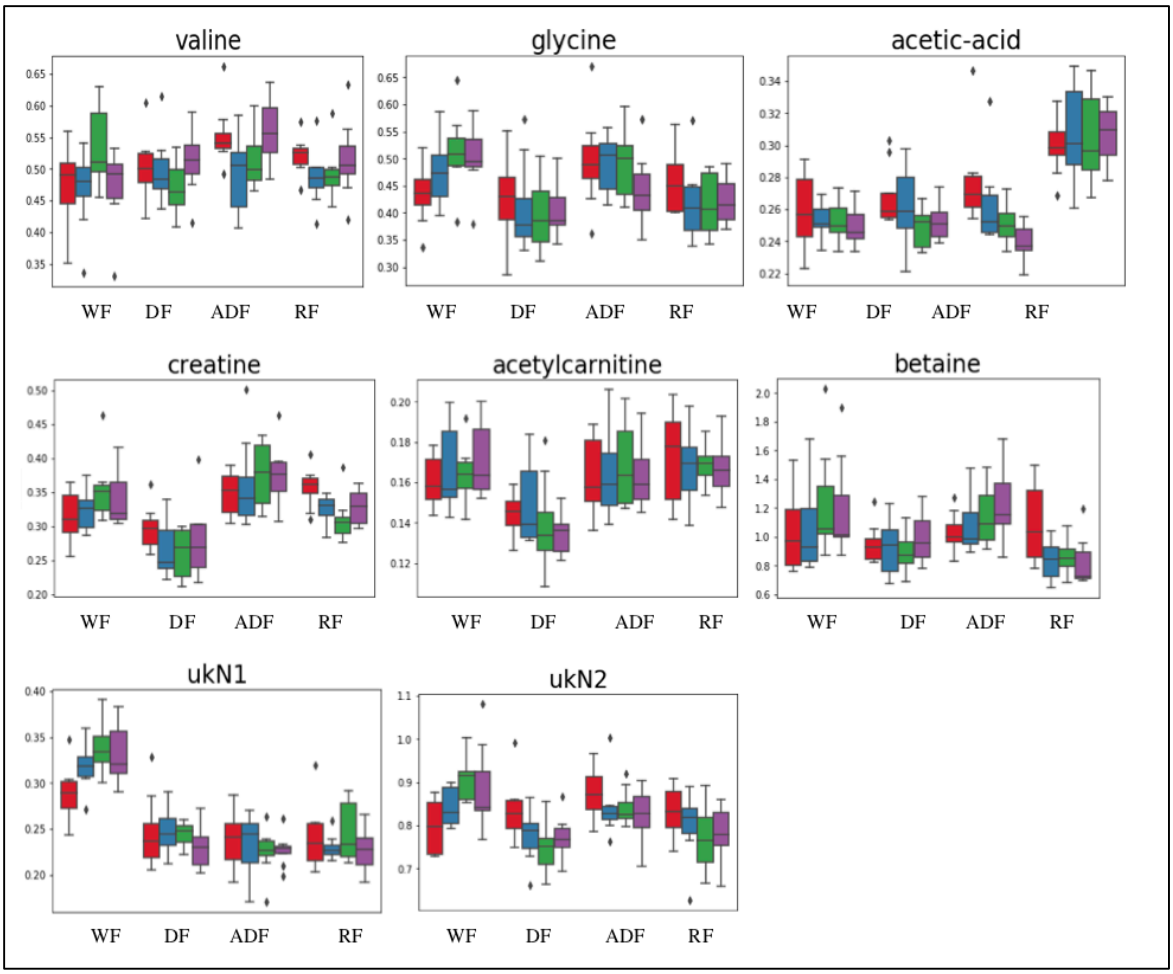


Figure 4.3. Boxplots showing the trends for each serum metabolite over the four-week feeding trial for all four diets. Box colors indicate different weeks: week 1 (red), week 2 (blue), week 3 (green) and week 4 (purple).

Abbreviations: WF – canned wet food; DF – dry kibble; ADF – air-dried; RF – mildly cooked/raw meat; ukN1 – unknown serum metabolite 1; ukN2 – unknown serum metabolite 2

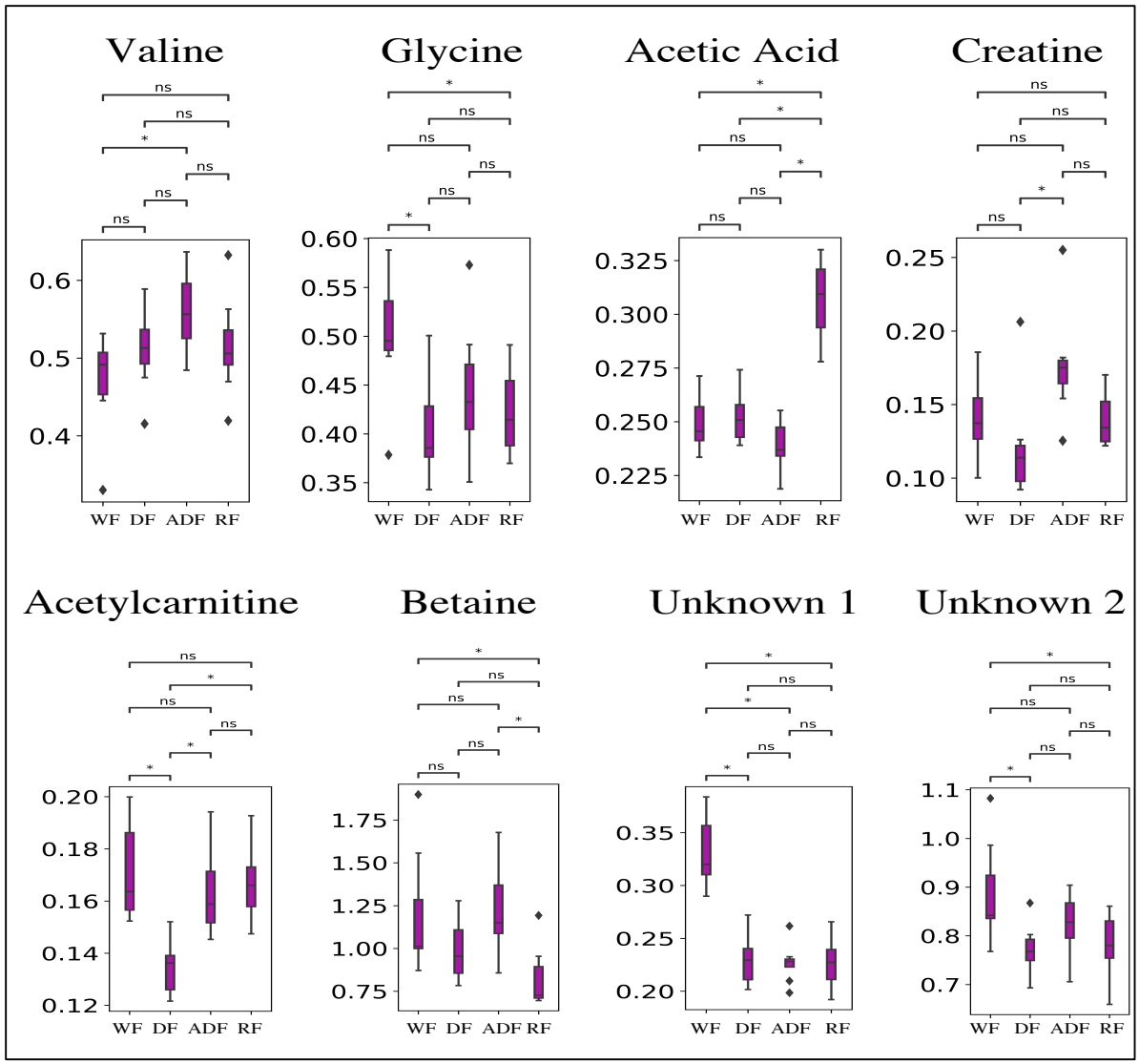


Figure 4.4. Boxplots showing the significant differences for each serum metabolite between all four diets at week four (end of feeding each diet).

* statistically significant difference family-wise error rate (FWER) < 0.1

ns – no significance

Abbreviations: WF – canned wet; DF – dry kibble; ADF – air-dried; RF – mildly cooked/raw

Overall trends over the four weeks for each diet are shown in the boxplots for each metabolite in Fig. 4.3. Valine is highest with the air-dried diet and lowest with the canned wet diet both in weeks one and four; however, no significant differences were found for weeks two and three. Glycine was significantly higher with the canned wet diet compared with the low amount in the dry kibble and mildly cooked/raw diets by the fourth week (Fig. 4.4). Acetic acid was consistently highest with the mildly cooked/raw diet for all four weeks and significantly different from levels in all other diets. Creatine was highest with the air-dried diet and lowest with the dry kibble diet, a significant difference observed over all four weeks when these diets were compared. Acetylcarnitine was lowest with the dry kibble for all four weeks compared to all other diets. Betaine was lowest with the mildly cooked/raw diet and was different from both the canned wet and air-dried diets by week four (Fig 4.4). The two unknown metabolites also showed differences between diets that were consistent over the feeding period. Unknown serum metabolite 1 was highest with the canned wet diet and was consistently different from all other diets as early as week two while unknown serum metabolite 2 increased with the canned wet diet by week two of feeding to show differences between the canned wet and mildly cooked/raw as well as canned wet and dry kibble diets by end of the dietary treatments.

4.3.2 Urine metabolic response to the four diets

Mean relative intensities of the discriminatory urine metabolites (Appendix P) as well as significant differences between diets for each of the four weeks (Appendix Q) were recorded. There were no differences in urine metabolite concentrations from weeks one to four of feeding on the same diet; however, the urine metabolites found to be significantly different between the diets at the end of feeding (week four) were acetic acid, dimethylamine, dimethyl-sulfone creatinine, carnitine, and methylnicotinamide. Means and standard deviations for the urine

metabolites for each diet at week four and the significant differences between diets are listed in Tables 4.6 and 4.7 respectively.

Table 4.6. Means and standard deviations (SD) of relative intensities for each urine metabolite at the end of the feeding trial.

Metabolites	Diets			
	Canned Wet	**Dry Kibble**	**Air Dried**	**Mildly cooked/Raw**
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Acetic acid	$0.56^{a,b} \pm 0.12$	$0.49^{a} \pm 0.06$	$0.64^{b} \pm 0.14$	$0.51^{a,b} \pm 0.08$
Carnitine	$0.85^{b} \pm 0.16$	$0.40^{a} \pm 0.07$	$1.01^{b} \pm 0.15$	$1.31^{c} \pm 0.22$
Dimethylamine	$0.34^{a,b} \pm 0.09$	$0.31^{a} \pm 0.07$	$0.40^{b} \pm 0.03$	$0.31^{a} \pm 0.03$
Dimethyl-sulfone	$0.37^{a} \pm 0.03$	$0.26^{b} \pm 0.07$	$0.39^{a} \pm 0.05$	$0.31^{b} \pm 0.01$
Creatinine	$3.07^{a} \pm 0.71$	$2.31^{b} \pm 0.65$	$3.55^{a} \pm 0.44$	$2.36^{b} \pm 0.22$
Methylnicotinamide	$0.28^{a} \pm 0.05$	$0.18^{b} \pm 0.07$	$0.25^{a,b} \pm 0.06$	$0.35^{a,c} \pm 0.10$

a, b and c - superscript letters within rows indicate significant differences at $p < 0.05$.

Table 4.7. Significant differences between diets for urine metabolites at week four after feeding.

Metabolite	ppm	Diet 1	Diet 2	Mean Difference	p-adj	95% Confidence Interval	
						lower	upper
Acetic Acid	1.9189	DF	ADF	0.15	0.036	0.01	0.30
Carnitine	2.4362	WF	DF	-0.45	0.001	-0.66	-0.23
		WF	RF	0.46	0.001	0.24	0.68
		DF	ADF	0.60	0.001	0.39	0.82
		DF	RF	0.91	0.001	0.69	1.13
		ADF	RF	0.31	0.0042	0.08	0.53
Dimethylamine	2.7205	DF	ADF	0.09	0.0552	0.00	0.17
		ADF	RF	-0.09	0.0603	-0.17	-0.01
Dimethyl sulfone	3.1483	WF	DF	-0.11	0.001	-0.18	-0.04
		WF	RF	-0.0625	0.0889	-0.1235	-0.0015
		DF	ADF	0.13	0.001	0.07	0.20
		ADF	RF	-0.09	0.0109	-0.16	-0.02
Creatinine	4.0454	WF	DF	-0.76	0.0452	-1.51	-0.01
		WF	RF	-0.7142	0.0796	-1.3965	-0.0319
		DF	ADF	1.24	0.001	0.49	1.99
		ADF	RF	-1.19	0.0014	-1.96	-0.41
Methylnicotinamide	4.4725	WF	DF	-0.10	0.037	-0.20	0.00
		DF	RF	0.17	0.001	0.07	0.28
		ADF	RF	0.11	0.0383	0.00	0.21

Abbreviations: WF – canned wet food; DF – dry kibble; ADF – air-dried; RF – mildly cooked/raw

Significant differences between diets at the end of feeding each diet are listed in Table 4.7 and depicted as boxplots showing trends over the four weeks of feeding (Fig. 4.5) and at four weeks at the end of feeding (Fig. 4.6). It was observed that the differences in response to diets were more consistent and occurred earlier in urine than for serum.

Acetic acid was consistently highest with the air-dried diet and was lowest with the dry kibble diet showing varied statistical differences between diets over the four weeks (Fig. 4.5). At the end of the four weeks, the only significant difference was between the air-dried diet and the dry kibble diet having the lowest acetic acid mean relative intensity by that time (Fig.4.6). Dimethylamine was also highest with the air-dried diet for all four weeks with variation in statistical differences between diets over the course of feeding except for air-dried and dry kibble comparison which, was observed to be significantly different for each week of feeding. Dimethyl-sulfone was consistently highest in the air-dried diet followed in decreasing order by the canned wet diet, the mildly cooked/raw diet and lowest with the dry kibble. Significant differences between diets showed some variation over feeding; however, for all four weeks differences were observed between the dry kibble and the canned wet diet as well as the dry kibble and air-dried diet. Creatinine was consistently highest with the air-dried diet as seen with other urine metabolites: dimethyl-sulfone, dimethylamine and acetic acid (Table 4.6). Differences in creatinine concentrations between all diets were variable over the four weeks but there were consistent statistically significant differences between the dry kibble and canned wet food, the dry kibble and air-dried as well as the air-dried and mildly cooked/raw diets.

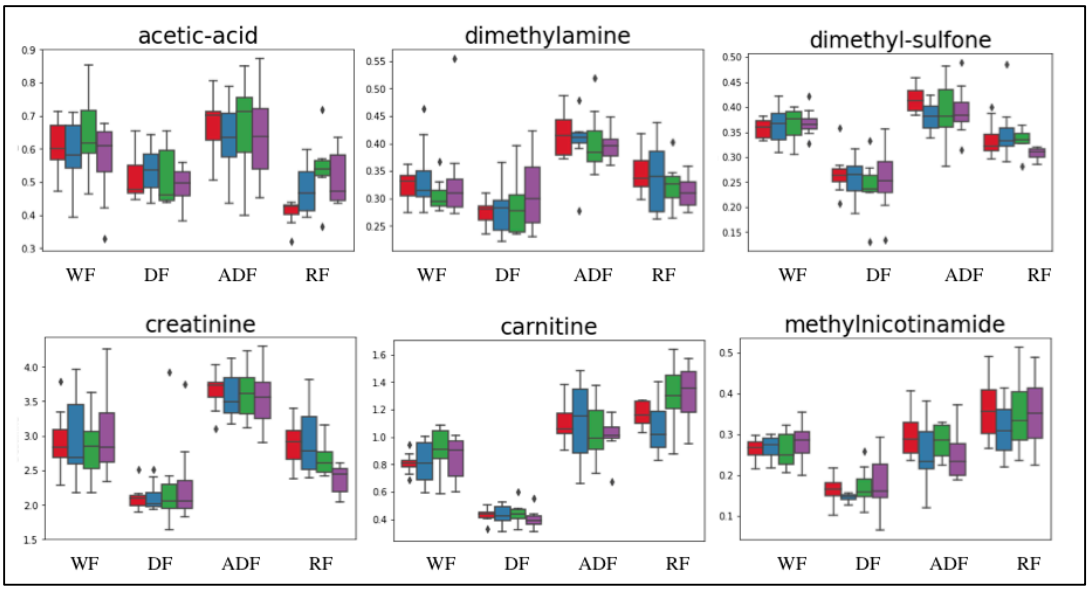


Figure 4.5. Boxplots showing the trends for each urine metabolite over the four-week feeding trial for all four diets. Box colors indicate different weeks: week 1 (red), week 2 (blue), week 3 (green) and week 4 (purple).

Abbreviations: WF – canned wet food; DF – dry kibble; ADF – air-dried food; RF – mildly cooked/raw meat

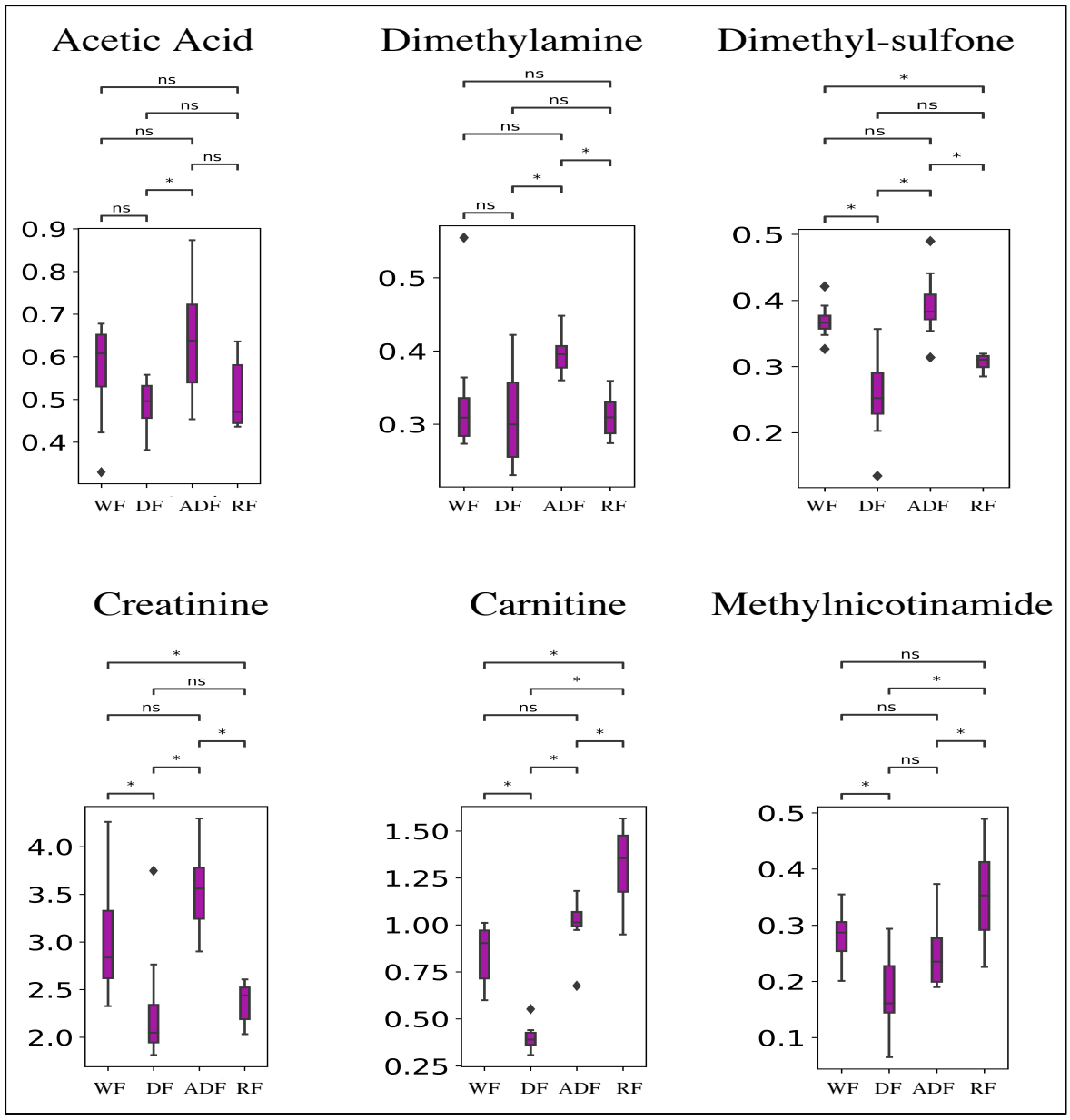


Figure 4.6. Boxplots showing the significant differences for each urine metabolite between all four diets at week four (end of feeding each diet).

* statistically significant difference family-wise error rate (FWER) < 0.1

ns – no significance

Abbreviations: WF – canned wet; DF – dry kibble; ADF – air-dried; RF – mildly cooked/raw

Carnitine was lowest with the dry kibble and about doubled with the canned wet food (Table 4.6). Significant differences for week four were observed between dry kibble and all other diets, between the air-dried and mildly cooked/raw as well as the canned wet and mildly cooked/raw diets (Fig. 4.6). Methylnicotinamide was highest with the mildly cooked/raw diet and lowest with the dry kibble showing significant differences in weeks three and four between the dry kibble and canned wet, dry kibble and mildly cooked/raw as well as air-dried and mildly cooked/raw diets (Fig. 4.6).

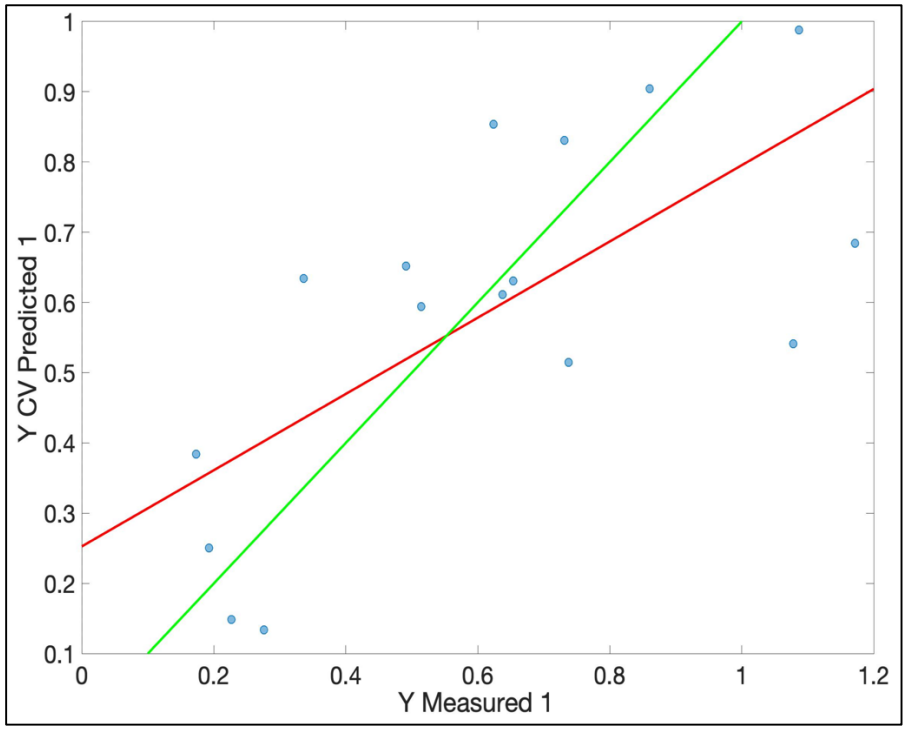
4.3.3 Partial Least Squares Regression (PLSR) Analysis

Partial least squares regression analysis was performed on VIP (variable importance in projection) score selected metabolites (serum - creatine, acetic acid and acetylcarnitine; urine - carnitine and methyl nicotinamide) resulting in $R^2 = 0.77$ for prediction of CML quantity in the diets (Fig. 4.7). In this model, these metabolites were selected to be good predictors of the dietary CML intake but did not produce similar results for CEL, MG-H1 or total dietary AGEs (Table 4.8).

Table 4.8. Coefficients of determination (R^2) values after performing partial least squares regression (PLSR) analysis on selected serum and urine metabolites for prediction of individual and grouped dietary advanced glycation end products (AGEs).

AGEs	R^2
CML	0.77
CEL	0.67
MG-H1	0.39
CML + CEL	0.60
CML + CEL + MG-H1	0.52

a



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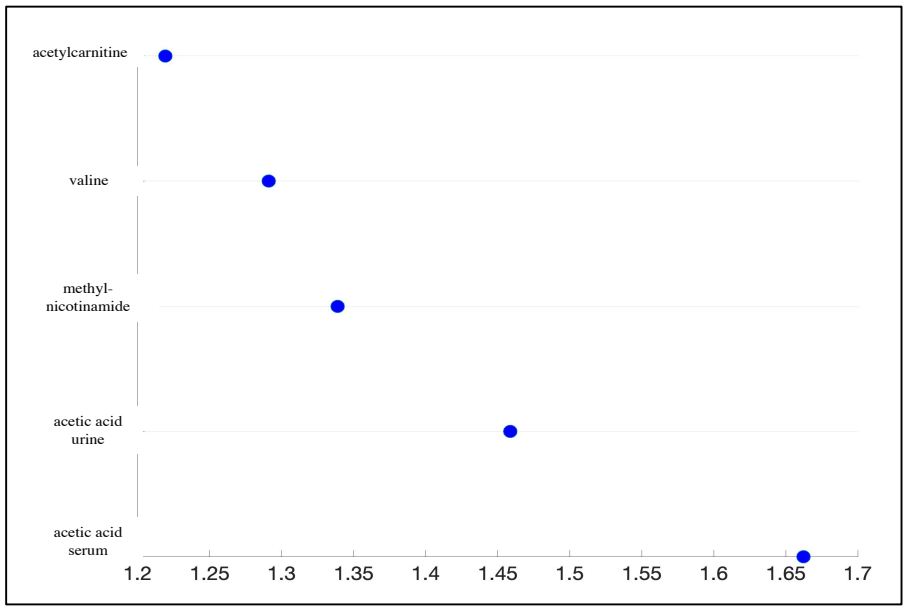

Figure 4.7. Model prediction plot (a) for carboxymethyllysine (CML) showing the fitted line for $R^2 = 0.77$ (red) vs. x=y line (green) as reference. Variable importance in projection (VIP) scores plot (b) – VIP scores (x-axis) for the top five selected metabolites (y-axis).

4.4 DISCUSSION

Both serum and urine metabolites changed in response to feeding these diets suggesting that different processing methods and possibly varied amounts of dietary AGEs can influence metabolic profiles in dogs on different diets. Additionally, while there were differences in serum and urine metabolome after four weeks of feeding each diet, it was observed that metabolite concentration within each four-week period corresponding to the consumption of the same diet were not statistically significant. In serum, only three known metabolites (valine, acetic acid and creatine) were different between week one, the start of a new diet, and other weeks (Table 4.3). In urine, there were no differences in metabolite concentrations between weeks one to four when the same diet was consumed. These findings suggest that after one week of a dietary change, the canine serum and urine metabolome responds to the new diet allowing metabolites to reach steady-state for the duration of feeding. Fluctuations as a result of biological variation or individual dog response may occur which, should be investigated in further canine metabolomic studies related to discrimination between diets.

At this time, there is no feasible way to determine the direct effect of dietary AGEs on the metabolome of biofluids because of many confounding variables such as the degree of AGE formation in the diets, individual response to diets, genetic predisposition to disease and the vast range of AGEs and MRPs that exist as part of the AGE pool. Research looking at the effects of several factors including metabolites found that extruded food was less digestible, increased circulating triglyceride levels and altered fecal metabolomics (Algya et al., 2018). Data on the effect of high heat processed foods on the canine blood and urine metabolome is sparse. Comparing the effects of the dry kibble with all the other diets provides an excellent opportunity to identify potential metabolic disturbances that may indicate how long-term feeding of this type

of diet may predispose susceptible dogs to certain diseases. In human beings and animals, metabolomics is proven to be a useful tool in determining the effect of diet on the body (Fave et al., 2009; A. Zhang et al., 2013), changes associated with the Western diet (Bouchard-Mercier et al., 2013) and revealing metabolic disturbances associated with dietary patterns that pose as risks for disease (Zabek et al., 2017).

4.4.1 Serum Metabolites

Amino acids found to be discriminatory between diets included valine, glycine and creatine. Valine is an essential amino acid and along with leucine and isoleucine form the group of branched-chain amino acids (BCAA) that were found to be higher in obese patients and positively correlated with insulin resistance (Newgard et al., 2009). The BCAA's have normal physiological roles in glucose and protein metabolism but may also have negative effects such as participating in glycation reactions with hemoglobin (valine) to form $HbA1_c$ and being negatively correlated with adiponectin suggesting interference with glucose homeostasis and fatty acid degradation (Nakamura et al., 2014). Valine was highest when dogs were fed the air-dried diet and lowest with the canned wet diet but was the only BCAA to show a difference between diets. Glycine is known as a conditional or non-essential amino acid with a vast range of biochemical functions. This amino acid was only significantly different between the two high-heat processed diets suggesting that elements of processing other than temperature and composition may play a role in influencing its serum levels. Creatine is obtained via the diet or made endogenously by the liver, pancreas and kidneys. Its primary role is in its phosphorylated form, phosphocreatine, used for regenerating adenosine triphosphate (ATP) in skeletal muscle after which, it is recycled and excreted by the kidneys as creatinine. After four weeks of ingestion of the diets, a significant difference in creatine levels was observed between the dry

kibble and air-dried diets only. The dry kibble was associated with persistently low creatine levels across all feeding treatments. Interestingly, urine creatinine levels were also lowest when the dry kibble was fed.

Acetic acid levels were greatest with the mildly cooked/raw diet and were distinct from the other diets. In the gut, acetic acid, propionic acid and butyric acid are the short-chain fatty acids (SCFAs) produced by the colonic microbiota in the ratio 60:20:20 to be assimilated by the host. The higher proportion of acetic acid in the blood in human beings allows it to be measured more readily than the smaller amounts of the other two acids (D. L. Topping & P. M. Clifton, 2001). This is likely the same for dogs. The colonocytes absorb ninety-five percent of SCFAs leaving only five percent to be excreted in feces (den Besten et al., 2013) indicating the importance and dependence on bacterial production of SCFAs by the host. Acetic acid in combination with coenzyme A is essential to carbohydrate and fat metabolism. The precise reason for the significantly higher acetic acid levels when the mildly cooked/raw diet was fed is unknown; however, the mildly cooked/raw diet was preserved using a product containing *Pediococcus acidilactici* which, is a lactic acid-producing bacteria (LAB). It is possible that high amounts of lactic acid fermentation by *Pediococcus* spp. led to acetic acid production by the bacteria themselves (Porto, Kuniyoshi, Azevedo, Vitolo, & Oliveira, 2017) or as a by-product of lactic acid fermentation in the mildly cooked/raw food. To the authors' knowledge, no metabolomics studies have investigated the effect of pediocin preserved raw diets on serum metabolites in dogs but if there is a shift to more natural diets for pets, this effect should be determined. Since acetic acid is key to energy metabolism, over-production by colonic microbiota and subsequent use of this substrate by the host may lead to obesity (Rahat-Rozenbloom, Fernandes, Gloor, & Wolever, 2014).

Acetylcarnitine is the acetic acid ester of carnitine involved in the oxidation of fatty acids by facilitating the movement of acetyl CoA (coenzymeA) into the matrices of mitochondria. Levels were lowest when the dogs were fed the dry kibble diet and different from all other diets. Carnitine measurements in the urine matched the serum acetylcarnitine findings although there were additional differences between diets. The main observation is that levels of acetylcarnitine in serum from ingestion of the dry kibble are significantly different from all other diets and correspond to measures of carnitine in urine which, is also lowest with this diet and is distinct from all other diets.

Betaine was lowest for the mildly cooked/raw diet being only significantly different from the air-dried diet by the fourth week of feeding. Betaine is obtained from the diet or is produced by the degradation of choline in mammalian cells along with dimethylglycine (DMG) (Xie et al., 2013). These three quaternary ammonium compounds are involved in several biological processes but are all metabolically linked in performing the enzymatic remethylation of homocysteine to methionine (Holm, Ueland, Kvalheim, & Lien, 2003). Betaine serves as the methyl group donor in this methylation reaction but also possesses anti-oxidant properties (M. Zhang et al., 2016), protects the kidney from inflammatory injury (Fan et al., 2014) and is known to increase carnitine production when added as a dietary supplement (Pekkinen et al., 2013). Deficiency of betaine in human beings and rodents predisposes to atherosclerosis and cardiovascular disease (Ganguly & Alam, 2015; Obeid, 2013), is associated with obesity, glucose and insulin insensitivity and other metabolic disorders (Sivanesan, Taylor, Zhang, & Bakovic, 2018). The mildly cooked/raw diet is associated with the lowest levels of betaine suggesting that the mildly cooked/raw diet itself is not a good source of betaine or its precursor

choline. Another suggestion is that betaine may be utilized in metabolic processes causing lowered serum levels.

Reasons for these differences in serum metabolites have not been determined but larger studies and longer feeding trials (Ryan et al., 2013) will be necessary to explore these differences between differently processed diets in dogs. We observed that serum metabolite concentrations associated with the canned wet diet, containing the highest total AGEs, was frequently determined to be statistically different from the other diets. This finding suggests that dietary AGEs may influence the serum metabolome and warrants further metabolomics studies to identity a possible metabolic signature.

4.4.2 Urine Metabolites

In dogs, acetic acid is a characteristic urinary metabolite that distinguishes it from the urine of human beings (Lee et al., 2019) making the presence of this compound a normal finding in the canine species that may change in response to diet. The biochemical role of acetic acid was outlined in earlier discussion since it was consistently highest in serum for all four weeks of feeding the mildly cooked/raw diet and was observed as a discriminatory metabolite between diets for both serum and urine. Excretion in the urine was highest with the air-dried diet for all weeks of feeding and by the fourth week, acetic acid level was only different from feeding the dry kibble. The mildly cooked/raw diet also was associated with low amounts of urinary acetic acid but was not statistically different from other diets. It is important to note that all mean relative intensity values of acetic acid in the serum, even for the highest numbers associated with the mildly cooked/raw diet, were lower than mean values for the urine. In the fourth week, acetic acid in the urine for the air-dried and dry kibble diets were 0.64 and 0.49 respectively while in serum, the highest mean relative intensity of this microbial metabolite was a much lower 0.31.

Furthermore, for the air-dried diet, the amount of acetic acid in the serum was measured as the lowest of all the diets at 0.24 by the end of feeding but was much higher at 0.64 in the corresponding urine sample. This finding indicates that far more acetic acid is excreted in the urine than exists in circulation in dogs.

Carnitine in the urine was significantly different between all diets except for the canned wet vs. the air-dried diet. It was highest with the mildly cooked/raw diet and over three times lower with the dry kibble. Carnitine can be obtained from the dogs' diet or synthesized as needed in the liver from lysine and methionine. It becomes esterified to acetylcarnitine to drive movement of acetyl CoA into the mitochondrial matrices to perform its key role in energy metabolism and fatty acid oxidation. A study using NMR plasma metabolomics in Labrador Retriever dogs to elucidate metabolic alterations related to overweightness found that obese dogs had lower plasma carnitine concentrations than lean dogs. It was proposed that lower carnitine may decrease lipid metabolism and that diet can affect plasma concentrations (Soder et al., 2019). In the serum of these dogs, acetylcarnitine was also significantly lowest with the dry kibble diet compared to all other diets. This study design could not confirm if high heat processing or dietary AGEs was the reason for carnitine and acetylcarnitine measurements being lowest in response to feeding the dry kibble diet in urine and serum respectively, but shows an inverse association with the high-heat extruded diet which, may be significant to lipid metabolism, obesity and its comorbidities in dogs. Carnitine has been implicated in dilated cardiomyopathy (DCM) in non-predisposed breeds of dogs possibly associated with consumption of grain-free diets (Freeman, Stern, Fries, Adin, & Rush, 2018) or as a result of high heat processing reducing digestibility and nutrient bioavailability. Whether reduced carnitine levels itself is the cause of DCM is debatable (Mansilla et al., 2019) but given the

distinct lower amounts of acetylcarnitine and carnitine associated with the grain-free, extruded, dry kibble diet used in this study, there is justification for further investigation into pet food processing and the Maillard reaction related to nutrient deficiency. Potential association of urine metabolomic profiles related to high heat processing, dog food ingredients and DCM was not a goal of this experiment; however, these findings may offer some insight into the current canine DCM discourse.

Dimethylamine (DMA) is another degradation product of choline but unlike betaine and DMG discussed earlier, is formed from by the gut microbiota along with trimethylamine (TMA) (Xie et al., 2013) and can be derived from other sources such as trimethylamine N-oxide (TMAO) and asymmetric dimethylarginine (ADMA) (Zabek et al., 2017). This secondary amine was highest in the urine for the air-dried diet, as was betaine in serum, but lowest with the dry kibble diet (betaine was lowest when the mildly cooked/raw diet was fed). This suggests that choline, as the precursor to these compounds, may have been high in the air-dried diet and that DMA excretion in the urine of dogs may be in response to diet. In rodents, gut microbial choline metabolism may play a role in diabetes (S. Zhang et al., 2008), nonalcoholic fatty liver disease (NAFLD) (Dumas et al., 2006) and cardiovascular disease (Z. Wang et al., 2011) all of which also have associations with either dietary AGEs, high heat processing of foods or the Western diet in human beings (Delgado-Andrade & Fogliano, 2018; Leung et al., 2016).

Like DMA excretion, dimethyl-sulfone and creatinine were both highest when the air-dried diet was fed and lowest with the dry kibble. However, unlike DMA, they were significantly different between the two high-heat as well as between the two low-heat processed diets. Dimethyl-sulfone ($DMSO_2$) is the primary metabolite of dimethyl sulfoxide (DMSO) known to be a normal compound in the plasma of human beings (Engelke et al., 2005) that is excreted in

urine. Creatinine is the excreted form of creatine often used as a measure of renal function since almost all is removed by glomerular filtration and proximal tubule secretion. Both creatine in the serum and creatinine in the urine displayed the same trends in mean relative intensities across the diets being highest with the air-dried diet with decreasing amounts with the canned wet, mildly cooked/raw and dry kibble diets in that order indicating their close metabolic relationship. Methylnicotinamide is the metabolic product of nicotinic acid (vitamin B3 – niacin) and is normally excreted in the urine of dogs (Lee et al., 2019). It was highest with the mildly cooked/raw diet and lowest when the dry kibble was fed. This suggests that vitamin B either had decreased bioavailability in the dry kibble diet or that ingestion of this type of processed food reduced the metabolism of nicotinic acid.

Despite the small sample size, these findings in serum and urine metabolomics imply that diet plays a role in blood and urine metabolic profiles in dogs and can be a reliable indicator of dietary effects in this species. Overall, the recurring observation is that consumption of the dry kibble diet relative to the other diets resulted in low amounts of metabolites in serum and urine that are needed for normal physiological function suggesting that extruded pet food may be deficient in nutrient bioavailability compared to the other diets in this feeding trial.

4.4.3 Prediction of advanced glycation end products in the diets based on metabolomics data.

Selected metabolites (Fig. 4.7b) were used to predict CML quantity in the diets using a regression model. The resultant R^2 value of 0.77 indicated that the model produced a favorable prediction and suggests that in this study, acetylcarnitine, valine and acetic acid in serum as well as methylnicotinamide and acetic acid in urine, correlate with dietary CML quantity. The model shows potential for use of canine serum and urine metabolomic profiles in determining

biomarkers for dietary AGE intake but did not hold for CEL and MG-H1 or total AGEs (Table 4.8).

Our hypothesis stated that the canine serum and urine metabolome will be altered in response to different thermally processed diets. These findings show that changes in metabolite concentrations were apparent between diets and that high heat processed diets, such as the canned wet that contained the highest total AGEs or the dry kibble that was associated with low metabolite concentrations, may have an etiologic role in development of diseases. However, our findings also revealed metabolic differences for comparisons between all pairs of diets indicating that additional studies are needed to explore such alterations. It is recommended that investigations utilize NMR spectroscopy to identify and quantify metabolites in dogs in response to differently processed diets. Future aims should include identifying a metabolic signature associated with high dietary AGE intake as well as the discovery of biomarkers related to diseases associated with high AGE levels in the canine body. Limitations of this study include the small sample size and relatively short feeding trial. Identification of serum and urine biomarkers will require more expansive and rigorous testing based on larger populations and longer-term experiments.

Footnotes

Animal Care and Use Approval:
This protocol with amendments was reviewed and approved by the Summit Ridge Farms' Institutional Animal Care and Use Committee (IACUC) in compliance with the Animal Welfare Act in addition to the IACUC at the University of Georgia.

Acknowledgements:
Mohammad Chegeni and Fariba Tayyari performed sample processing and preliminary data acquisition and analyses.

a – Summit Ridge Farms, Susquehanna, PA

CHAPTER 5

THE EFFECT OF FOUR DIFFERENTLY PROCESSED DIETS ON THE CANINE FECAL MICROBIOME

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[1] Siobhan Bridglalsingh, Joseph W. Bartges, Ruth Schade, Claire B. de La Serre, Parastoo Azadi, Arthur S. Edison, Rebecca L. Remillard. To be submitted to the *Journal of Animal Physiology and Animal Nutrition*.

Contributors

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Ruth Schade	– sample processing, data acquisition and statistical analyses
Joseph W. Bartges	– project design
Claire B. de La Serre	– data analyses and editing
Parastoo Azadi	– editing
Arthur S. Edison	– editing
Rebecca L. Remillard	– project design and editing

Abstract

Traditional pet food processing employs high temperatures to decrease microbial load of ingredients, increase shelf-life and storage while maintaining desirable texture and taste. High heat processed foods of the Western diet are associated with increased intake of dietary advanced glycation end products (AGEs) and diseases such as obesity, type 2 diabetes mellitus, cardiovascular diseases and atherosclerosis, and inflammatory conditions. Dysbiosis, known as an imbalance of gut microbiota, has been identified in obese human beings and rodents. We hypothesized that the canine fecal microbiome would be altered in response to differently processed diets that vary in AGE content. The four diets were two high heat processed - canned wet (WF) and dry kibble (DF), and two low heat processed - air-dried (ADF) and mildly cooked/raw (RF) that differed in quantities of three AGEs: carboxymethyllysine, carboxyethyllysine and methylglyoxal hydroimidazolone-1. A Latin square experimental design was used for eight laboratory colony dogs that were paired and fed each diet for four weeks in

random sequence over 16 weeks. Fecal and food samples were collected for each four-week feeding period. 16S rRNA gene sequencing data obtained via the Illumina MiSeq platform were analyzed using METAGENassist and Galaxy online platforms to reveal five predominant phyla: *Firmicutes*, *Bacteroidetes*, *Fusobacterium*, *Proteobacteria* and *Actinobacteria*. Several bacterial genera were different between diet groups and dysbiosis was identified as a decrease in α-diversity associated with the dry kibble diet. Only the low heat processed diets contained bacterial organisms, none of which were pathogenic, that were more numerous in the air-dried diet compared with the mildly cooked/raw diet and principal component analysis (PCA) showed that the microbial populations were distinctly different between these two diets. These results show that different processing methods influence the fecal microbiota and further studies are warranted to determine possible negative impacts on health.

5.1 INTRODUCTION

The canine gastrointestinal microbiome comprises an estimated trillion microorganisms, the majority of which are harbored in the distal gut (Hooda et al., 2012; Suchodolski, 2016). These organisms consist of bacteria, archaea, protozoa, fungi and viruses forming an intimate and symbiotic relationship with the host and are essential for normal host physiological processes (Suchodolski, 2011a). The colonic microbiota takes on multiple functions including metabolism, energy homeostasis, immunological processes, gut epithelial health and neurodevelopment (Cho & Blaser, 2012; Shreiner, Kao, & Young, 2015; Turnbaugh et al., 2007). Various factors can alter the intestinal microbiota resulting in effects in both the intestinal ecology and the host. Some of these factors include diet, medicine e.g. antibiotics, diseases especially gastrointestinal

illnesses, age of the host and several genetic and environmental influences (Blake & Suchodolski, 2016). The bacterial members as part of the gastrointestinal microbiota have been studied far more than other organisms, through the use of high-throughput sequencing platforms complemented by comparative metagenomics software.

The five predominant phyla in the adult canine intestinal tract are *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria* and *Actinobacteria*. Of these, *Firmicutes* and *Bacteroidetes* are in the highest proportion, similar to rats and human beings, whereas *Fusobacteria* may be either predominant or codominant with the aforementioned two in dogs only (Barko et al., 2018; Garcia-Mazcorro et al., 2012; Hand et al., 2013; Handl et al., 2011; Middelbos et al., 2010; Panasevich et al., 2015; Swanson et al., 2011). The primary role of colonic bacteria is the fermentation of non-digestible carbohydrates and resistant starches that host enzymes are unable to degrade. Fermentation is mediated by saccharolytic bacteria to produce straight-chain short-chain fatty acids (SCFAs) that decrease the pH of the colon. The SCFAs produced are acetate, propionate and butyrate normally in the ratio 3:1:1 to function in lipogenesis (systemic circulation), gluconeogenesis (liver) and as the major energy source for colonocytes, respectively (Cummings, 1981; Scott, Duncan, & Flint, 2008; Scott, Gratz, Sheridan, Flint, & Duncan, 2013; D. L. Topping & P. M. Clifton, 2001). Wild canids are carnivorous suggesting that fermentation should not play an essential role in meeting the caloric needs but the domesticated dog consumes commercial pet foods comprised of processed protein sources, added fiber and an array of components designed to ensure a balanced diet and healthy digestive system. It follows that pet dogs being fed commercial diets may not depend on fermentation to meet metabolic requirements but rather, depend on this process for maintaining a balanced gut microbial community and overall health (Hooda et al., 2012).

An imbalance of the microbial population is termed “dysbiosis”. It is defined as an alteration in the composition or richness (number) of the intestinal microbiota (Suchodolski, 2016). Several diseases are associated with dysbiosis in animals and human beings. Inflammatory bowel disease (IBD) (Vazquez-Baeza, Hyde, Suchodolski, & Knight, 2016) is one of the most common of the chronic enteropathies that has been extensively studied in dogs as well as human beings to elucidate changes in the microbiome in the hope of designing therapeutic protocols involving manipulation of the gastrointestinal microbiome (Minamoto et al., 2015; Redfern, Suchodolski, & Jergens, 2017; Suchodolski, 2016). A dysbiosis index (DI), as a single numerical value, has been developed as an assessment tool to identify deviation from normobiosis. The higher the value, the greater the degree of dysbiosis allowing its use as a monitor for response to therapy and return to normobiosis (AlShawaqfeh et al., 2017). This exploration is ongoing and it remains unclear as to whether dysbiosis is the cause or the effect of gastrointestinal diseases (Redfern et al., 2017).

Pet food processing began at the time of the First World War and evolved as the availability of meat and resources for processing changed (Gentzel, 2013). Presently, commercial pet food processing is done via a range of methods that give rise to many forms of food (van Rooijen et al., 2013). The common element to most of these methods is heat processing with varied degrees of moisture and additives. In general, heat processing of foods is not only necessary for food safety via reduction of bacterial numbers and stability for storage but also adds desirable flavor, aroma and color (ALjahdali & Carbonero, 2017; Poulsen et al., 2013). The reaction involved in the latter, as a result of heat application, is known as the Maillard reaction after the French chemist by whom it was first discovered. It is a non-enzymatic glycation reaction between the carbonyl group of reducing sugars and the amino groups in proteins. The

extensive range of heterogeneous compounds formed are known as Maillard reaction products (MRPs), and are further characterized based on the extent to which the reaction has proceeded. The reaction occurs in stages: early, intermediate, advanced and final, giving rise to compounds at every stage and at completion, produces melanoidins (ALjahdali & Carbonero, 2017).

For the purposes of this study, the focus was on the products at the advanced stage of the reaction known as advanced glycation end products (AGEs). The major AGEs most often used as biomarkers for the Maillard reaction are: carboxymethyllysine (CML), carboxyethyllysine (CEL), pentosidine, methylglyoxal-hydroimidazalone (MG-H1) and pyrraline (Poulsen et al., 2013; Snelson & Coughlan, 2019). Consumption of thermally processed foods by human beings has grown tremendously over the past few decades because of their convenience, cost and flavor. This has led to the concept of the "Western diet" consisting of high fats, simple sugars and thermally treated foods (Martinez et al., 2017) with increased consumption of AGEs known to be present in such foods (Delgado-Andrade, 2016). High consumption of AGEs in heat treated diets is related to diseases such as obesity and its comorbidities: diabetes mellitus, metabolic syndrome, cardiac and kidney disease, in addition to degenerative and neoplastic conditions (Uribarri et al., 2015). Several animal studies suggest that diets high in AGEs cause inflammation and oxidative stress leading to the development of these diseases but findings in people have been inconsistent (Kellow & Coughlan, 2015; Snelson & Coughlan, 2019). The Western diet is known to affect structure and function of the human gut microbiota (Martinez et al., 2017) and since obesity is also associated with microbiota alterations, especially the *Firmicutes* to *Bacteroidetes* ratio (Li, Lauber, Czarnecki-Maulden, Pan, & Hannah, 2017), there is likely an association between AGEs, obesity and the Western diet due to the common factor,

the high consumption of thermally processed diets (ALjahdali & Carbonero, 2017; Martinez et al., 2017).

This is particularly important for dogs who are fed commercially heat processed pet foods for long periods. Currently, no studies are investigating the direct effects of AGEs on the microbiota of dogs but work has been done *in vitro* as well as *in vivo* for both humans and other animals. Unfortunately, all the work completed so far have had inconsistent results and discrepancies making it difficult to arrive at definitive conclusions about the effect of AGEs on the gastrointestinal microbiome (Snelson & Coughlan, 2019).

This study aims to investigate the effect of varied quantities of AGEs in four diets, of the same nutritional composition but manufactured by different methods of processing, on the fecal microbiome of eight laboratory dogs. The four processing methods are retorting (canned wet), extrusion (dry kibble), air drying (air-dried) and slow, low heat cooking (mildly cooked/raw) applied to kangaroo meat and sweet potato fiber. Macronutrient sources and nutritional composition were kept similar to avoid variability due to components so that differences would likely be related only to the diets. The hypothesis stated for this experiment is that the fecal microbiota changes in response to differently processed diets. The results of this study will not establish causation but will be the first, according to the authors' knowledge, of its nature seeking to establish an association between dietary AGE quantity and the canine fecal microbiome.

5.2 MATERIALS AND METHODS

5.2.1 Animals

Eight purpose-bred laboratory colony Beagle dogs[a], 4 males and 4 females, all intact, ranging from 3 -7 years old were used. Their health status was confirmed through physical examination, complete blood cell counts, serum biochemical analyses and urinalyses (Appendices A – D). The study employed a Latin square experimental design (Appendix G) and ran for 16 weeks. Cages and bowls were cleaned and sanitized daily. Fresh tap water fit for human consumption was available *ad libitum* for the duration of the study period. Dogs were housed individually in cages under a 12-hour light-dark cycle with temperature range maintained within 50^{o} to 85^{o}F.

5.2.2 Study foods

Four diets of similar nutritional composition consisting of kangaroo meat and sweet potato but differing in AGE quantities were evaluated: 1 – high heat processed canned wet food (WF); 2 – high heat processed dry kibble (DF); 3 – an air-dried, low heat processed food (ADF); 4 – a low heat, mildly cooked/raw diet (RF). In this study, the high heat processing was performed at temperatures over 200^{o}F and low heat processing was done under 150^{o}F. Table 5.1 shows maximum temperatures used for processing of each diet. Both low heat processed diets were treated with a *Pediococcus acidilactici* fermentation product as a biological preservative and bactericidal agent. Nutritional composition of each diet in the study is provided in Appendix E.

Table 5.1. Method of processing and maximum temperatures used for all four diets.

Diet	Process	Maximum temperature (^{o}F)	Additional details
Canned Wet	Retorting	254	Treated with high temperatures for 60 – 90 minutes
Dry Kibble	Extrusion	265	After extrusion, food is dried to <10% moisture
Air-dried	Dehydration	140	Dried for 12 hours until <12% moisture
Mildly cooked/Raw	Light and slow cooking	105	Mildly cooked for 10 hours then frozen until feeding

5.2.3 Study Design

A Latin-square design (Appendix G) allowed for minimization of differences enabling comparison among diets consumed by the same dog. Four diet sequences were created for feeding to avoid differences due to the order of consumption of the diets. Pairs of dogs were assigned to one of four diet group sequences based on a table of random numbers. Each pair received the first diet in their sequence for four weeks, samples collected, dogs were then crossed over to the next diet until all dogs completed all diets. Samples were collected prior to beginning the sequence of feeding to obtain "baseline" record of clinical parameters. Feeding quantities were based on daily caloric requirement determined by body weight which was measured weekly and feeding amount adjusted accordingly (see Appendices H and J for weekly body weights and food consumption respectively) to maintain body weight within 5% of baseline. The canned wet and mildly cooked/raw diets were offered twice daily while the dry kibble and air-dried diets were offered once daily, all for a minimum of one hour.

5.2.4 Sample collection

The sample collection schedule for the study is provided in Appendix I.

5.2.4.1 Diets

For this experiment, all the food was processed at the same time in the same batch and stored for use during the feeding protocol. Diet samples were collected weekly, just as the stool samples, to be able to determine differences among feeding batches, if any. Food samples were stored in bags, frozen at -70^{o}C and shipped to the laboratory on dry ice. Samples were stored at -80^{o}C until laboratory analysis. Food samples were analyzed by the same methods as described for the feces.

5.2.4.2 Feces

Feces (5g) were collected four hours after the morning feeding directly from dogs' rectum via loop or gloved finger. If no sample was available, the overnight sample was collected and 5g extracted from the center of the specimen. Fecal samples were stored in 50ml centrifuge tubes, frozen at -70^{o}C, and shipped to the laboratory on dry ice. Samples were stored at -80^{o}C until analysis.

5.2.5 DNA extraction, 16S rDNA amplicon sequencing and raw data processing

Fecal and food DNA extraction was performed using the *Quick*-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA, USA) according to manufacturers' instructions with the modification of using 100 – 150mg of fecal sample and processing for ten minutes to ensure maximum cell lysis. The quality of the DNA was checked spectrophotometrically using a NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA)

PCR amplification was performed using the primer pairs 341F and 785R targeting the V4 region of the bacterial 16S rRNA gene. A MiSeq platform (Illumina, San Diego, CA, USA) at the

Georgia Genomics and Bioinformatics Core (GGBC) at the University of Georgia was used to perform sequencing of 2 x 250 to obtain FASTQ files that were processed using the Quantitative Insights into Microbial Ecology (QIIME) (Caporaso et al., 2010) pipeline (version 1.9.1) to establish operational taxonomic units (OTUs) and taxonomy assignments.

5.2.6 Statistical analyses

Taxonomic abundance data were normalized by log transformation and METAGENassist (http://www.metagenassist.ca/METAGENassist/faces/Home.jsp), a web server designed for comparative metagenomics analysis (Arndt et al., 2012), was used to determine phylogenetic quantification, differences in fecal communities between diet groups, fold change as well as principal component analysis for both diets and fecal microbiota. The Galaxy (Segata et al., 2011) online platform was used to perform linear discriminant analyses of the diets and feces. IBM SPSS® (Statistical Package for the Social Sciences) statistics software (version 23) was used to determine statistical differences between phylum and genus relative abundances, Shannon and Simpson indices as well as *Firmicutes*:*Bacteroidetes* ratio by one way analysis of variance (ANOVA) for repeated measures with Bonferroni adjustment for pairwise comparisons. Statistically significant differences were determined at $p < 0.05$.

5.3 RESULTS

5.3.1 Fecal microbiota analysis

5.3.1.1 Composition of the fecal communities of diet groups

The bacterial signature of each of the four diets was determined by linear discriminant analysis (LDA) and represented at a linear discriminant analysis effect size (LEfSe) plot (Fig. 5.1a). At

genus taxa level, the fecal community associated with the canned wet diet featured *Phascolarctobacterium*, *Prevotella*, *Clostridium* spp. and *Clostridium perfringens* all of which had high abundances for this diet group (Fig 5.2). The mildly cooked/raw diet group featured *Pediococcus acidilactici* as the signature of this fecal community (Fig. 5.1a) and was the organism with the highest abundance (Fig. 5.2). *Megamonas* had the highest abundance (Fig. 5.2) and together with *Lactobacillus*, were the signature organisms in the dry kibble diet group (Fig. 5.1a and b). The air-dried diet group featured *Fusobacterium* (Fig. 5.1a and b), having the highest abundance (Fig. 5.2) for this group, along with *Dorea* and *Turicibacter* (Fig. 5.1a and b). The cladogram based on these differences in communities (Fig. 5.1b) shows the clades formed by the ancestral relations among these organisms. The mildly cooked/raw group features abundance of *Bacilli* members, the dry kibble and canned wet diets were characterized by abundance of *Clostridia*, both classes of the phylum *Firmicutes* and the air-dried diet was associated with highest abundance of the class *Fusobacteriia* of the phylum *Fusobacteria*. Differences in bacterial members of the fecal microbiome between diet groups were determined using ANOVA as seen in Table 5.2. Original and normalized abundances are shown as boxplots in Fig. 5.2., for the 17 bacteria that were statistically different in the fecal microbiome among the four diet groups.

(a)

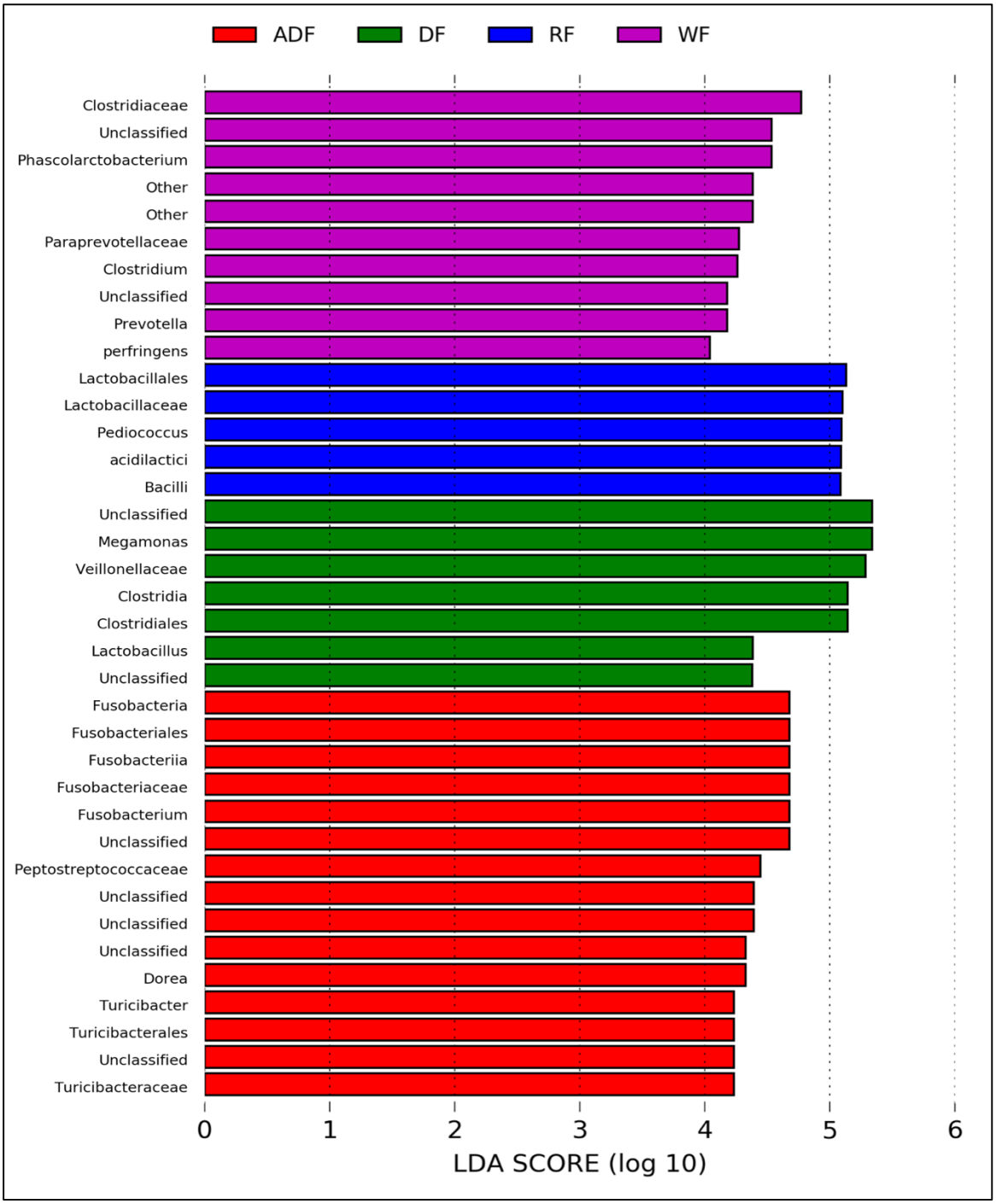

Figure 5.1a. Linear discriminant analysis effect size (LEfSe) plot constructed from LDA score of 4.0, showing the differences in fecal bacterial communities among the four diet groups.

(b)

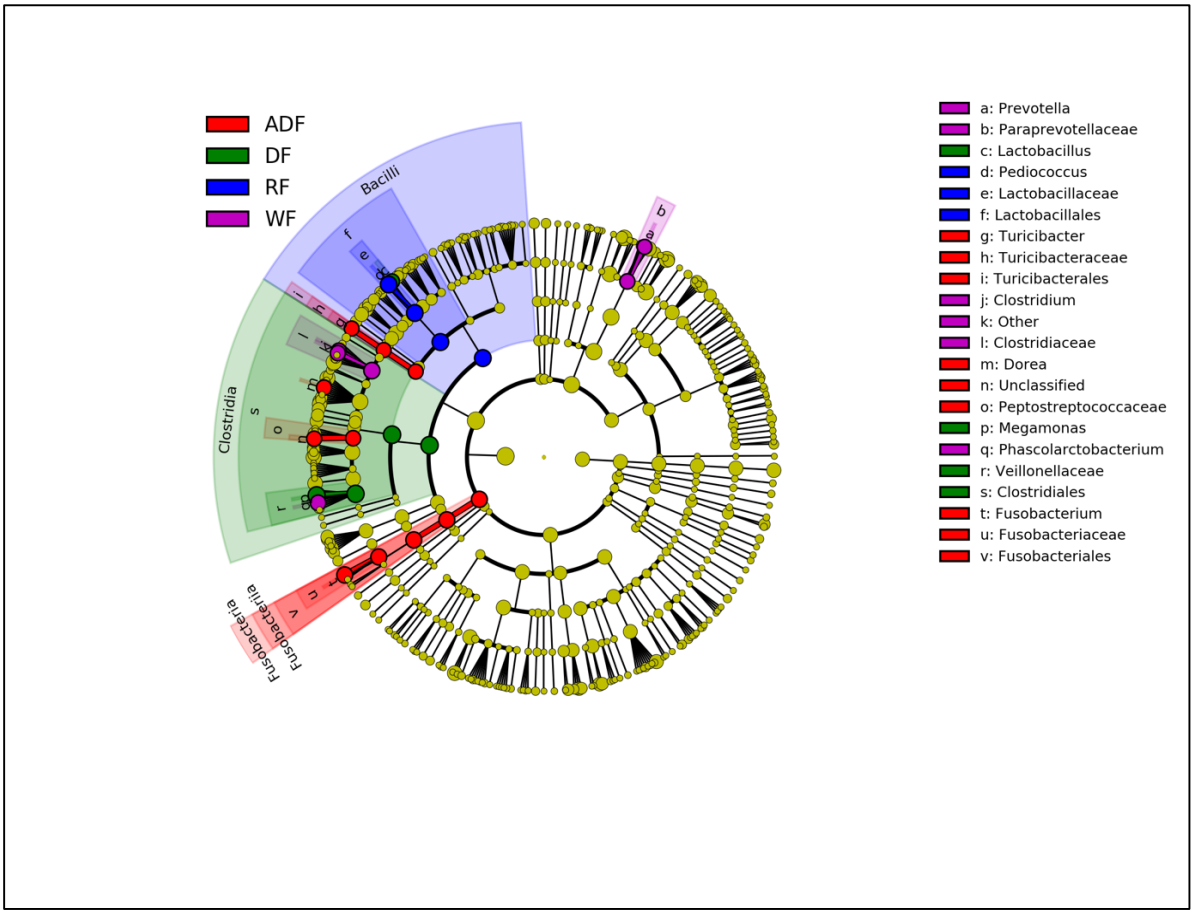

Figure 5.1(b). Cladogram constructed from LDA score of 4.0 showing ancestral relations of bacteria in different diet groups: ADF (red), DF (green), RF (blue) and WF (purple). Abbreviations: LDA – linear discriminant analysis; ADF – air-dried; DF – dry kibble; RF – mildly cooked/raw; WF – canned wet

Table 5.2 Differences in bacterial members among the four differently processed diets: air-dried, dry kibble, mildly cooked/raw and canned wet. Statistical analyses were performed using METAGENassist – ANOVA with significance set at $p < 0.05$.

Bacteria (genus)	**p value**	**Fisher's LSD post hoc comparison**	
		Diet 1	**Diet 2**
Pediococcus	<0.0001	ADF	DF
		RF	ADF
		ADF	WF
		RF	DF
		RF	WF
Megamonas	<0.0001	DF	ADF
		DF	RF
		DF	WF
Coprococcus	<0.0001	WF	ADF
		WF	DF
		WF	RF
Clostridium	<0.0001	WF	ADF
		WF	DF
		WF	RF
Oscillospira	<0.0001	WF	ADF
		WF	DF
		WF	RF
Phascolarctobacterium	0.002	WF	ADF
		WF	DF
		WF	RF
Peptococcus	0.003	WF	ADF
		WF	DF
		WF	RF
Coprobacillus	0.004	WF	ADF
		WF	DF
		WF	RF
Lactococcus	0.011	RF	ADF
		RF	DF
		RF	WF
Turicibacter	0.013	ADF	RF
		WF	DF
		WF	RF

Table 5.2 (continued) Differences in bacterial members among the four differently processed diets: air-dried, dry kibble, mildly cooked/raw and canned wet. Statistical analyses were performed using METAGENassist – ANOVA with significance set at $p < 0.05$.

Bacteria (genus)	**p value**	**Fisher's LSD post hoc comparison**	
		Diet 1	**Diet 2**
Sutterella	0.016	WF	ADF
		WF	DF
		WF	RF
Fusobacterium	0.018	ADF	DF
		RF	DF
		WF	DF
Prevotella	0.035	DF	ADF
		WF	ADF
Dorea	0.036	ADF	DF
Vagococcus	0.041	RF	DF
		RF	WF
Lactobacillus	0.046	DF	ADF
		DF	WF
Catenibacterium	0.048	DF	ADF
		DF	WF

Abbreviations: ADF – air-dried; DF – dry kibble; RF – mildly cooked/raw; WF – canned wet

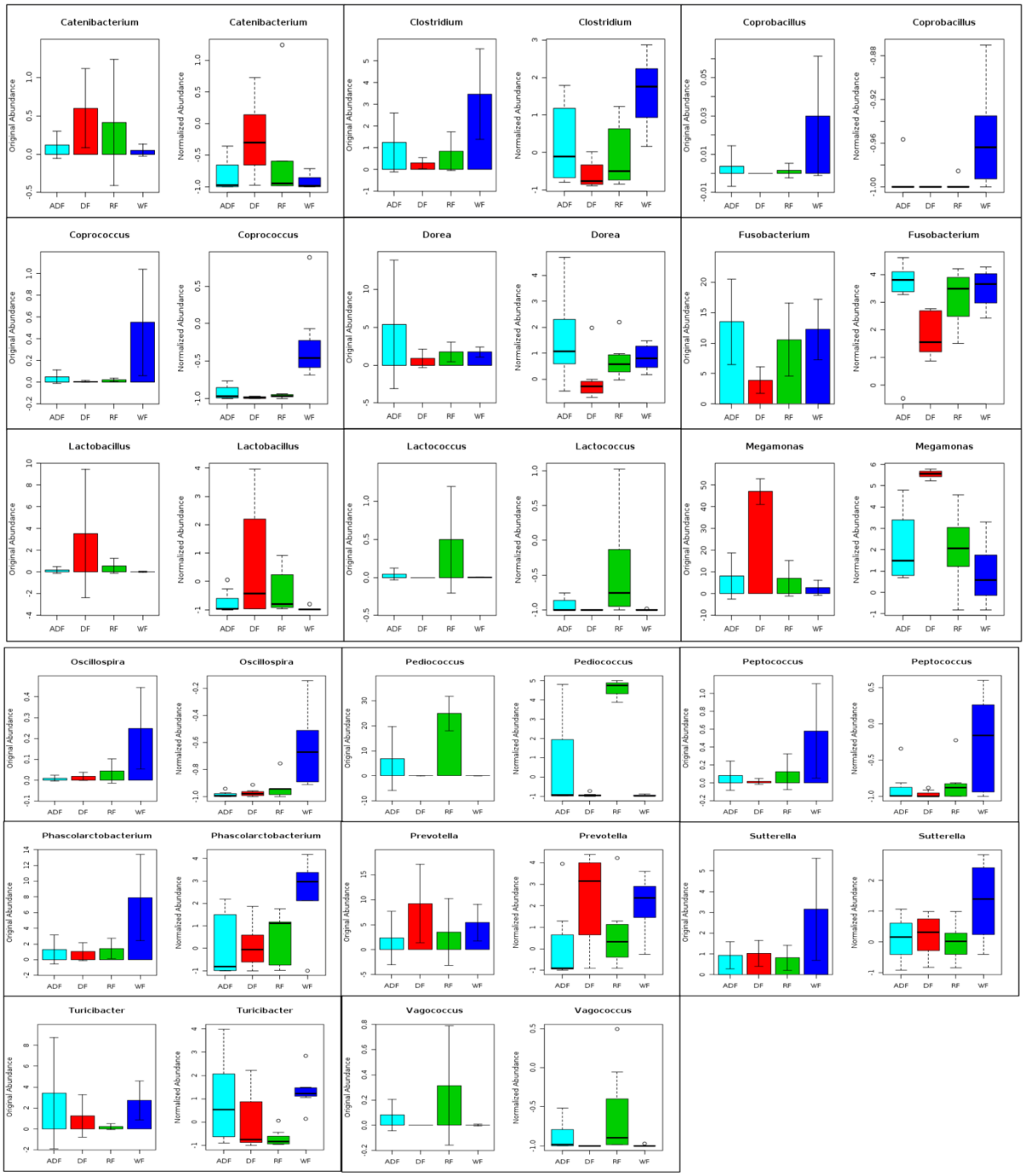

Figure 5.2. Boxplots showing original (left) and normalized (right) abundances of different bacteria (genus) in the fecal communities between diet groups determined by one-way ANOVA with signficance level set at $p < 0.05$

Abbreviations: ADF – air-dried; DF – dry kibble; RF – mildly cooked/raw; WF – canned wet

Relative abundances (%) were compared at two taxonomic levels: phylum and genus. The relative abundance at phylum taxa level is shown in Figure 5.3 which, illustrates the main phyla as *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, *Proteobacteria*, *Actinobacteria* and other phyla with an average of < 0.1% across diets consisting of *Chloroflexi*, *Spirochaetes*, *OD1*, *Synergistetes*, *TM7*, *Verrucomicrobia*, *Cyanobacteria*, *Tenericutes* and *Deferribacteres*.

Relative abundance data are provided in Appendix T. Differences in phyla among the diets were tested using one-way ANOVA for repeated measures and revealed no significant differences among the four diet groups at phyla taxa level.

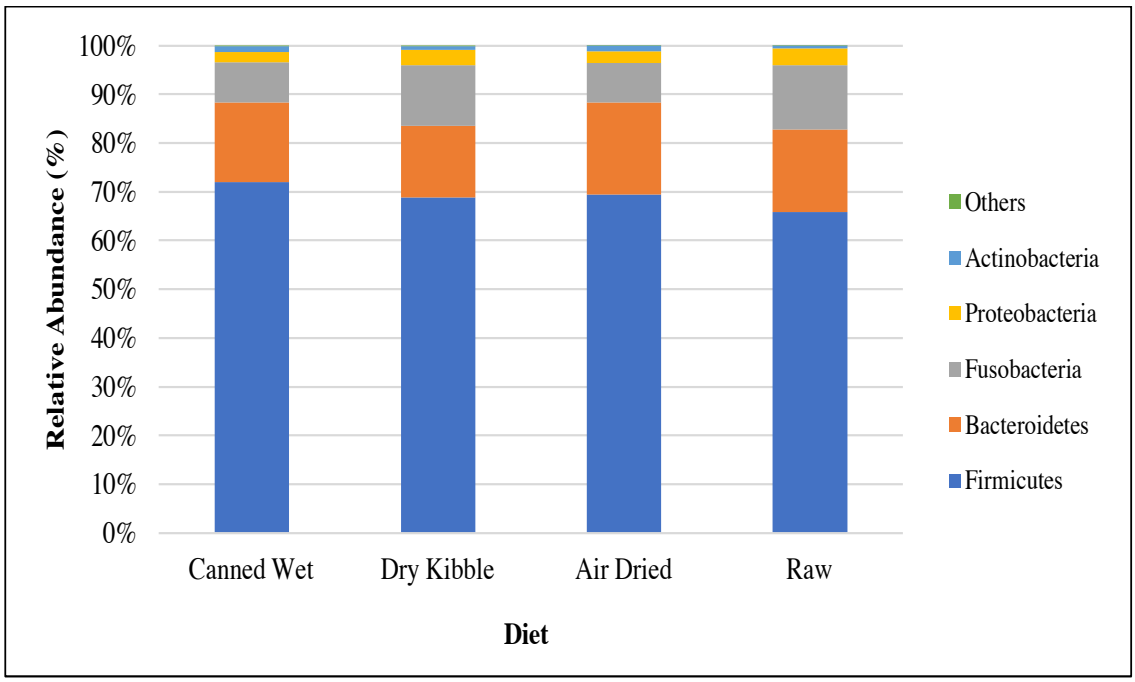

Figure 5.3. Stacked bar graph showing the relative abundances of five phyla: *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, *Proteobacteria* and *Actinobacteria* for each diet group.

At the genus taxa level 33 different genera were identified (relative abundance data – Appendix U) across samples from all diets (Fig. 5.4). Table 5.3 shows these genera and the phyla to which they are assigned. It is noteworthy that the phylum *Firmicutes* has the largest variation in organisms for a total of 21 out of the 33 identified bacteria as well as the highest relative abundance in comparison to the other phyla (Figure 5.3).

Table 5.3. The five dominant phyla and the lists of corresponding genera identified in the fecal samples of dogs in all diet groups. (The table shows all genera except *Mucispirillum* since it has been classified as "Other")

Firmicutes	***Bacteriodetes***	***Fusobacteria***	***Proteobacteria***	***Actinobacteria***
Megamonas	*Bacteroides*	*Fusobacterium*	*Helicobacter*	*Slackia*
Blautia	*Prevotella*		*Sutterella*	*Bifidobacterium*
Streptococcus			*Anaerobiospirillum*	*Corynebacterium*
Lactobacillus				*Actinomyces*
Pediococcus				*Collinsella*
Turicibacter				
Catenibacterium				
Allobaculum				
Ruminococcus				
Faecalibacterium				
Dorea				
Phascolarctobacterium				
Clostridium				
Eubacterium				
Peptococcus				
Oscillospira				
Roseburia				
Epulopiscium				
Enterococcus				
Coprococcus				
Vagococcus				

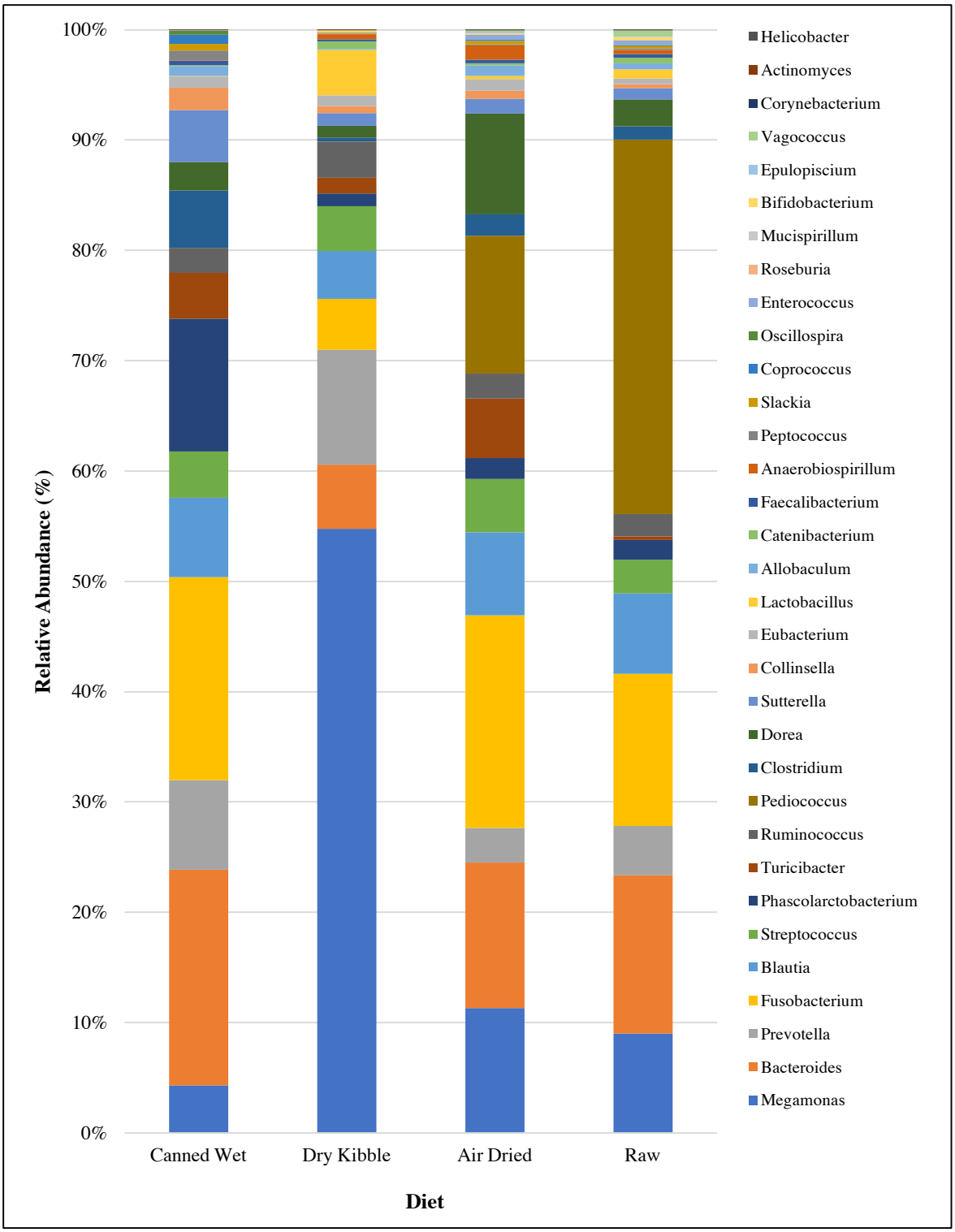

Figure 5.4. Stacked bar graph showing the 33 genera identified in fecal samples for each diet group.

One-way ANOVA for repeated measures showed significant differences (Table 5.4) between the diets for seven of the 33 bacterial genera for which relative abundances are presented as boxplots in Figure 5.5. A comparison of the WF and DF identified significant differences for *Bacteroides*, *Dorea*, *Fusobacterium* and *Megamonas*. When WF was compared to ADF significant difference was found for *Clostridium*. Testing WF against RF, there were significant differences for *Pediococcus* and *Turicibacter*. The low heat processed diet (ADF and RF) were processed using a *Pediococcus acidilactici* fermentation product so significant differences compared to other diets were expected. The only bacteria found to be significantly different between DF and ADF was *Megamonas* while DF against RF showed *Fusobacterium*, *Megamonas* and *Pediococcus* to be significantly different.

Table 5.4 List of seven genera showing significant differences among the four diet groups.

Bacteria (Genus)	Diets		Mean Difference (A-B)	Standard Error	p-value	95% C.I.	
	A	B				lower	upper
Bacteroides	WF	DF	11.69	2.94	0.044	0.36	23.03
Clostridium	WF	ADF	3.67	0.77	0.018	0.70	6.64
Dorea	WF	DF	2.06	0.40	0.013	0.52	3.60
Fusobacterium	WF	DF	14.35	3.35	0.031	1.43	27.28
	DF	RF	-9.55	1.93	0.016	-17.01	-2.08
Megamonas	DF	WF	51.38	3.14	<0.0001	39.23	63.52
	DF	ADF	43.42	7.13	0.005	15.89	70.95
	DF	RF	47.01	4.55	<0.0001	29.42	64.59
Pediococcus	RF	WF	29.66	5.69	0.007	8.97	50.36
	RF	DF	29.65	5.69	0.007	8.97	50.33
Turicibacter	WF	RF	4.27	1.05	0.039	0.23	8.30

Abbreviations: WF – Canned wet food; DF – Dry Kibble; ADF – Air-dried; RF – Mildly cooked/Raw

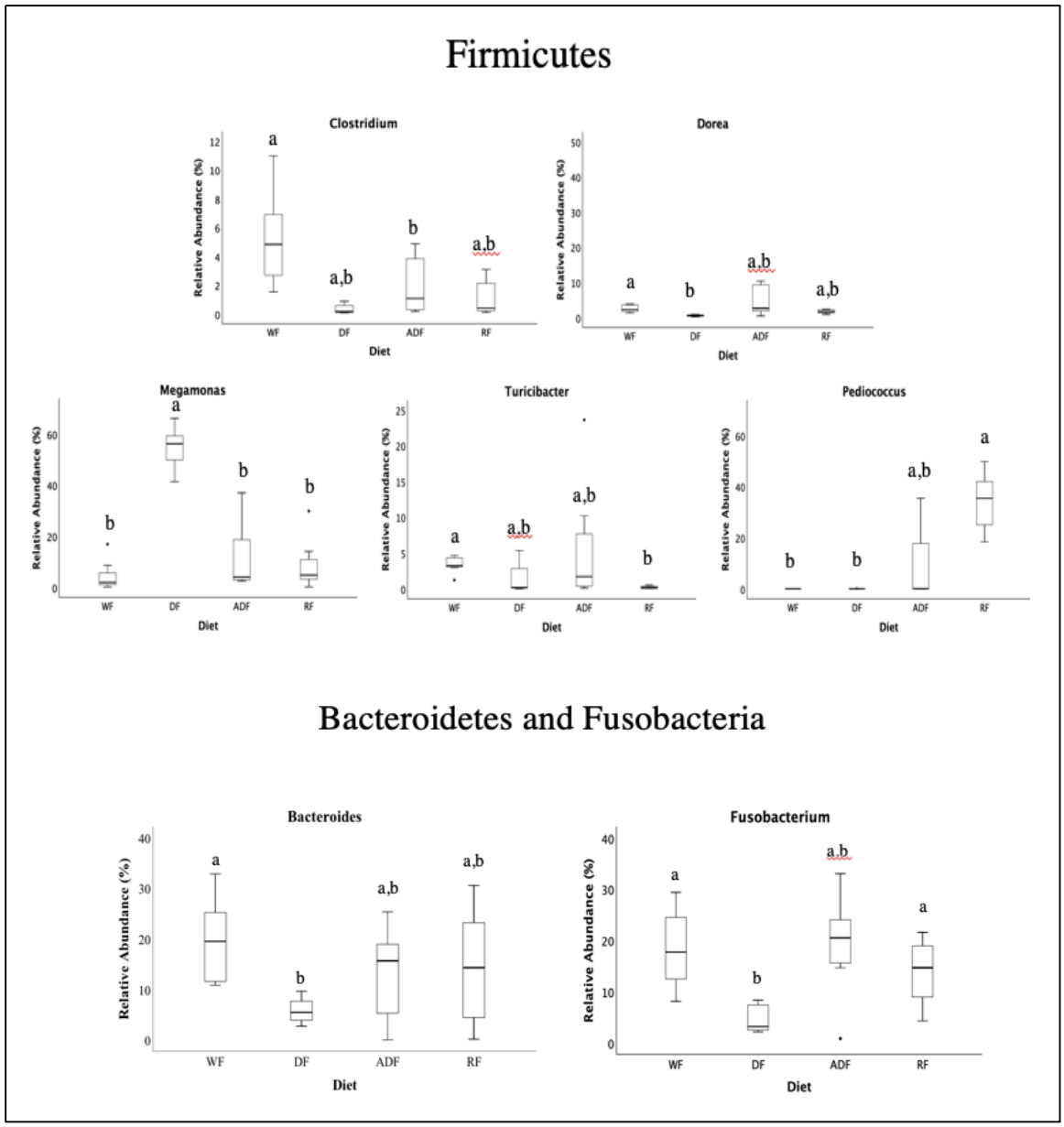

Figure 5.5 Boxplots showing relative abundances of seven bacteria (genus) that were significantly different between fecal microbial communities of the four diet groups by one-way analysis of variance (ANOVA) for repeated measures, significance set at $p < 0.05$.

a, b – superscript letters indicate significant differences between diet groups

Abbreviations: WF – canned wet; DF – dry kibble; ADF – air dried; RF – mildly cooked/raw

5.3.1.2 Diversity

Alpha diversity within fecal communities was determined based on calculation of the Shannon and Simpson indices to compare the richness and evenness of the groups based on diet. Raw data for these indices is available in Appendix V. Mean values for both Shannon (Table 5.5) and Simpson (Table 5.7) indices show that the WF diet had the highest mean value when compared to all the other diets (Fig. 5.6). A comparison of the communities between all diets revealed significant differences for both indices between all pairs of diets except for when ADF was compared to RF (Table 5.6 and 5.8).

Table 5.5. Descriptive statistics for the Shannon diversity index across diet groups.

Diet	Mean	Standard Deviation	Standard Error	95% Confidence Interval	
				lower	upper
Canned Wet	2.99	0.10	0.04	2.90	3.08
Dry Kibble	2.09	0.13	0.05	1.98	2.20
Air Dried	2.59	0.22	0.08	2.37	2.74
Mildly cooked/Raw	2.53	0.14	0.05	2.40	2.66

Table 5.6. Pairwise comparisons with Bonferroni adjustment ($p < 0.05$) between the means of the Shannon indices for microbial communities of fecal samples.

Diet A	Diet B	Mean Difference (A – B)	Standard Error	p - value	95% C.I.	
					lower	upper
Canned Wet	Dry Kibble	0.90	0.08	< 0.001	0.68	1.11
	Air Dried	0.43	0.08	< 0.001	0.22	0.64
	Mildly cooked/Raw	0.46	0.08	< 0.001	0.24	0.68
Dry Kibble	Canned Wet	-0.90	0.08	< 0.001	-1.11	-0.68
	Air Dried	-0.47	0.08	< 0.001	-0.68	-0.25
	Mildly cooked/Raw	-0.44	0.08	< 0.001	-0.66	-0.22
Air Dried	Canned Wet	-0.43	0.08	< 0.001	-0.64	-0.22
	Dry Kibble	0.47	0.08	< 0.001	0.25	0.68
	Mildly cooked/Raw	0.03	0.08	0.987	-0.19	0.25
Mildly cooked/ Raw	Canned Wet	-0.46	0.08	< 0.001	-0.68	-0.24
	Dry Kibble	0.44	0.08	< 0.001	0.22	0.66
	Air Dried	-0.03	0.08	0.987	-0.25	0.19

Table 5.7. Descriptive statistics for the Simpson diversity index across diet groups.

Diet	Mean	Standard Deviation	Standard Error	95% Confidence Interval	
				lower	upper
Canned Wet	0.92	0.01	0.00	0.91	0.93
Dry Kibble	0.74	0.04	0.02	0.71	0.78
Air Dried	0.88	0.03	0.01	0.85	0.90
Mildly cooked/Raw	0.86	0.02	0.01	0.85	0.88

Table 5.8. Pairwise comparisons with Bonferroni adjustment ($p < 0.05$) between the means of the Simpson indices for microbial communities of fecal samples.

Diet A	Diet B	Mean Difference	Standard Error	p - value	95% Confidence Interval	
					lower	upper
Canned Wet	Dry Kibble	0.18	0.02	< 0.001	0.14	0.22
	Air Dried	0.05	0.02	0.013	0.01	0.09
	Mildly cooked/Raw	0.06	0.02	0.002	0.02	0.10
Dry Kibble	Canned Wet	-0.18	0.02	< 0.001	-0.22	-0.14
	Air Dried	-0.13	0.02	< 0.001	-0.17	-0.09
	Mildly cooked/Raw	-0.12	0.02	< 0.001	-0.16	-0.08
Air Dried	Canned Wet	-0.05	0.02	0.013	-0.09	-0.01
	Dry Kibble	0.13	0.02	< 0.001	0.09	0.17
	Mildly cooked/Raw	0.01	0.02	0.83	-0.03	0.05
Mildly cooked/ Raw	Canned Wet	-0.06	0.02	0.002	-0.10	-0.02
	Dry Kibble	0.12	0.02	< 0.001	0.08	0.16
	Air Dried	-0.01	0.02	0.83	-0.05	0.03

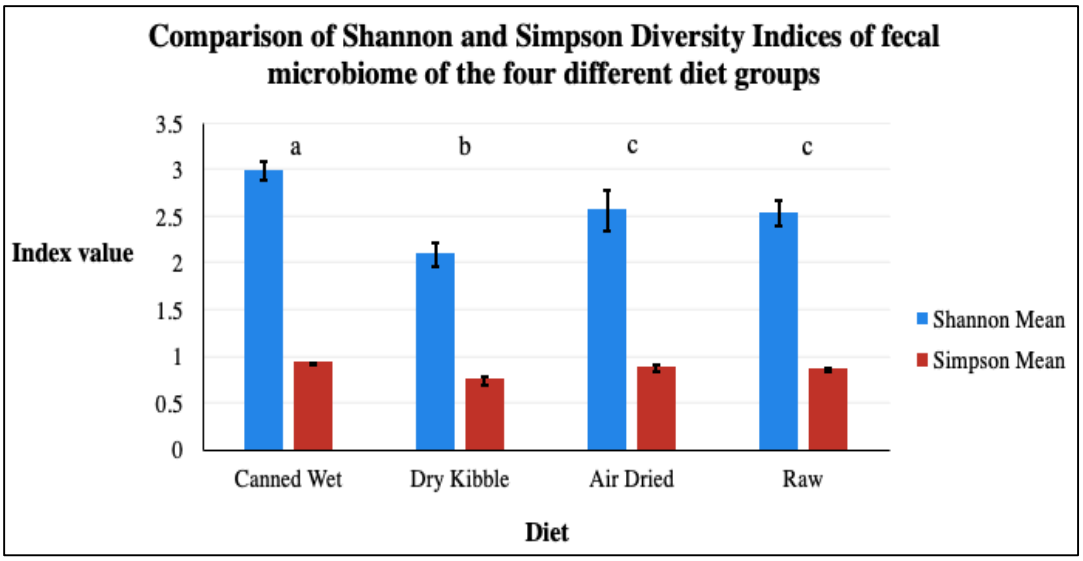

Figure 5.6. Clustered bar graph with standard deviation showing the Shannon and Simpson diversity indices for the fecal microbial communities for each diet.

a, b and c – letters above bars indicate significant differences between diets for both Shannon and Simpson indices at $p < 0.05$.

Beta diversity between fecal communities was investigated using principal component analysis (PCA) analysis. The two-dimensional (2D) PCA plot (Fig. 5.7) shows no overall distinct clustering of each of the four diet groups from each other although there was some separation observed between the RF (turquoise) and DF (blue) diets (PCA scores and loadings for diet groups are available in Appendix W). This finding suggests a difference between the microbiome of the dogs when being fed these two diets although the small sample size serves as a limitation to drawing this conclusion. A sequencing error occurred for one of the fecal samples belonging to the mildly cooked/raw diet group so results are depicted for seven samples only.

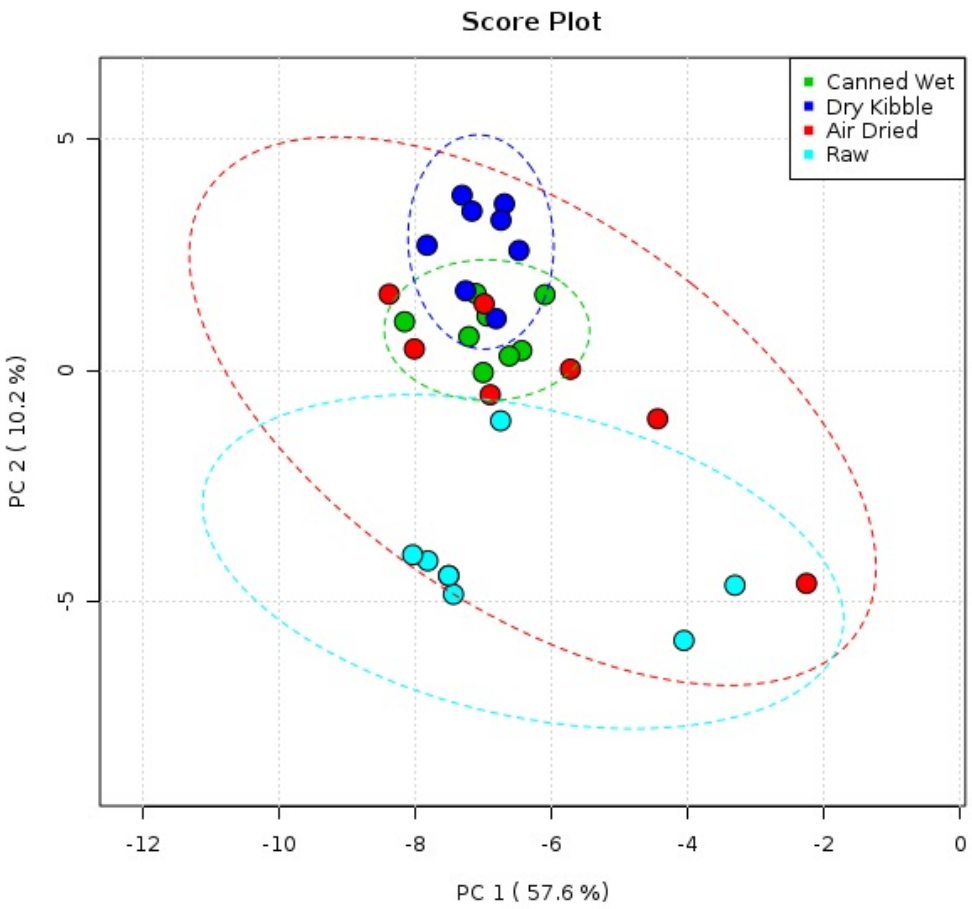

Figure 5.7. Two-dimensional (2D) principal component analysis (PCA) score plot of the fecal microbiome at genus taxa level for each diet group: canned wet (green), dry kibble (blue), air-dried (red) and mildly cooked/raw (turquoise). Each dot represents fecal samples from each dog within a group. *There is missing data for one fecal sample of the mildly cooked/raw diet group as a result of sequencing error*.

5.3.2 Differences in the microbial content of the air-dried and mildly cooked/raw diets

All diets were subjected to the same sample processing and sequencing as the fecal samples. No bacterial organisms were identified in the canned wet and dry kibble diets while analysis revealed a greater number of bacterial organisms in the ADF compared with the RF.

Two-dimensional (2D) PCA of each sample of the diet (Fig. 5.8) shows distinct clustering of these samples indicating that the microbial composition of the diets remained distinct from each other during storage (PCA loadings and scores – Appendix X). There was a sequencing error for one of the ADF samples so the results reflect the first two principal components based on data from only three ADF samples and the full complement of four RF samples.

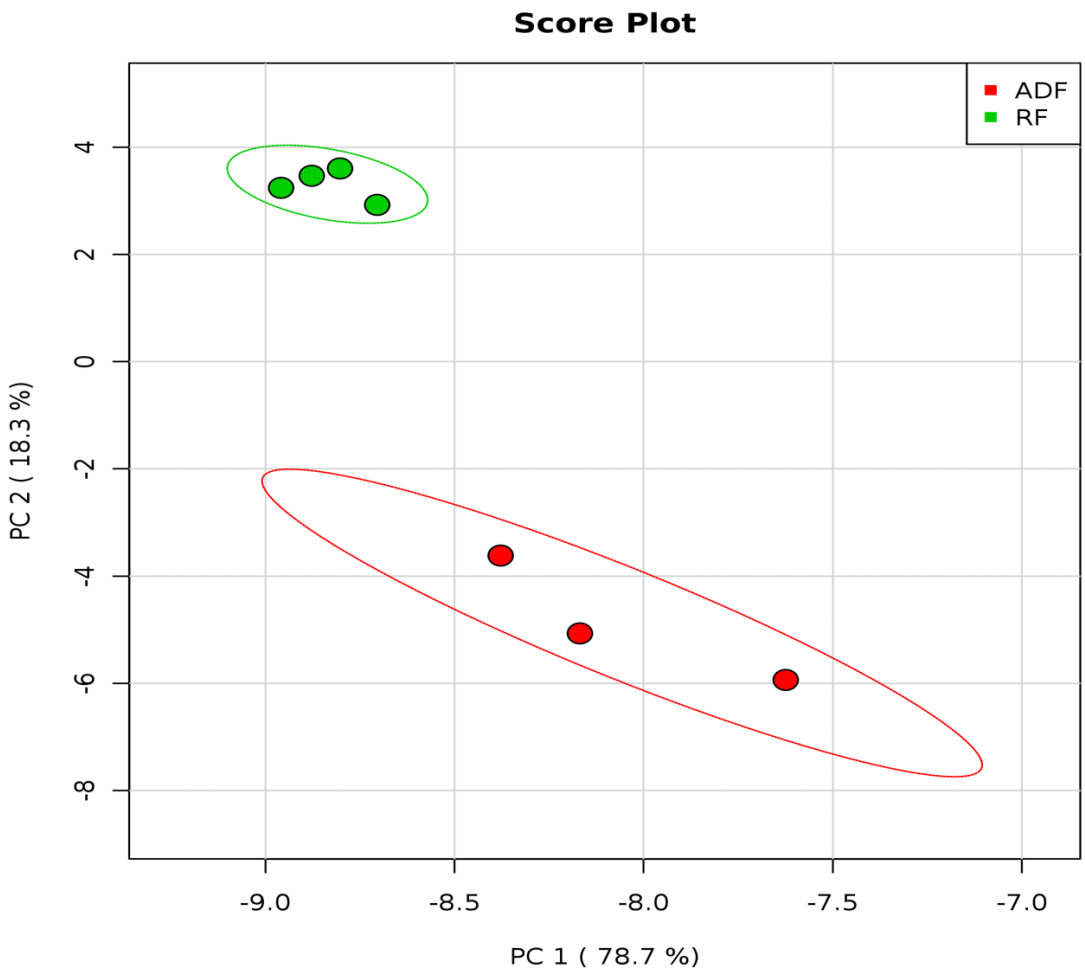

Figure 5.8. Two-dimensional (2D) principal component analysis (PCA) plot showing distinct clustering of the three samples of ADF (red) from the four RF samples (green) at genus taxa level.

(Note: There was a sequencing error in one ADF sample – no data available)

Abbreviations: ADF – air-dried diet; RF – mildly cooked/raw diet

A comparison of identified bacterial organisms using the Wilcoxon rank sum test revealed significant differences in 25 genera (Table 5.9). The fold change analysis of these microbial communities showed that 15 genera were overrepresented in ADF compared to two overrepresented in RF (Fig. 5.9).

Table 5.9. List of significantly different bacterial genera between the air-dried (ADF) and mildly cooked/raw (RF) diets determined by Wilcoxon rank sum test ($p < 0.05$).

Bacteria (genus)	**p-value**
Enhydrobacter	0.025
Sphingomonas	0.030
Brachybacterium	0.030
Bacteroides	0.030
Macrococcus	0.032
Geobacillus	0.032
Bacillus	0.032
Corynebacterium	0.032
Myroides	0.032
Erwinia	0.032
Kocuria	0.032
Peptoniphilus	0.032
Janthinobacterium	0.032
Staphylococcus	0.032
Salinicoccus	0.032
Enterobacter	0.032
Microbacterium	0.032
Carnobacterium	0.044
Psychrobacter	0.044
Brochothrix	0.044
Serratia	0.044
Cloacibacterium	0.044
Pseudomonas	0.050
Delftia	0.050
Streptococcus	0.050

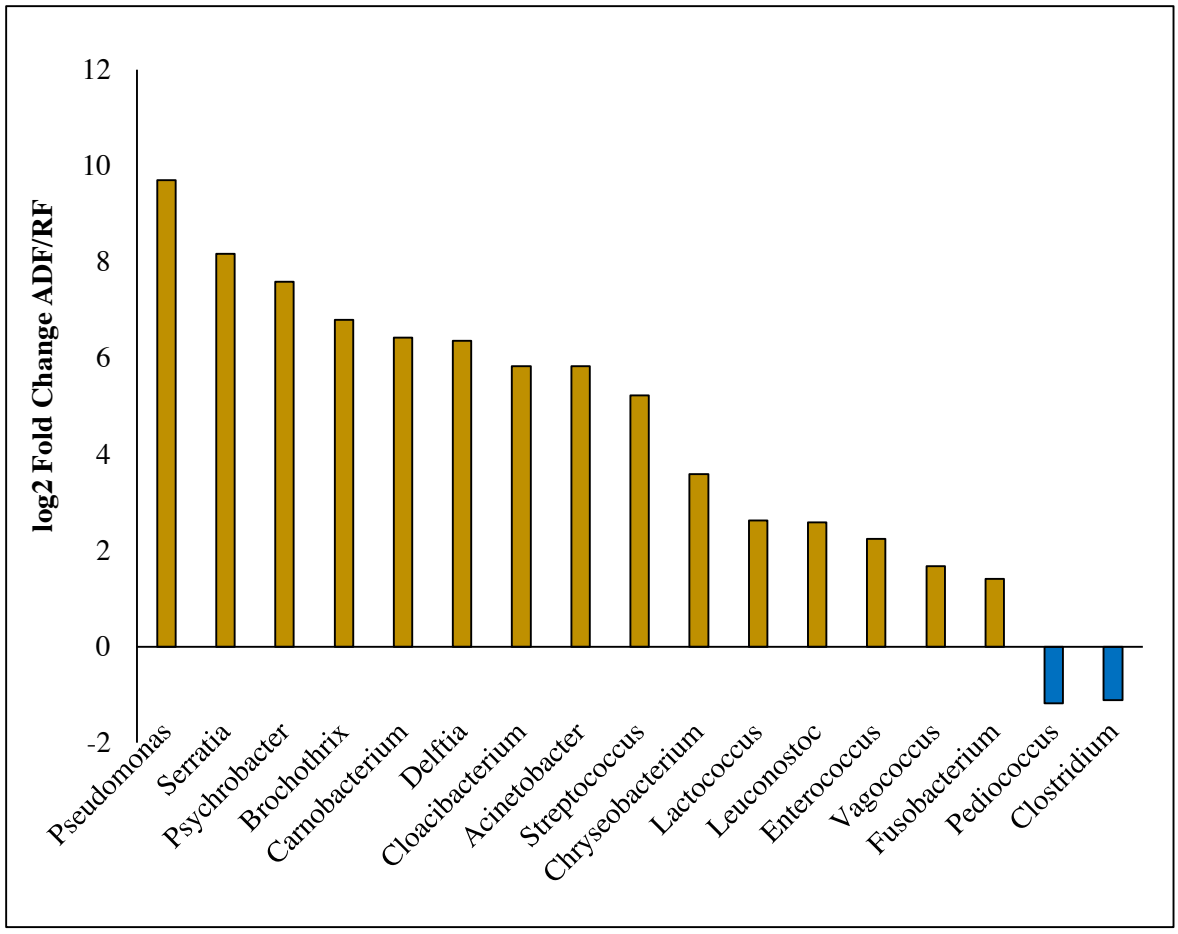

Figure 5.9. Bar graph showing log2 fold change (FC) ADF/RF in bacteria (genus) between the air-dried (ADF) and mildly cooked/raw (RF) diets. Bacteria are overrepresented in ADF as yellow and in RF as blue colored columns.

Pediococcus acidilactici and *Clostridium* are the only two genera overrepresented in the RF while *Pseudomonas*, *Serratia*, *Psychrobacter*, *Brochothrix*, *Carnobacterium*, *Delftia*, *Acinetobacter*, *Streptococcus*, *Chryseobacterium*, *Lactococcus*, *Leuconocstoc*, *Enterococcus*, *Vagococcus* and *Fusobacterium* were all overrepresented in the ADF (See Appendix Y for values).

5.4 DISCUSSION

5.4.1 The four differently processed diets and AGE levels

The diets used in this study were subjected to different methods of heat processing to determine the effect on the fecal microbiome when these diets were consumed by dogs for four weeks. All diets were similarly formulated to have the similar nutrient composition and ingredients so that differences observed will likely be due differences in thermal processing methods.

MRPs and AGEs have been documented in high heat processed dog foods (van Rooijen et al., 2014a) related to the reduction in availability of lysine in commercial pet foods as a result of glycation reactions (van Rooijen et al., 2014b). Thermal processing of foods, as a characteristic of the Western diet, has been linked to AGE levels in these foods and dysbiosis, all of which have been implicated in inflammatory and degenerative diseases such as obesity, diabetes mellitus and atherosclerosis (Miclotte & Van de Wiele, 2019). To the authors' knowledge, there have been no investigations into the effect of AGEs on the fecal microbiome of healthy dogs.

Three dietary AGEs were measured: CML, CEL and MG-H1 in the four diets. For this discussion, the total amount of AGEs in each diet is considered since the contribution of dietary AGEs to the AGE pool of the body determines the glycoxidant burden associated with diseases (Snelson & Coughlan, 2019). The mean total quantity of AGEs (mg/100kcal of diet as fed) are shown in Fig. 5.10 to depict the variation in levels across the differently processed diets. If high temperature exposure for prolonged time is regarded as the driving force behind AGE formation in the foods, then the relative amount of AGEs in the diets correspond well. The canned wet diet contained the highest total AGEs and was the diet that was processed using a maximum temperature of 254^{o}F for an about an hour that was significantly higher from the total AGE amounts in all the other diets. No other statistical differences were observed among the diets.

The lower total AGE quantity in the dry kibble compared with that of the canned wet diet may be a reflection of the process of extrusion characterized by short exposure times to high temperatures preventing significant AGE formation relative to retorting which, involves processing ingredients for longer times, under high temperature and pressure. The low heat processed diets in this study were formulated with dextrose, a reducing sugar, intended as a substrate for the *Pediococcus acidilactici* organisms. It is possible that any dextrose that was not used by the bacteria, was available for participation in glycation reactions during processing resulting in similar quantities of AGEs compared with the extruded diet. To the authors' knowledge, no research investigating the effects of bacterial fermentation products as biological preservatives in food related to AGE formation has been done so these findings support the need for additional research.

There is evidence that high heat processed diets cause a reduction in fecal microbial diversity (Z. Zhang & Li, 2018) and since high heat processing also contributes to AGE formation, there may be a connection between dietary AGEs, contributing to the body's AGE pool, and a lack of diversity of the gastrointestinal microbiome. One study demonstrated a positive correlation between plasma CML and CEL in a heat treated, high fat diet in mice and specific genera but was not found to be statistically significant (Marungruang et al., 2016).

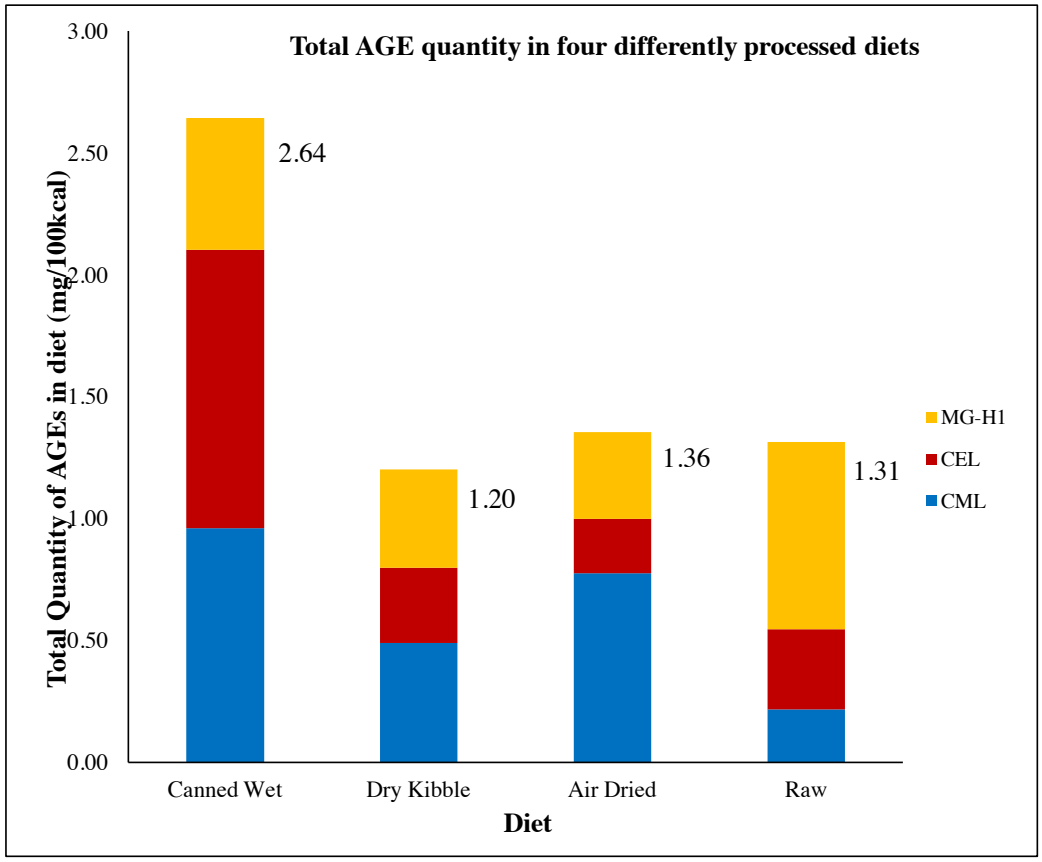

Figure 5.10. Stacked bar graph showing mean quantities (mg/100kcal) as fed of dietary advanced glycation end products (AGEs) in the four diets.

Abbreviations: CML – carboxymethyllysine; CEL – carboxyethyllysine; MG-H1 – methylglyoxal hydroimidazolone-1

High throughput 16S rRNA sequencing of the diets revealed bacterial loads in the low heat processed foods but not the canned wet or dry kibble diets. This is not surprising since high temperatures destroy bacterial cells and disrupt nucleic acids (Ramesh, 2003, 2007) limiting identification. In contrast, the air-dried and mildly cooked/raw diets contained several bacterial organisms; 25 bacterial genera that were significantly different between the diets (Table 5.9) and

17 bacteria are overrepresented (Fig. 5.9). Comparison of the fold change data for these genera between the two diets revealed that only two genera, *Clostridium* and *Pediococcus*, were overrepresented in the mildly cooked/raw diet. It is important to note that a *Pediococcus acidilactici* fermentation product was added to the low heat processed diets (ADF and RF) causing *Pediococcus* counts to be higher in the mildly cooked/raw food indicating that *Clostridium* the only organism naturally overrepresented in the mildly cooked/raw diet but was not statistically significant. The higher abundance of *Pediococcus* in the mildly cooked/raw diet compared with the air-dried diet may be attributed to the light cooking conditions that encouraged the growth of these organisms in the mildly cooked/raw diet. Differences in microbial content between these two diets were also demonstrated by PCA analysis (Fig. 5.8) showing separation based on the first two principal components into distinct groups. The mildly cooked/raw diet samples clustered together while the air-dried diet was somewhat varied in its components seen as samples with greater distance between them on the plot.

The method of processing directly affects the survival of bacterial cells and spores in the food. Dehydration removes water from the food but preserves bacterial cellular proteins thereby maintaining viability (Chitrakar, Zhang, & Adhikari, 2019). The mildly cooked/raw food possibly had lower numbers of bacterial organisms because it was frozen after being lightly cooked, a process associated with disruption of cellular structure and likely destruction of bacterial nucleic acids (Archer, 2004). It should be noted that no pathogenic bacteria were identified in any of these diets despite the higher bacterial load of the low-heat processed diets. There were no apparent negative effects of the bacteria in the low heat processed diets on the health of subjects since all dogs had normal stool quality and normal clinical parameters for the

duration of the study. It is possible that genetic material was present in the diets but microorganisms were inactivated or dead.

5.4.2 Fecal microbiota analysis

Each diet was characterized by a different microbial signature (Fig. 5.1a and b) observed as differences among the fecal bacterial communities. The air-dried diet had a high abundance of *Fusobacteria*, the dry kibble and canned wet diets featured *Clostridia* and the mildly cooked/raw diet was marked by high numbers of *Pediococcus acidilactici* that was a known dietary additive. Our hypothesis stated that the canine fecal microbiome changes in response to differently processed diets and these findings support this claim. These data provide a foundation for additional experiments investigating such fecal microbial alterations in response to high and low heat processing methods used in the pet food industry. Furthermore, the discovery that addition of bacterial products to low heat processed foods alters the fecal microbiome is significant and warrants additional investigation into fecal microbiome as well as fecal metabolomics of animals eating these diets to determine functional changes within the communities.

Diversity measures of the fecal microbiome across diets were calculated to determine whether there were differences within and between groups. Alpha diversity was measured using the Shannon and Simpson indices taking into consideration richness and evenness respectively. Analysis of the alpha diversity of the fecal microbiome samples using these indices gave similar results. The most diverse fecal microbiomes were in response to the canned wet diet followed in descending order by the air-dried, mildly cooked/raw and dry kibble. This was true for both Shannon and Simpson indices suggesting that the canned wet diet, despite being a high heat processed diet, still allows for a more diverse fecal microbiome than the low heat processed diets. In contrast, the dry kibble had the lowest values for both Shannon and Simpson indices

indicating decreased diversity of the fecal microbiome when dogs consumed this diet. Dysbiosis is described as an imbalance of the gastrointestinal microbiota related to the richness of the community (Suchodolski, 2016). It takes into consideration numbers and members as a means of determining cause or effect of illness. Evidence of a dysbiotic state was observed as alteration of the relative abundances at the genus level (Fig. 5.4) for the dry kibble group where over 50% of the fecal microbiome comprised *Megamonas* spp. The metric known as the dysbiosis index has been developed to aid in diagnosis and follow-up in recovery of dogs with intestinal inflammation (AlShawaqfeh et al., 2017) but does not acknowledge the contribution of these microbes to the disease process. Determining the metabolic role of *Megamonas* within the fecal community was beyond the scope of this work but future investigations into the metabolic activity of the fecal microbiota in dysbiosis is recommended. Alpha diversity was found to be significantly different between the fecal microbiome associated with all diets except the mildly cooked/raw and air-dried diets. Other studies investigating diversity in fecal microbiome in response to thermally processed diets have produced inconsistent results (Herstad et al., 2017; Schmidt et al., 2018). Limitations, much like those faced in this study, include small sample size and relatively short-term feeding protocols. While four weeks used for feeding trial used in this study is relatively short compared to the lifespan of dogs, the human gastrointestinal microbiome (Singh et al., 2017), as a reference for this comparative work, can respond to dietary changes within a few days to two weeks suggesting that four weeks should be sufficient, in dogs, to identify changes in the fecal microbiome.

Beta diversity as a measure of differences in fecal communities between groups was not clear among all four diets as seen in Fig. 5.7. However, if only the mildly cooked/raw (turquoise) and dry kibble (blue) diet fecal samples are considered, samples from each diet cluster together

and the two groups are distinct from each other. Given the small sample size in this study it is difficult to make a firm assertion about beta diversity between groups. Longer duration experiments are needed with larger populations to be able to draw conclusions about differences in diversity in fecal communities after consumption of these diets. Further research is recommended to explore the loss of diversity and resultant dysbiosis that may occur in association with extruded diets.

The predominant phyla of the fecal microbial communities were consistent with findings in previous studies on the canine microbiota of normal dogs (Hooda et al., 2012; Suchodolski et al., 2008). Average relative abundances across diets showed *Firmicutes* (68%) to be the dominant phyla in these dogs with smaller percentages of *Bacteroidetes* (16%), *Fusobacteria* (10%), *Proteobacteria* (3%), *Actinobacteria* (1%) and other phyla making up <1% of bacteria at this taxonomic level. No differences were observed between diets for phyla-level taxonomic classifications of bacterial in the fecal communities.

At genus taxa level, significant differences between abundances among groups showed that seven genera are distinct. Five of these are members of *Firmicutes* (*Megamonas*, *Clostridium*, *Dorea*, *Pediococcus* and *Turicibacter*) and the other two belong to *Bacteroidetes* (*Bacteroides*) and *Fusobacteria* (*Fusobacterium*). Recall that *Pediococcus acidilactici* fermentation product was added to the low heat processed diets so its difference is due to it being an additive thereby changing its abundance. It cannot be confirmed whether the bacterial load of the air-dried and mildly cooked/raw diets affected the fecal numbers other than for *Pediococcus* although, it seems unlikely since none of the other overrepresented genera in the air-dried or mildly cooked/raw diets had significantly higher abundances in the feces of dogs when compared to the other diets. The comparison of fecal communities at phyla is too broad to determine

differences between treatments; however, even at genus taxa level classification, it should be noted that there are differences in metabolic capabilities between species and strains so the specific roles of these statistically different genera within each diet group is unknown (Vieira-Silva et al., 2016). While methods used have enabled identification of the members of the communities and the alteration of genera among diets, knowing the functional roles of these microorganisms provides a complementary assessment of the response to the four diets.

Obesity is associated with a dysbiotic fecal microbiome in rodents characterized by an increase in the *Firmicutes*:*Bacteroidetes* ratio in response to Western-style diets (Sen et al., 2017). Since there was no difference in this ratio in the fecal microbiota of the diet groups (Appendix Z), it was observed that different processing conditions and dietary AGE quantities in this study did not influence the abundances of these two phyla.

Another form of assessment of the distal gut microbes is done by comparison of saccharolysis versus proteolysis in the colonic environment. Fermentation of substrates to release straight-chain short chain fatty acids (SCFAs) such as butyrate, acetate and propionate are essential for maintaining a healthy gut and host. Putrefaction is detrimental to the host producing branched-chain SCFAs and depends on the proteolytic function of certain bacteria, e.g. members of family Desulfovibrionaceae (Jackson & Jewell, 2019). An imbalance favoring putrefaction shifts a healthy gut to an inflammatory environment resulting in gastrointestinal disease. One way of determining the metabolic activity of the colonic bacteria is to explore the fecal metabolome. The identification of SCFA's gives an indication of bacterial activity and can provide insight as to the balance between fermentation and putrefaction. Fecal metabolomic investigation was beyond the scope of this work but could have provided information beyond the presumptive roles of different bacterial genera. Metagenomic analysis of 16S data did not reveal

any differences between saccharolytic versus proteolytic metabolic activity among diets (Appendix AA). This suggests that balance between the two processes was maintained, did not fluctuate with diet change during the course of the experiment and, high heat processed diets did not shift the fecal microbiome to proteolytic activity. It is worth mentioning that for domestic dogs, the findings in this study suggest that feeding a mildly cooked/raw diet does not increase proteolytic activity of colonic microbes. Domestication has shifted dogs from carnivorous natural diet of ancestral wild canids to commercial dog food with added fiber. As a result, gut microbial fermentation in dogs is not necessary for caloric requirements but instead, serves to maintain gut health and normal stool quality (Hooda et al., 2012; Jackson & Jewell, 2019). Future experiments should combine metagenomic with metabolomic data on the canine distal gut to be able to describe the effects raw diets may have on microbial function of domestic carnivores.

The aim of this investigation was to determine the changes if any, to the fecal microbial communities of these dogs in response to differently processed diets with varied levels of total AGEs. Analysis of the 16S data has shown differences in bacterial signatures, alpha diversity, some degree of beta diversity and specific differences in relative abundances of genera between diets that may be more apparent in a larger sample size. These observations create a foundation for future studies that should include not only identification of members of the microbial community but also serum and fecal metabolomic data that can describe bacterial metabolism in the colon and the effects on the host.

Footnotes

Animal Care and Use Approval:
This protocol with amendments was reviewed and approved by the Summit Ridge Farms' Institutional Animal Care and Use Committee (IACUC) in compliance with the Animal Welfare Act in addition to the IACUC at the University of Georgia.

a – Summit Ridge Farms, Susquehanna, PA.

CHAPTER 6

SUMMARY AND CONCLUSION

The Maillard reaction (MR) is a spontaneous, non-enzymatic glycation reaction between the carbonyl group of a reducing sugar and the free amine group of an amino acid, peptides or proteins (Poulsen et al., 2013) producing heterogeneous compounds when foods are cooked. The MR is favored under conditions of high heat and low moisture but many factors influence the extent of formation of Maillard reaction products in foods such as pH, mineral and vitamin content, availability of precursors (Poulsen et al., 2013), presence of fats and lipids and cooking conditions. The stages of the MR are early, intermediate and advanced; the final product of which, are dark pigmented melanoidins that give foods color, taste and aroma (Friedman, 1996). Since products at all stages of the MR are present, there are also potentially harmful compounds in the food at the end of cooking. Dietary sources of advanced glycation end products (AGEs), formed in the late intermediate stage of the Maillard reaction, have been implicated in diseases such as obesity, type 2 diabetes mellitus, renal diseases, neurodegenerative diseases such as Alzheimer's and Parkinson's disease, cardiovascular diseases and atherosclerosis (ALjahdali & Carbonero, 2017; Poulsen et al., 2013; Uribarri et al., 2015) in human beings. No causal role has been established for AGEs for any disease but they have been associated with numerous inflammatory and degenerative conditions as a result of their actions at the cellular level. AGEs may cause tissue inflammation and oxidative stress by altering structure and function to slow turn-over proteins, e.g. collagen, accumulating locally in tissues causing oxidative stress, and most significantly, AGEs bind to the receptor for AGEs (RAGE) initiating cellular signaling and

activation of sustained and perpetuated inflammation (Delgado-Andrade, 2014; Ramasamy et al., 2012; Uribarri et al., 2015). Cellular RAGE is bound to the cell membrane and attached to a cytoplasmic tail that is responsible for cellular signaling (Bierhaus et al., 2005). There are isoforms of cellular RAGE known as soluble RAGE formed by alternative pathways that act as decoy receptors binding to circulating AGEs and preventing AGE-RAGE interaction (Ciccocioppo et al., 2015; Maillard-Lefebvre et al., 2009; L. Zhang et al., 2008). Endogenous AGEs are formed from glycation processes and are indistinguishable from exogenous dietary sources. Both exogenous and endogenous AGEs form the circulating AGE pool so that excessive amounts from either source or both cumulatively, may overwhelm the homeostatic mechanisms of the body inciting inflammation and tissue damage (Kellow & Coughlan, 2015).

In human beings, high heat processing of foods is associated with high dietary intake of AGEs as a characteristic of the Western diet. All of the aforementioned human diseases have also been associated with consuming the Western diet suggesting that AGEs may play an etiological role in the pathogenesis of inflammatory and degenerative conditions (Nowotny et al., 2018; Sebekova & Sebekova, 2019). Pet food processing is similar to the processing methods used for the Western diet that employ high heat to manufacture a product that is of desirable palatability and texture (van Rooijen et al., 2013) while having a reduced microbial load and being safe for storage with a longer shelf-life. The implication is that high heat used in processing human foods may increase inflammatory diseases in human beings associated with their diet and similarly, pets consuming high heat processed foods may also be at risk for these diseases. In addition to dietary AGE intake associated with the Western diet, there have been investigations in human beings related to determining a metabolic signature (Bouchard-Mercier et al., 2013) and gastrointestinal microbiome alterations (Moran-Ramos et al., 2017) in response

to this type of diet. These studies provide valuable details as to the metabolic transit and biological effect of dietary AGEs which, despite on-going research over the last few decades, still remain elusive. In this study, we investigated dietary AGE intake of four differently processed diets, the effect on plasma AGE level, serum sRAGE concentrations, differences in serum and urine metabolome, and the canine microbiome in response to these diets as comparative work with studies done in human beings. The information gained will serve to guide the pet food industry, veterinary clinical practice and possibly draw attention to dogs as an animal model for further dietary AGE experiments.

The diets used for this study were a canned wet and dry kibble that were high heat processed as well as air-dried and mildly cooked/raw diets produced under low heat conditions. Diet and plasma samples were analysed using liquid chromatography-mass spectrometry by methods that were successfully applied to a human dietary AGE (Scheijen et al., 2016) and a rat plasma AGE study (Hashimoto et al., 2013). We hypothesized that diets higher in AGEs will influence total plasma AGE levels. The AGEs measured in the diets were carboxymethyllysine (CML), carboxyethyllysine (CEL) and methylglyoxal hydroimidazolone-1 (MG-H1). CML was highest in the $WF^{a} > ADF^{a} > DF^{b} > RF^{c}$, CEL was highest in the $WF^{b} > RF^{a} > DF^{a} > ADF^{a}$ and MG-H1 was highest in the $RF^{a} > DF^{a} > WF^{a} > ADF^{a}$. Total dietary AGEs were highest in the $WF^{b} > ADF^{a} > DF^{a} > RF^{a}$. Significant differences are indicated by superscript letters. We observed that the canned wet diet had the highest total amount of dietary AGEs (2.55mg/100kcal) as fed and attributed this to retorting as the method of processing where components are under high temperature (maximum temperature for retorting of this diet – $254^{o}F$) for over an hour, which is the longest high-heat exposure than any of the other processing

methods used for these diets. The mildly cooked/raw diet had the lowest total AGEs (1.25mg/100kcal) and was processed using the lowest heat of all four diets. In plasma, the three AGEs measured in the diets in addition to glyoxal hydroimidazolone-1 (GH-1) and argpyrimidine (AP) were quantified. We found that in plasma, CML was highest with the $ADF^{a} > DF^{a} > WF^{a} > RF^{b}$, CEL was highest with the $WF^{a} > DF^{b} > RF^{b} > ADF^{b}$, MG-H1 was highest with the $WF^{a} > DF^{b} > ADF^{b,c} > RF^{b,d}$, GH-1 was highest with the $ADF^{a} > WF^{a} > DF^{a,b} > RF^{b}$ and AP was highest with the $RF^{b} > WF^{a} > DF^{a} > ADF^{a}$. Total plasma AGE quantity revealed the highest amounts with the $WF^{a} > DF^{b} > ADF^{b,c} > RF^{b,d}$. Here we observe that the diet with the highest total AGE amount, the canned wet diet, was associated with the highest total plasma AGE levels (9.99nM/50µL) compared with the lowest heat processed diet, the mildly cooked/raw diet that was associated with the lowest total plasma AGE quantity (2.50nM/50µL). This result indicates that dietary AGEs in dog foods influence total plasma AGE burden and may be a risk for development of the range of inflammatory and degenerative diseases seen in people consuming high heat processed diets. In this study, serum sRAGE concentrations were measured using a commercial ELISA kit and no differences between diets were observed. A longer feeding trial or an experimental design that includes chronically ill dogs is recommended to further investigate the response of sRAGE to differently processed diets.

The changes in serum and urine metabolome in response to the four differently processed diets were measured using nuclear magnetic resonance (NMR) spectroscopy for each week of the study to determine trends over the four weeks of consuming each diet and the differences between each diet at the end of feeding. Our hypothesis stated that serum and urine metabolites would change in response to diet. Eight serum metabolites: valine, glycine, creatine, acetic acid,

acetylcarnitine, betaine and two unknown metabolites were identified as discriminatory between diets. There were differences in serum metabolite concentrations between all the diets suggesting that further investigation is needed to compare types of high heat processed diets with types of low heat processed diets and to look for distinct metabolic profiles. However, some differences were of particular interest such as the high acetic acid associated with the mildly cooked/raw diet and generally lower metabolite concentrations relative to the other diets when the dry kibble was fed. In urine, six discriminatory metabolites were identified between diets: acetic acid, carnitine, creatinine, dimethylamine, dimethyl-sulfone and methylnicotinamide. Similar trends were observed with urine as for serum. Acetic acid was common for both serum and urine but was lowest in the urine with the mildly cooked/raw diet and all metabolites were lower, especially carnitine and creatinine, when the dry kibble was fed. Overall, over the four week feeding trial for each diet, both serum and urine metabolic profiles did not change significantly providing evidence for rapid change in metabolome in response to dietary changes. The first sample was taken seven days after switching to a new diet and metabolite concentrations fluctuated but was not significantly different from measurements taken at 28 days, at the end of feeding.

A partial least squares model was able to predict the amount of CML in the diets based on variable importance in projection (VIP) scores > 1.0 of acetylcarnitine, valine and acetic acid in serum and methylnicotinamide in urine but was not a good predictor for the other individual AGEs or total AGE quantity. This work shows that differently processed diets, varied in AGE content, influence the canine serum and urine metabolome. Free AGEs are absorbed into circulation to be deposited in tissues or excreted in urine and may influence cellular biochemical functions that manifest in the metabolome. Further dietary trials are warranted to identify a

metabolic signature in canine biofluids that may indicate a high dietary AGE intake or predisposition to diseases.

Dietary AGEs that are too large to enter circulation across the gastrointestinal barrier remain in the digestive tract where they are either excreted in feces or used as substrates by colonic microbial fermenters (Kellow & Coughlan, 2015; Poulsen et al., 2013). In this way, the presence of high molecular weight (HMW) AGEs in the distal gut can influence the canine fecal microbiome. We hypothesized that the fecal microbiome will be altered in response to the four differently processed diets. Microbiome data and microbial load of the diets were obtained by high-throughput 16S rRNA gene sequencing via the MiSeq platform and analyzed using QIIME (Caporaso et al., 2010), METAGENassist (Arndt et al., 2012) and Galaxy (Segata et al., 2011) online metagenomic tools. Dysbiosis is characterized by decreased diversity (Kriss, Hazleton, Nusbacher, Martin, & Lozupone, 2018), changes in relative abundances (Hang et al., 2012), increased *Firmicutes* to *Bacteroidetes* ratio (Ley et al., 2005) and a shift in metabolism from fermentation to putrefaction (Jackson & Jewell, 2019). In this study, a decrease in α-diversity, as measured by the Shannon and Simpson diversity indices of the fecal microbial communities was observed with the dry kibble while β-diversity, assessed by principal component analysis, was minimal between diet groups. There were more bacterial members of the phylum *Firmicutes* in these samples and statistical analyses revealed seven bacterial genera that were significantly different between diet groups; five belonging to the phyla *Firmicutes*, one from *Bacteroidetes* and one from *Fusobacteria*. There were no changes in the *Firmicutes* to *Bacteroidetes* ratio and no change from fermentation to putrefaction as dogs were fed the different diets. Based on these results, we observe that the dry kibble was associated with a dysbiotic fecal microbiome and that there were changes in the bacterial members of the communities in response to these diets. Fecal

metabolomics should be included in this type of study in future so that changes in microbiome membership can be correlated with the metabolic activity within the communities. There were no bacterial organisms in the high heat processed foods as was expected but several organisms were present in the low heat processed air-dried and mildly cooked/raw diets. Principal component analysis (PCA) showed that bacterial load of the two low heat processed diets were distinct from each other. Log2 fold change results indicated that there were 15 organisms overrepresented in the air-dried diet and two in the mildly cooked/raw diet: *Pediococcus* spp. and *Clostridium* spp. *Pediococcus* fermentation product was added to the low heat processed diets as a bactericidal agent; however, processing conditions for the air-dried diet did not allow the organisms to thrive as much as in the mildly cooked/raw diet. We observed that *Pediococcus* was not only present in the fecal microbiome of the mildly cooked/raw diet group, but also was found to be different in relative abundance from both high heat processed diets. None of the bacteria that were present in the low heat processed diets, except for *Pediococcus* as an additive, affected the relative abundances of the fecal communities. In this study, the diets did not contain any pathogenic organisms and did not appear to negatively affect the gut microbial balance or the host's health. The canine fecal microbiome is responsive to differently processed diets containing varied amounts of AGEs and has demonstrated a change towards dysbiosis when the dry kibble diet is fed. This has long-term implications for dogs consuming only extruded diets and should be further investigated to determine if the dysbiosis observed in this study is a consistent finding when dogs are switched to a dry kibble diet. Fecal metabolomics should be performed in addition to metagenomic analyses to determine if the imbalance in the fecal community is associated with bacterial metabolic activity that may be harmful to the canine host.

This study was designed to be a preliminary investigation into the fate of dietary AGEs in the normal canine body related to their influence on plasma circulating AGE quantities, effects on inflammation indicated by sRAGE values, alterations in biochemical processes in the body that may be evident in the serum and urine metabolome and lastly, effects on the gut microbiota that could lead to long-term negative effects. We observed potentially negative effects of the two high heat processed diets associated with higher total dietary AGE quantity. The canned wet diet contained the highest amount of dietary AGEs and was associated with the highest plasma burden of total AGEs while the dry kibble resulted in lower metabolite concentrations in both serum and urine and a dysbiotic fecal microbiome characterized by low diversity. These findings support the likelihood of the effects of high heat processed pet foods being detrimental to their health if they are fed these types of diets for long periods but needs further investigation to determine if our findings are consistent across diets and dogs with concurrent illness or of differently physiological states. Urine AGE analyses and fecal metabolomics should also be included in future studies since urine AGEs appear to be a better indicator of circulating AGEs in human beings (Scheijen et al., 2018) and fecal metabolomics complements microbiome metagenomic investigations by providing additional details of bacterial member metabolism within the fecal microbial communities of different diet groups (Hamer, De Preter, Windey, & Verbeke, 2012).

Dietary AGEs in pet foods and the effects on their health are still largely unknown. The results of this research inform the discussion of traditional high heat versus low heat methods of pet food processing outlining AGE quantities as a factor that affects choice of diet. More research into dietary AGEs in pet foods is needed before changes can be made to the range of commercial pet foods offered to veterinarians and pet owners but if mildly cooked/raw diets

prove to be a superior option as a result of decreased AGE quantity then there may be a drive to increase the availability of this type of product. The veterinarians' role will be as a liaison between the pet food industry and pet owners so that clients are aware of product options, attributes and potential negative effects of differently processed diets.

In summary, differently processed diets contain varied levels of dietary AGEs as a result of choice of ingredients, pre-processing and processing conditions, biochemical properties and availability of precursors in individual components and possibly microbial activity when low heat methods are used. The different extents of AGE formation in the diets influence the metabolic transit of the AGEs, host metabolism and fecal microbiome. Further investigations are needed to compare a wider range of diets, processing methods, biofluids and feces between healthy and chronically ill dogs in longer term feeding trials to be able to draw definitive conclusions about the possible etiological role of AGEs in dogs.

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APPENDICES

Appendix A

Hematology

Hematology - baseline

Parameter	Dog No.								Mean	SD
	1	2	3	4	5	6	7	8		
WBC (10^3/mm3)	13.1	12.4	6.7	7.9	5.8	5.9	8.8	13.9	9.3	3.34
RBC (10^6/mm3)	7.6	7.4	7.6	8.5	7.3	8.4	6.4	6.8	7.5	0.72
Hemoglobin (g/dL)	16.6	17.3	16.3	19.9	17.1	20.8	14.4	16.2	17.3	2.08
Hematocrit (%)	55	56	55	57	51	59	48	54	54	3.5
MCV (um^3)	73	75	72	68	70	70	75	80	73	3.8
MCH (uug)	22.0	23.4	21.5	23.6	23.5	24.9	22.5	24.0	23.2	1.11
MCHC (g/dl)	30	31	30	35	34	36	30	30	32	2.6
Platelets (10^3/mm3)	363	320	299	213	207	281	367	322	297	60.7
Absolute Polys	10087	10044	5092	5530	4234	3835	5984	10981	6973	2906.6
% Polys	77	81	76	70	73	65	68	79	74	5.6
Absolute Bands	0	0	0	0	0	0	0	0	0	0.0
% Bands	0	0	0	0	0	0	0	0	0	0.0
Absolute Lymphs	2227	1612	1139	1975	1276	1770	1936	1529	1683	367.1
% Lymphs	17	13	17	25	22	30	22	11	20	6.3
Absolute Monos	524	620	335	237	232	177	616	834	447	236.0
% Monos	4	5	5	3	4	3	7	6	5	1.4
Absolute Eos	262	124	134	158	58	118	264	556	209	157.1
% Eos	2	1	2	2	1	2	3	4	2	1.0
Absolute Basos	0	0	0	0	0	0	0	0	0	0.0
% Basos	0	0	0	0	0	0	0	0	0	0.0

Appendix A continued

Hematology – test diet 1 (canned wet)

Parameter	Dog No.								Mean	SD
	1	2	3	4	5	6	7	8		
WBC (10^3/mm3)	11.5	6.9	6.0	22.7	5.0	5.9	20.8	9.3	11.0	6.97
RBC (10^6/mm3)	6.6	6.2	6.6	6.6	6.3	8.1	6.1	7.3	6.7	0.67
Hemoglobin (g/dL)	15.0	15.1	14.9	15.1	14.4	19.0	14.3	17.8	15.7	1.72
Hematocrit (%)	45	44	49	51	49	63	44	56	50	6.6
MCV (um^3)	69	71	75	77	78	77	71	76	74	3.4
MCH (uug)	22.8	24.3	22.7	22.8	23.0	23.4	23.3	24.2	23.3	0.63
MCHC (g/dl)	33	34	31	30	30	30	33	32	32	1.6
Platelets (10^3/mm3)	319	407	378	242	224	231	381	333	314	73.5
Absolute Polys	8510	5313	3960	19295	3300	3776	18096	7440	8711	6431.0
% Polys	74	77	66	85	66	64	87	80	75	8.9
Absolute Bands	0	0	0	0	0	0	0	0	0	0.0
% Bands	0	0	0	0	0	0	0	0	0	0.0
Absolute Lymphs	2300	1242	1620	2043	1400	1652	2080	1302	1705	394.4
% Lymphs	20	18	27	9	28	28	10	14	19	7.9
Absolute Monos	460	207	300	681	200	236	624	465	397	189.4
% Monos	4	3	5	3	4	4	3	5	4	0.8
Absolute Eos	230	138	120	681	100	236	0	93	200	208.8
% Eos	2	2	2	3	2	4	0	1	2	1.2
Absolute Basos	0	0	0	0	0	0	0	0	0	0.0
% Basos	0	0	0	0	0	0	0	0	0	0.0

Appendix A continued

Hematology – test diet 2 (dry kibble)

Parameter	Dog No.								Mean	SD
	1	2	3	4	5	6	7	8		
WBC (10^3/mm3)	14.0	9.2	4.7	8.5	6.4	7.0	10.6	8.0	8.6	2.84
RBC (10^6/mm3)	6.1	5.7	6.5	6.9	5.9	7.7	5.4	6.4	6.3	0.73
Hemoglobin (g/dL)	13.7	13.7	14.3	15.8	13.6	18.6	12.3	14.6	14.6	1.91
Hematocrit (%)	44	43	48	50	45	59	41	49	47	5.6
MCV (um^3)	73	75	73	72	75	77	76	77	75	1.9
MCH (uug)	22.6	23.8	22.1	22.9	23.0	24.4	22.8	23.1	23.1	0.71
MCHC (g/dl)	31	32	30	32	30	32	30	30	31	1.0
Platelets (10^3/mm3)	420	400	336	162	235	288	447	264	319	99.4
Absolute Polys	10080	6808	3102	6205	4544	4760	7208	5680	6048	2102.4
% Polys	72	74	66	73	71	68	68	71	70	2.8
Absolute Bands	0	0	0	0	0	0	0	0	0	0.0
% Bands	0	0	0	0	0	0	0	0	0	0.0
Absolute Lymphs	2800	1840	1222	1785	1472	1680	2332	1760	1861	494.6
% Lymphs	20	20	26	21	23	24	22	22	22	2.1
Absolute Monos	840	460	235	170	256	210	742	320	404	255.6
% Monos	6	5	5	2	4	3	7	4	5	1.6
Absolute Eos	280	92	141	340	128	350	318	240	236	102.8
% Eos	2	1	3	4	2	5	3	3	3	1.2
Absolute Basos	0	0	0	0	0	0	0	0	0	0.0
% Basos	0	0	0	0	0	0	0	0	0	0.0

Appendix A continued

Hematology – test diet 3 (air-dried)

Parameter	Dog No								Mean	SD
	1	2	3	4	5	6	7	8		
WBC (10^3/mm3)	11.2	8.8	6.6	7.1	7.2	9.2	10.3	6.1	8.3	1.85
RBC (10^6/mm3)	6.3	6.4	6.6	7.6	6.8	7.6	5.1	7.0	6.7	0.80
Hemoglobin (g/dL)	13.8	15.0	15.0	17.9	15.5	18.4	11.7	15.8	15.4	2.14
Hematocrit (%)	47	50	44	52	49	56	39	53	49	5.4
MCV (um^3)	75	78	68	69	72	73	77	76	74	3.7
MCH (uug)	22.0	23.5	22.9	23.6	22.8	24.2	23.0	22.8	23.1	0.66
MCHC (g/dl)	29	30	34	34	32	33	30	30	32	2.0
Platelets (10^3/mm3)	389	353	372	228	234	427	406	341	344	74.8
Absolute Polys	8064	6688	4950	5325	5184	6348	6489	4148	5900	1234.4
% Polys	72	76	75	75	72	69	63	68	71	4.4
Absolute Bands	0	0	0	0	0	0	0	0	0	0.0
% Bands	0	0	0	0	0	0	0	0	0	0.0
Absolute Lymphs	2576	1584	1122	1420	1584	2300	2884	1403	1859	638.6
% Lymphs	23	18	17	20	22	25	28	23	22	3.6
Absolute Monos	336	264	330	213	288	368	515	244	320	94.2
% Monos	3	3	5	3	4	4	5	4	4	0.8
Absolute Eos	224	264	198	142	144	184	412	305	234	91.1
% Eos	2	3	3	2	2	2	4	5	3	1.1
Absolute Basos	0	0	0	0	0	0	0	0	0	0.0
% Basos:	0	0	0	0	0	0	0	0	0	0.0

Appendix A continued

Hematology – test diet 4 (mildly cooked/raw)

Parameter	Dog No.								Mean	SD
	1	2	3	4	5	6	7	8		
WBC (10^3/mm3)	11.1	8.1	14.3	13.5	8.4	6.4	9.9	7.6	9.9	2.85
RBC (10^6/mm3)	7.0	7.0	7.1	7.9	6.1	7.3	5.9	7.0	6.9	0.64
Hemoglobin (g/dL)	15.4	16.6	16.0	18.2	13.8	17.6	13.5	16.5	16.0	1.67
Hematocrit (%)	51	53	51	57	45	55	45	55	52	4.5
MCV (um^3)	72	76	71	73	75	76	78	79	75	2.8
MCH (uug)	22.0	23.6	22.5	23.0	22.8	24.3	23.1	23.5	23.1	0.71
MCHC (g/dl)	30	31	32	32	30	32	30	30	31	1.0
Platelets (10^3/mm3)	360	366	419	304	350	357	471	318	368	53.9
Absolute Polys	7770	5913	10725	10260	6384	3904	6633	5168	7095	2380.3
% Polys	70	73	75	76	76	61	67	68	71	5.3
Absolute Bands	0	0	0	0	0	0	0	0	0	0.0
% Bands	0	0	0	0	0	0	0	0	0	0.0
Absolute Lymphs	2442	1539	2288	2565	1596	1984	2277	1596	2036	414.6
% Lymphs	22	19	16	19	19	31	23	21	21	4.5
Absolute Monos	555	324	858	405	252	192	594	456	455	214.4
% Monos	5	4	6	3	3	3	6	6	5	1.4
Absolute Eos	333	324	429	270	168	320	396	380	328	81.6
% Eos	3	4	3	2	2	5	4	5	4	1.2
Absolute Basos	0	0	0	0	0	0	0	0	0	0.0
% Basos	0	0	0	0	0	0	0	0	0	0.0

Appendix B

Serum chemistry

Serum chemistry - baseline

Parameter	Dog No.								Mean	SD
	1	2	3	4	5	6	7	8		
Total Protein (g/dL):	6.4	6.3	7.8	6.9	6.4	6.2	7.7	6.3	6.8	0.65
Albumin (g/dL):	3.4	3.7	2.9	3.2	3.6	3.5	3.2	3.5	3.4	0.26
Globulin (g/dL):	3.0	2.6	4.9	3.7	2.8	2.7	4.5	2.8	3.4	0.89
A/G Ratio:	1.1	1.4	0.6	0.9	1.3	1.3	0.7	1.3	1.1	0.31
AST (U/L):	39	19	27	23	20	25	25	16	24	7.0
ALT (U/L):	33	26	33	27	85	68	28	31	41	22.3
Alkaline Phosphatase (U/L):	73	27	58	60	23	32	51	95	52	24.7
GGTP (U/L):	5	5	5	4	4	6	4	4	5	0.7
Total Bilirubin (mg/dL):	0.2	0.1	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.05
Urea Nitrogen (mg/dL):	11	9	13	15	10	12	13	9	12	2.1
Creatinine (mg/dL):	0.5	0.4	0.5	0.6	0.5	0.6	0.7	0.4	0.5	0.10
BUN/Creatinine Ratio:	22	23	26	25	20	20	19	23	22	2.5
Phosphorus (mg/dL):	3.7	3.7	3.2	3.7	3.4	3.2	3.3	4.0	3.5	0.29
Glucose (mg/dL):	97	117	97	90	106	95	99	99	100	8.2
Calcium (mg/dL):	9.6	10.0	10.2	9.9	9.7	9.9	10.4	10.0	10.0	0.26
Magnesium (mEq/L):	1.7	1.7	1.5	1.6	1.6	1.5	1.6	1.4	1.6	0.10
Sodium (mEq/L):	146	148	146	149	149	149	145	148	148	1.6
Potassium (mEq/L):	4.2	4.1	4.1	4.2	4.0	4.8	5.1	4.5	4.4	0.39
Chloride (mEq/L):	112	113	112	117	112	114	112	114	113	1.8
Cholesterol (mg/dL):	187	180	153	147	176	183	301	276	200	56.6
Triglycerides (mg/dL)	19	42	38	40	39	48	35	67	41	13.4
CPK(U/L)	207	92	91	76	91	104	74	57	99	46.0

Appendix B continued

Serum chemistry – test diet 1 (canned wet)

Parameter	Dog No.								Mean	SD
	1	2	3	4	5	6	7	8		
Total Protein (g/dL):	6.3	6.0	7.6	6.7	6.5	6.4	6.8	7.0	6.7	0.49
Albumin (g/dL):	3.0	3.5	2.8	2.6	3.2	3.2	2.8	3.5	3.1	0.33
Globulin (g/dL):	3.3	2.5	4.8	4.1	3.3	3.2	4.0	3.5	3.6	0.70
A/G Ratio:	0.9	1.4	0.6	0.6	1.0	1.0	0.7	1.0	0.9	0.27
AST (U/L):	27	19	25	24	17	22	27	27	24	3.9
ALT (U/L):	34	21	30	28	28	67	50	41	37	14.9
Alkaline Phosphatase (U/L):	99	16	49	117	29	34	24	67	54	36.9
GGTP (U/L):	5	7	4	3	5	4	4	5	5	1.2
Total Bilirubin (mg/dL):	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.04
Urea Nitrogen (mg/dL):	18	11	20	18	13	15	14	21	16	3.5
Creatinine (mg/dL):	0.5	0.6	0.6	0.6	0.6	0.6	0.8	1.0	0.7	0.16
BUN/Creatinine Ratio:	36	18	33	30	22	25	18	21	25	6.9
Phosphorus (mg/dL):	3.3	3.6	2.7	3.3	2.9	3.2	3.5	5.0	3.4	0.70
Glucose (mg/dL):	92	90	87	63	94	91	89	95	88	10.3
Calcium (mg/dL):	10.1	10.0	10.2	10.0	9.9	9.9	9.8	9.7	10.0	0.16
Magnesium (mEq/L):	1.6	1.9	1.5	1.6	1.6	1.7	1.4	1.8	1.6	0.16
Sodium (mEq/L):	147	148	145	146	150	150	145	147	147	2.0
Potassium (mEq/L):	4.6	4.8	4.4	4.1	4.7	4.9	4.8	4.8	4.6	0.27
Chloride (mEq/L):	113	112	113	116	114	114	113	114	114	1.2
Cholesterol (mg/dL):	214	172	158	134	181	158	158	204	172	26.5
Triglycerides (mg/dL)	53	37	30	24	31	32	51	59	40	12.9
CPK(U/L)	111	134	79	68	83	64	70	134	93	29.2

Appendix B continued

Serum chemistry – test diet 2 (dry kibble)

Parameter	Dog No.								Mean	SD
	1	2	3	4	5	6	7	8		
Total Protein (g/dL):	6.6	6.0	7.1	6.0	6.1	6.4	8.0	6.7	6.6	0.68
Albumin (g/dL):	3.1	3.5	2.6	2.7	3.2	3.2	2.8	3.1	3.0	0.30
Globulin (g/dL):	3.5	2.5	4.5	3.3	2.9	3.2	5.2	3.6	3.6	0.87
A/G Ratio:	0.9	1.4	0.6	0.8	1.1	1.0	0.5	0.9	0.9	0.28
AST (U/L):	30	16	25	24	16	19	24	20	22	4.9
ALT (U/L):	30	22	29	31	25	49	41	38	33	8.9
Alkaline Phosphatase (U/L):	108	18	58	104	52	40	129	98	76	39.0
GGTP (U/L):	5	7	6	4	5	4	5	4	5	1.1
Total Bilirubin (mg/dL):	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.04
Urea Nitrogen (mg/dL):	21	14	20	14	12	11	19	15	16	3.8
Creatinine (mg/dL):	0.4	0.5	0.5	0.5	0.4	0.6	0.6	0.5	0.5	0.08
BUN/Creatinine Ratio:	53	28	40	28	30	18	32	30	32	10.3
Phosphorus (mg/dL):	6.1	5.4	4.1	4.5	5.4	4.2	3.5	4.6	4.7	0.85
Glucose (mg/dL):	91	105	87	67	96	93	82	85	88	11.2
Calcium (mg/dL):	9.8	9.7	10.1	9.8	9.9	10.2	10.3	9.9	10.0	0.21
Magnesium (mEq/L):	1.8	1.8	1.5	1.6	1.5	1.5	1.6	1.5	1.6	0.13
Sodium (mEq/L):	147	146	147	148	147	148	142	147	147	1.9
Potassium (mEq/L):	5.1	5.4	4.4	4.2	4.7	5.1	4.8	4.7	4.8	0.39
Chloride (mEq/L):	114	111	114	116	113	111	109	111	112	2.3
Cholesterol (mg/dL):	225	145	137	99	185	155	374	182	188	84.1
Triglycerides (mg/dL)	54	41	45	31	26	36	89	63	48	20.4
CPK(U/L)	117	89	81	81	74	66	65	60	79	18.1

Appendix B – continued

Serum chemistry – test diet 3 (air-dried)

Parameter	Dog No.								Mean	SD
	1	2	3	4	5	6	7	8		
Total Protein (g/dL):	6.4	6.0	6.9	6.6	6.2	6.4	7.6	6.9	6.6	0.50
Albumin (g/dL):	3.1	3.5	2.8	3.0	3.4	3.4	2.6	3.0	3.1	0.32
Globulin (g/dL):	3.3	2.5	4.1	3.6	2.8	3.0	5.0	3.9	3.5	0.81
A/G Ratio:	0.9	1.4	0.7	0.8	1.2	1.1	0.5	0.8	0.9	0.29
AST (U/L):	28	23	30	20	20	28	22	17	24	4.7
ALT (U/L):	36	36	39	31	33	53	28	29	36	8.0
Alkaline Phosphatase (U/L):	69	36	38	55	15	23	44	51	41	17.4
GGTP (U/L):	4	5	4	3	8	7	5	4	5	1.7
Total Bilirubin (mg/dL):	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.04
Urea Nitrogen (mg/dL):	10	11	16	18	21	21	20	13	16	4.5
Creatinine (mg/dL):	0.6	0.5	0.6	0.7	0.6	0.8	0.7	0.6	0.6	0.09
BUN/Creatinine Ratio:	17	22	27	26	35	26	29	22	26	5.4
Phosphorus (mg/dL):	3.2	3.6	3.1	3.9	4.8	5.0	3.0	3.7	3.8	0.75
Glucose (mg/dL):	82	83	79	62	93	95	86	83	83	10.1
Calcium (mg/dL):	9.8	9.8	10.1	10.1	10.0	9.8	10.4	10.3	10.0	0.23
Magnesium (mEq/L):	1.6	2.0	1.5	1.6	1.6	1.6	1.6	1.7	1.7	0.15
Sodium (mEq/L):	147	147	146	148	148	149	147	150	148	1.3
Potassium (mEq/L):	4.7	4.5	4.3	4.1	4.7	5.5	4.7	4.6	4.6	0.41
Chloride (mEq/L):	114	112	114	117	115	117	113	114	115	1.8
Cholesterol (mg/dL):	175	188	150	132	175	153	253	202	179	37.5
Triglycerides (mg/dL)	25	45	50	32	56	94	38	40	48	21.2
CPK(U/L)	137	151	117	93	87	124	57	79	106	31.8

Appendix B continued

Serum chemistry – test diet 4 (mildly cooked/raw)

Parameter	Dog No.								Mean	SD
	1	2	3	4	5	6	7	8		
Total Protein (g/dL):	6.7	5.8	7.2	6.7	6.4	6.5	7.6	6.8	6.7	0.54
Albumin (g/dL):	3.0	3.2	2.8	3.0	3.2	3.1	2.8	3.1	3.0	0.16
Globulin (g/dL):	3.7	2.6	4.4	3.7	3.2	3.4	4.8	3.7	3.7	0.68
A/G Ratio:	0.8	1.2	0.6	0.8	1.0	0.9	0.6	0.8	0.8	0.20
AST (U/L):	34	25	33	27	21	26	25	23	27	4.6
ALT (U/L):	47	31	63	35	41	131	25	76	56	34.7
Alkaline Phosphatase (U/L):	79	26	104	69	60	63	61	77	67	22.0
GGTP (U/L):	3	5	6	4	4	5	4	3	4	1.0
Total Bilirubin (mg/dL):	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.04
Urea Nitrogen (mg/dL):	10	14	39	25	11	14	19	13	18	9.7
Creatinine (mg/dL):	0.6	0.5	0.8	0.7	0.5	0.6	0.7	0.6	0.6	0.10
BUN/Creatinine Ratio:	17	28	49	36	22	23	27	22	28	10.2
Phosphorus (mg/dL):	2.8	3.9	3.8	4.2	3.0	3.0	3.1	3.9	3.5	0.54
Glucose (mg/dL):	70	82	84	81	90	106	89	85	86	10.2
Calcium (mg/dL):	9.8	9.9	10.1	10.0	9.4	10.2	11.0	10.4	10.1	0.47
Magnesium (mEq/L):	1.7	1.9	1.5	1.6	1.6	1.7	1.7	1.8	1.7	0.12
Sodium (mEq/L):	149	150	144	146	147	149	146	148	147	2.0
Potassium (mEq/L):	4.6	4.6	4.7	4.5	4.8	5.3	4.8	4.6	4.7	0.25
Chloride (mEq/L):	113	113	114	116	112	113	109	109	112	2.4
Cholesterol (mg/dL):	227	180	197	157	243	200	375	268	231	68.1
Triglycerides (mg/dL)	35	44	76	38	50	48	52	60	50	13.0
CPK(U/L)	122	131	103	98	97	60	64	104	97	24.9

Appendix C

Urinalysis

Urinalysis – baseline

Dog No.	Method of Collection	Color	Appearance	Specific Gravity	pH	Protein	Glucose	Ketone	Bilirubin
1	Free catch	Dark Yellow	Cloudy	1.046	6.5	Neg	Neg	Neg	1+
2	Free catch	Orange	Turbid	1.047	6.0	1+	Neg	Neg	1+
3	Free catch	Orange	Cloudy	1.057	7.5	2+	Neg	Neg	1+
4	Free catch	Orange	Cloudy	1.059	7.0	1+	Neg	Neg	1+
5	Free catch	Orange	Cloudy	1.055	6.5	2+	Neg	Neg	1+
6	Free catch	Dark Yellow	Turbid	1.029	6.5	2+	Neg	Neg	1+
7	Free catch	Dark Yellow	Cloudy	1.051	6.5	1+	Neg	Neg	1+
8	Free catch	Orange	Cloudy	1.051	6.5	3+	Neg	Neg	1+
			Mean:	1.049	6.6				
			SD:	0.0094	0.44				

Dog No.	Blood	WBC (hpf)	RBC (hpf)	Casts (lpf)	Crystals (hpf)	Bacteria (hpf)	Epithelial Cells (hpf)	Other
1	Neg	None	0-1	None	Present	None	0-1	Struvite Crystals 4-10, Squamous Epithelia, Fat Droplets 2-3
2	Neg	0-1	0-1	None	None	None	0-1	Squamous Epithelia, Fat Droplets 4-10
3	Neg	4-10	None	None	Present	None	2-3	Struvite Crystals 11-20, Squamous Epithelia, Sperm
4	Neg	2-3	None	None	Present	None	None	Struvite Crystals 11-20, Ca Oxalate Dihydrate Crystals 2-3, Sperm
5	1+	None	4-10	None	Present	None	2-3	Struvite Crystal 2-3, Squamous Epithelia, Fat Droplets 2-3
6	Neg	0-1	None	None	None	None	0-1	Sqaumous Epithelia, Sperm
7	Neg	4-10	None	None	Present	None	2-3	Struvite Crystals 4-10, Squamous Epithelia, Fat Droplets 2-3
8	Neg	None	None	None	Present	None	None	Struvite Crystals 2-3, Ca Oxalate Crystals 21-50

Appendix C continued

Urinalysis – test diet 1 (canned wet)

Dog No.	Method of Collection	Color	Appearance	Specific Gravity	pH	Protein	Glucose	Ketone	Bilirubin
1	Free catch	Yellow	Slightly cloudy	1.020	6.0	Neg	Neg	Neg	Neg
2	Free catch	Yellow	Cloudy	1.026	6.0	Neg	Neg	Neg	Neg
3	Free catch	Yellow	Clear	1.024	6.0	Trace	Neg	Neg	Neg
4	Free catch	Yellow	Cloudy	1.024	6.0	Neg	Neg	Neg	Neg
5	Free catch	Yellow	Cloudy	1.021	6.5	Neg	Neg	Neg	Neg
6	Free catch	Yellow	Hazy	1.025	6.5	1+	Neg	Neg	Neg
7	Free catch	Dark Yellow	Cloudy	1.029	8.0	1+	Neg	Neg	1+
8	Cystocentesis	Dark Yellow	Slightly hazy	1.047	7.5	2+	Neg	Neg	1+
			Mean:	1.027	6.6				
			SD:	0.0086	0.78				

Dog No.	Blood	WBC (hpf)	RBC (hpf)	Casts (lpf)	Crystals (hpf)	Bacteria (hpf)	Epithelial Cells (hpf)	Other
1	Neg	0-1	None	None	Present	Present	None	Struvite Crystals 0-1, Bacteria Rods 26-50, Fat Droplets 0-1
2	Neg	0-1	None	None	None	None	None	Fat Droplets 4-10
3	Neg	None	None	None	None	Present	None	Bacteria Cocci 51-100, Sperm
4	Neg	4-10	None	None	None	Present	None	Bacteria Rods >100
5	Neg	None	None	None	Present	Present	None	Amorphous Crystals 21-50, Bacteria Rods>100
6	Neg	None	None	None	Present	Present	None	Struvite Crystals 0-1, Bacteria Cocci and Rods 10-25
7	Neg	4-10	0-1	None	Present	Present	2-3	Struvite Crystals 4-10, Bacteria Rods >100, Squamous Epithelia
8	Neg	None	11-20	None	None	None	None	Fat Droplets 21-50

Appendix C continued

Urinalysis – test diet 2 (dry kibble)

Dog No.	Method of Collection	Color	Appearance	Specific Gravity	PH	Protein	Glucose	Ketone	Bilirubin
1	Free catch	Yellow	Clear	1.040	8.5	1+	Neg	Neg	Neg
2	Free catch	Yellow	Clear	1.031	8.5	1+	Neg	Neg	Neg
3	Free catch	Dark Yellow	Clear	1.055	8.5	1+	Neg	Neg	Neg
4	Free catch	Yellow	Cloudy	1.040	7.5	Trace	Neg	Neg	Neg
5	Free catch	Dark Yellow	Cloudy	1.051	8.0	2+	Neg	Neg	1+
6	Free catch	Dark Yellow	Slightly Cloudy	1.042	8.0	2+	Neg	Neg	Neg
7	Free catch	Yellow	Cloudy	1.036	8.0	1+	Neg	Neg	Neg
8	Free catch	Dark Yellow	Cloudy	1.054	8.5	3+	Trace	Neg	1+
			Mean:	1.044	8.2				
			SD:	0.0088	0.37				

Dog No.	Blood	WBC (hpf)	RBC (hpf)	Casts (lpf)	Crystals (hpf)	Bacteria (hpf)	Epithelial Cells (hpf)	Other
1	Neg	None	2-3	None	Present	None	0-1	Struvite Crystals 21-50, Squamous Epithelia, Fat Droplets 4-10
2	Neg	0-1	0-1	None	Present	Present	None	Struvite Crystals 0-1 Bacteria Rods 10-25, Fat Droplets 11-20
3	Neg	0-1	None	None	Present	Present	None	Struvite Crystals 2-3, Bacteria Cocci 10-25
4	Neg	None	None	None	Present	Present	2-3	Struvite Crystals 11-20, Bacteria Cocci and Rods 10-25, Squamous Epithelia, Sperm
5	Neg	0-1	None	None	Present	None	None	Struvite Crystals 2-3, Amorphous Phosphate Crystals 0-1, Fat Droplets 4-10
6	Neg	0-1	None	None	Present	None	None	Struvite Crystals 2-3, Fat Droplets 2-3, Sperm
7	Neg	None	None	None	Present	Present	None	Struvite Crystals 4-10, Bacteria Cocci and Rods 26-50
8	Neg	None	None	None	Present	Present	None	Struvite Crystals 11-20, Bacteria Cocci 51-100, Fat Droplets 4-10

Appendix C continued

Urinalysis – test diet 3 (air-dried)

Dog No.	Method of Collection	Color	Appearance	Specific Gravity	PH	Protein	Glucose	Ketone	Bilirubin
1	Free catch	NA	Cloudy	1.042	8.5	1+	Neg	Neg	Neg
2	Free catch	Yellow	Turbid	1.050	8.5	1+	Trace	Neg	Neg
3	Free catch	Dark Yellow	Cloudy	1.051	8.5	3+	Neg	Neg	Neg
4	Free catch	Dark Yellow	Cloudy	1.056	6.5	1+	Neg	Neg	1+
5	Free catch	Yellow	Hazy	1.047	8.5	2+	Neg	Neg	1+
6	Free catch	Yellow	Cloudy	1.028	8.5	2+	Neg	Neg	Neg
7	Free catch	Yellow	Hazy	1.057	7.0	1+	Neg	Neg	Neg
8	Free catch	Yellow	Turbid	1.050	8.5	2+	Neg	Neg	Neg
			Mean:	1.048	8.1				
			SD:	0.0092	0.82				

Dog No.	Blood	WBC (hpf)	RBC (hpf)	Casts (lpf)	Crystals (hpf)	Bacteria (hpf)	Epithelial Cells (hpf)	Other
1	Neg	None	None	None	Present	Present	None	Struvite Crystals >50, Bacteria Cocci >100
2	Neg	0-1	0-1	None	Present	Present	None	Struvite Crystals 4-10, Bacteria Cocci >100
3	Neg	2-3	None	None	Present	None	None	Struvite Crystals 2-3, Amorphous Phosphate Crystals 0-1, Fat Droplets 2-3, WBC Clumps 0-1, Sperm
4	Neg	0-1	None	None	Present	None	None	Struvite Crystals 2-3, Amorphous Crystals 0-1, Fat Droplets 4-10
5	Neg	None	None	None	Present	Present	0-1	Struvite Crystals 2-3, Bacteria Rods <10, Squamous Epithelia, Fat Droplets 11-20
6	Neg	0-1	0-1	None	Present	Present	0-1	Struvite Crystals 2-3, Bacteria Rods >100, Squamous Epithelia
7	Neg	11-20	2-3	None	Present	Present	0-1	Struvite Crystals 2-3, Bacteria Cocci 10-25, Squamous Epithelia
8	Neg	None	None	None	Present	None	None	Amorphous Phosphate Crystals >50

Appendix C continued

Urinalysis – test diet 4 (mildly cooked/raw)

Dog No.	Method of Collection	Color	Appearance	Specific Gravity	PH	Protein	Glucose	Ketone	Bilirubin
1	Free catch	Colorless	Cloudy	1.025	7.5	Neg	Neg	Neg	Neg
2	Free catch	Yellow	Cloudy	1.022	8.5	Neg	Neg	Neg	Neg
3	Free catch	Yellow	Slightly hazy	1.040	8.5	2+	Neg	Neg	Neg
4	Free catch	Yellow	Slightly hazy	1.025	8.0	Trace	Neg	Neg	Neg
5	Free catch	Yellow	Cloudy	1.015	7.0	Neg	Neg	Neg	Neg
6	Free catch	Yellow	Cloudy	1.022	7.0	1+	Neg	Neg	Neg
7	Free catch	Yellow	Cloudy	1.023	7.0	Trace	Neg	Neg	Neg
8	Free catch	Yellow	Cloudy	1.022	7.5	1+	Neg	Neg	Neg
			Mean:	1.024	7.6				
			SD:	0.0071	0.64				

Dog No.	Blood	WBC (hpf)	RBC (hpf)	Casts (lpf)	Crystals (hpf)	Bacteria (hpf)	Epithelial Cells (hpf)	Other
1	Neg	None	None	None	None	Present	None	Bacteria Rods >100
2	Neg	None	None	None	Present	Present	None	Struivte Crystals 2-3, Bacteria Cocci and Rods 10-25
3	Neg	2-3	0-1	None	Present	None	None	Struvite Crystals 0-1, Amorphous Phosphate Crystals 21-50
4	Neg	11-20	2-3	None	None	Present	0-1	Bacteria Rods 10-25, Transitional Epithelia, Squamous Epithelia
5	Neg	None	None	None	None	Present	None	Bacteria Rods >100
6	Neg	None	None	None	Present	Present	None	Struvite Crystals 0-1, Bacteria Rods >100
7	Neg	0-1	None	None	None	Present	None	Bacteria Rods 51-100, Fat Droplets 4-10
8	Neg	0-1	0-1	None	Present	Present	0-1	Struvite Crystals 0-1, Bacteria Rods 51-100, Squamous Epithelia, Fat Droplets 2-3

Appendix D

Normal reference ranges for hematology and serum chemistry

Parameter	**Normal Reference Ranges**
Total Protein (g/dL):	5.0 – 7.4 g/dL
Albumin (g/dL):	2.7 – 4.4 g/dL
Globulin (g/dL):	1.6 – 3.6 g/dL
A/G Ratio:	0.8 – 2.0 Ratio
AST (U/L):	15 – 66 U/L
ALT (U/L):	12 – 118 U/L
Alkaline Phosphatase (U/L):	5 – 131 U/L
GGTP (U/L):	1 – 12 U/L
Total Bilirubin (mg/dL):	0.1 – 0.3 mg/dL
Urea Nitrogen (mg/dL):	6 – 31 mg/dL
Creatinine (mg/dL):	0.5 – 1.6 mg/dL
BUN/Creatinine Ratio:	4 – 27 Ratio
Phosphorus (mg/dL):	2.5 – 6.0 mg/dL
Glucose (mg/dL):	70 – 138 mg/dL
Calcium (mg/dL):	8.9 – 11.4 mg/dL
Magnesium (mEq/L):	1.5 – 2.5 mEq/L
Sodium (mEq/L):	139 – 154 mEq/L
Potassium (mEq/L):	3.6 – 5.5 mEq/L
Chloride (mEq/L):	102 – 120 mEq/L
Cholesterol (mg/dL):	92 – 324 mg/dL
Triglycerides (mg/dL)	29 – 291 mg/dL
CPK(U/L)	59 – 895 U/L
WBC(10^3/mm3):	4.0 – 15.5 10^3/mm3
RBC(10^6/mm3):	4.8 – 9.3 10^6/mm3
Hemoglobin (g/dL):	12.1 – 20.3 g/dL
Hematocrit (%):	36 – 60%
MCV (um^3):	58 – 79 um^3
MCH (uug):	19 – 28 uug
MCHC (g/dl):	30 – 38 g/dL
Platelets (10^3/mm3):	170 – 400 10^3/mm3
Absolute Polys:	2060 – 10600
Absolute Bands:	0 – 300
Absolute Lymphs:	690 – 4500
Absolute Monos:	0 – 840
Absolute Eos:	0 – 1200
Absolute Basos:	0 – 150

Appendix E

Ingredients and nutritional analyses of test diets (100% dry matter basis)

Ingredients list - test diet 1 (canned wet)

Ingredient Name	**Formula%**
Water (MDF-2/11)	37.670
MD Kangaroo VIP (MDF - 4/16)	30.000
Sweet Potato (USDA-2/11)	14.000
Potato Starch (MDF - 12/11)	6.850
Kangaroo Hearts VIP (MDF - 4/16)	5.200
Kangaroo Liver VIP (MDF 4/16)	5.000
Guar Gum (MDF 10/11)	0.300
VIP Dog/Cat Vitamin-TM Premix (5/16)	0.260
Sunflower Oil-65% LA (USDA-2/11)	0.220
Tricalcium Phosphate Food Grade (MDF 8/1	0.150
FOS (MDF-12/10)	0.150
Sea Salt (MDF 8/10)	0.100
Potassium Chloride (MDF 8/10)	0.100
	100.000

Appendix E continued

Nutrient analysis - test diet 1 (canned wet)

No.	Nutrient Name	Formula Amount	100.0 %DM Amount	Units
1	ME (AAFCO - 3.5, 8.5)	754.19	3717.29	kcal/kg
3	Moisture	79.71		%
4	Protein	8.01	39.48	%
5	Fat	1.75	8.61	%
6	Ash	1.18	5.82	%
7	Crude Fiber	0.22	1.11	%
10	Carbohydrates	9.30	45.82	%
12	LA 18:2 n-6	0.24	1.17	%
13	ALA 18:3 n-3	0.03	0.13	%
14	AA 20:4 n-6	0.03	0.15	%
16	EPA 20:5 n-3	0.01	0.04	%
18	DHA 22:6 n-3	0.00	0.01	%
19	Total LCPUFA	0.02	0.09	%
21	Total n-6	0.27	1.33	%
22	Total n-3	0.06	0.31	%
24	Calcium	0.17	0.86	%
25	Phosphorus	0.16	0.76	%
27	Available Phosphorus	0.15	0.74	%
28	Magnesium	0.02	0.08	%
29	Potassium	0.21	1.04	%
30	Sodium	0.10	0.48	%
31	Chloride	0.18	0.87	%
33	Iron	47.14	232.34	mg/kg
34	Zinc	39.90	196.66	mg/kg
35	Manganese	2.56	12.60	mg/kg
36	Copper	2.57	12.66	mg/kg
37	Iodine	0.42	2.05	mg/kg
38	Selenium	0.17	0.82	mg/kg
42	Vitamin A	8815.70	43451.13	IU/kg
43	Vitamin D	182.00	897.05	IU/kg
44	Vitamin E	69.06	340.37	IU/kg
45	Vitamin K	0.00	0.01	mg/kg
46	Thiamin	26.85	132.36	mg/kg
47	Riboflavin	4.59	22.60	mg/kg
48	Niacin	43.97	216.70	mg/kg

Appendix E continued

Nutrient analysis - test diet 1 (canned wet) continued

No.	Nutrient Name	Formula Amount	100.0 %DM Amount	Units
49	Pantothenic Acid	7.88	38.85	mg/kg
50	Pyridoxine	5.87	28.94	mg/kg
51	Folic Acid	0.82	4.04	mg/kg
52	Biotin	0.03	0.17	mg/kg
53	Vitamin B12	44.46	219.16	mcg/kg
54	Vitamin C	27.58	135.94	mg/kg
55	Choline	760.14	3746.60	mg/kg
56	Carnitine	206.30	1016.80	mg/kg
58	Alanine	0.51	2.51	%
59	Arginine	0.51	2.49	%
60	Aspartic Acid	0.83	4.10	%
61	Glutamic Acid	1.26	6.21	%
62	Glycine	0.49	2.39	%
63	Histidine	0.24	1.16	%
64	Isoleucine	0.39	1.91	%
65	Leucine	0.70	3.43	%
66	Lysine	0.71	3.48	%
67	Methionine	0.20	0.98	%
68	Met-Cystine	0.26	1.30	%
69	Phenylalanine	0.38	1.87	%
70	Phe-Tyrosine	0.68	3.34	%
71	Proline	0.38	1.88	%
72	Serine	0.37	1.83	%
73	Threonine	0.41	2.01	%
74	Tryptophan	0.11	0.55	%
75	Valine	0.41	2.04	%
76	Taurine	717.90	3538.41	mg/kg
81	FOS	0.11	0.55	%

Appendix E continued

Ingredients list - test diet 2 – dry kibble

Ingredient Name	**Formula %**
Dried Potato (MDF-8/16)	18.500
Kangaroo Slurry (MDF - 2/17)	18.500
Dried Chickpeas (MDF 10/16)	18.500
Pea Protein 50% CP (MDF - 11/15)	11.600
Potato Protein 75% (MDF-11/15)	11.600
Field Peas - Green (MDF-2/17)	9.717
Swt Pot Flour-US (MDF-2/11)	3.000
Liquid Digest Premium (MDF-11/10)	2.500
Sunflower Oil-65% LA (USDA-2/11)	2.233
Coconut Oil (USDA-2/11)	1.420
Calcium Carbonate (MDF-3/11)	0.945
Dicalcium Phosphate (MDF-3/11)	0.550
Salt (MDF 8/10)	0.300
FOS (MDF-12/10)	0.250
Rayne Dry Dog Vitamin Premix (MDF 8/13)	0.120
Choline Chloride 60% (MDF 8/10)	0.100
Bern Trace Mineral Premix #95 (MDF-8/10)	0.060
Naturox Plus Dry (MDF-9/10)	0.050
Taurine (MDF 8/10)	0.035
Bern Chel Min - Albion IMZ (MDF-8/10)	0.020
	100.000

Appendix E continued

Nutrient analysis - test diet 2 (dry kibble)

No.	Nutrient	100.0% DM Amount	Units
1	ME (AAFCO - 3.5, 8.5 kcal/kg)	3,628.08	kcal/kg
3	Moisture	0.00	%
4	Protein	33.94	%
5	Fat	8.40	%
6	Ash	6.09	%
7	Crude Fiber	2.25	%
8	ADF	3.35	%
9	NDF	6.22	%
10	Carbohydrates	49.32	%
12	LA 18:2 n-6	3.38	%
13	ALA 18:3 n-3	0.16	%
14	AA 20:4 n-6	0.01	%
16	EPA 20:5 n-3		%
18	DHA 22:6 n-3		%
19	Total LCPUFA		%
21	Total n-6	3.39	%
22	Total n-3	0.16	%
24	Calcium	0.79	%
25	Phosphorus	0.66	%
27	Available Phosphorus		%
28	Magnesium	0.16	%
29	Potassium	1.39	%
30	Sodium	0.20	%
31	Chloride	0.38	%
33	Iron	267.81	mg/kg
34	Zinc	215.09	mg/kg
35	Manganese	71.11	mg/kg
36	Copper	23.30	mg/kg
37	Iodine	1.27	mg/kg
38	Selenium	0.63	mg/kg
42	Vitamin A	31,571.70	IU/kg
43	Vitamin D	2,321.24	IU/kg
44	Vitamin E	494.43	IU/kg
45	Vitamin K		mg/kg
46	Thiamin	8.93	mg/kg
47	Riboflavin	11.40	mg/kg
48	Niacin	79.89	mg/kg
49	Pantothenic Acid	45.39	mg/kg
50	Pyridoxine	10.75	mg/kg
51	Folic Acid	1.08	mg/kg
52	Biotin	0.24	mg/kg

Appendix E continued

Nutrient analysis - test diet 2 (dry kibble) continued

No.	Nutrient	100.0% DM Amount	Units
53	Vitamin B12	77.26	mcg/kg
54	Vitamin C		mg/kg
55	Choline	1,763.30	mg/kg
56	Carnitine		mg/kg
58	Alanine	1.58	%
59	Arginine	2.33	%
60	Aspartic Acid	4.24	%
61	Glutamic Acid	4.83	%
62	Glycine	1.54	%
63	Histidine	0.80	%
64	Isoleucine	1.64	%
65	Leucine	2.86	%
66	Lysine	2.56	%
67	Methionine	0.56	%
68	Met-Cystine	0.97	%
69	Phenylalanine	1.85	%
70	Phe-Tyrosine	3.18	%
71	Proline	1.53	%
72	Serine	1.65	%
73	Threonine	1.52	%
74	Tryptophan	0.39	%
75	Valine	1.88	%
76	Taurine	572.77	mg/kg
81	FOS		%

Appendix E continued

Ingredients list - test diet 3 (air-dried)

Ingredient Name	**Formula %**
MD Kangaroo VIP (MDF - 4/16)	59.230
Sweet Potato (USDA-2/11)	26.840
Water (MDF-2/11)	8.530
Dextrose (MDF-9/10)	3.660
VIP Dog/Cat Vitamin-TM Premix (5/16)	0.380
Sunflower Oil-65% LA (USDA-2/11)	0.320
Calcium Carbonate (MDF-3/11)	0.250
Tricalcium Phosphate Food Grade (MDF 8/1	0.220
FOS (MDF-12/10)	0.220
Sea Salt (MDF 8/10)	0.150
Potassium Chloride (MDF 8/10)	0.150
LHP	0.050
	100.000

Appendix E continued

Nutrient analysis – test diet 3 (air-dried)

No.	Nutrient Name	Formula Amount	100.0 %DM Amount	Units
1	ME (AAFCO - 3.5, 8.5)	950.48	3746.83	kcal/kg
3	Moisture	74.63		%
4	Protein	11.61	45.77	%
5	Fat	2.78	10.97	%
6	Ash	2.10	8.27	%
7	Crude Fiber	0.42	1.65	%
10	Carbohydrates	8.79	34.65	%
12	LA 18:2 n-6	0.33	1.30	%
13	ALA 18:3 n-3	0.04	0.16	%
14	AA 20:4 n-6	0.03	0.12	%
16	EPA 20:5 n-3	0.01	0.04	%
18	DHA 22:6 n-3			%
19	Total LCPUFA	0.02	0.08	%
21	Total n-6	0.36	1.43	%
22	Total n-3	0.09	0.36	%
24	Calcium	0.40	1.59	%
25	Phosphorus	0.23	0.92	%
27	Available Phosphorus	0.23	0.89	%
28	Magnesium	0.03	0.11	%
29	Potassium	0.33	1.30	%
30	Sodium	0.14	0.57	%
31	Chloride	0.26	1.04	%
33	Iron	58.06	228.89	mg/kg
34	Zinc	60.52	238.59	mg/kg
35	Manganese	3.60	14.20	mg/kg
36	Copper	3.39	13.35	mg/kg
37	Iodine	0.61	2.40	mg/kg
38	Selenium	0.23	0.91	mg/kg
42	Vitamin A	6080.00	23967.51	IU/kg
43	Vitamin D	266.00	1048.58	IU/kg
44	Vitamin E	101.75	401.09	IU/kg
45	Vitamin K	0.00	0.02	mg/kg
46	Thiamin	39.28	154.85	mg/kg
47	Riboflavin	5.07	19.97	mg/kg
48	Niacin	57.76	227.69	mg/kg
49	Pantothenic Acid	6.72	26.51	mg/kg
50	Pyridoxine	8.55	33.69	mg/kg
51	Folic Acid	0.99	3.91	mg/kg
52	Biotin	0.05	0.19	mg/kg

Appendix E continued

Nutrient analysis – test diet 3 (air-dried) continued

No.	Nutrient Name	Formula Amount	100.0 %DM Amount	Units
53	Vitamin B12	23.25	91.64	mcg/kg
54	Vitamin C	52.87	208.43	mg/kg
55	Choline	966.69	3810.70	mg/kg
56	Carnitine	370.31	1459.76	mg/kg
58	Alanine	0.75	2.97	%
59	Arginine	0.77	3.02	%
60	Aspartic Acid	1.25	4.91	%
61	Glutamic Acid	1.91	7.53	%
62	Glycine	0.73	2.87	%
63	Histidine	0.35	1.37	%
64	Isoleucine	0.57	2.23	%
65	Leucine	1.00	3.94	%
66	Lysine	1.07	4.22	%
67	Methionine	0.30	1.17	%
68	Met-Cystine	0.39	1.54	%
69	Phenylalanine	0.54	2.13	%
70	Phe-Tyrosine	0.97	3.84	%
71	Proline	0.56	2.22	%
72	Serine	0.54	2.14	%
73	Threonine	0.60	2.38	%
74	Tryptophan	0.16	0.63	%
75	Valine	0.60	2.35	%
76	Taurine	771.15	3039.89	mg/kg
81	FOS	0.16	0.65	%

Appendix E continued

Ingredient list – test diet 4 (mildly cooked/raw)

Ingredient Name	**Formula %**
MD Kangaroo VIP (MDF - 4/16)	40.080
Water (MDF-2/11)	38.060
Sweet Potato (USDA-2/11)	18.160
Dextrose (MDF-9/10)	2.470
VIP Dog/Cat Vitamin-TM Premix (5/16)	0.260
Sunflower Oil-65% LA (USDA-2/11)	0.220
Calcium Carbonate (MDF-3/11)	0.200
Tricalcium Phosphate Food Grade (MDF 8/1	0.150
FOS (MDF-12/10)	0.150
Sea Salt (MDF 8/10)	0.100
Potassium Chloride (MDF 8/10)	0.100
LHP	0.050
	100.000

Appendix E continued

Nutrient analysis – test diet 4 (mildly cooked/raw)

No.	Nutrient Name	Formula Amount	100.0 %DM Amount	Units
1	ME (AAFCO - 3.5, 8.5)	643.87	3741.33	kcal/kg
3	Moisture	82.79		%
4	Protein	7.86	45.66	%
5	Fat	1.89	10.96	%
6	Ash	1.45	8.42	%
7	Crude Fiber	0.28	1.65	%
10	Carbohydrates	5.96	34.62	%
12	LA 18:2 n-6	0.23	1.31	%
13	ALA 18:3 n-3	0.03	0.16	%
14	AA 20:4 n-6	0.02	0.12	%
16	EPA 20:5 n-3	0.01	0.03	%
18	DHA 22:6 n-3			%
19	Total LCPUFA	0.01	0.08	%
21	Total n-6	0.25	1.44	%
22	Total n-3	0.06	0.36	%
24	Calcium	0.29	1.66	%
25	Phosphorus	0.16	0.92	%
27	Available Phosphorus	0.15	0.89	%
28	Magnesium	0.02	0.11	%
29	Potassium	0.22	1.29	%
30	Sodium	0.10	0.57	%
31	Chloride	0.18	1.03	%
33	Iron	39.65	230.37	mg/kg
34	Zinc	41.21	239.43	mg/kg
35	Manganese	2.47	14.33	mg/kg
36	Copper	2.31	13.41	mg/kg
37	Iodine	0.42	2.42	mg/kg
38	Selenium	0.16	0.91	mg/kg
42	Vitamin A	4160.00	24172.59	IU/kg
43	Vitamin D	182.00	1057.55	IU/kg
44	Vitamin E	69.58	404.31	IU/kg
45	Vitamin K	0.00	0.02	mg/kg
46	Thiamin	26.87	156.11	mg/kg
47	Riboflavin	3.45	20.03	mg/kg
48	Niacin	39.34	228.61	mg/kg
49	Pantothenic Acid	4.57	26.56	mg/kg
50	Pyridoxine	5.81	33.74	mg/kg
51	Folic Acid	0.68	3.95	mg/kg

Appendix E continued

Nutrient analysis – test diet 4 (mildly cooked/raw) continued

No.	Nutrient Name	Formula Amount	100.0 %DM Amount	Units
52	Biotin	0.03	0.20	mg/kg
53	Vitamin B12	15.82	91.90	mcg/kg
54	Vitamin C	35.78	207.88	mg/kg
55	Choline	658.43	3825.94	mg/kg
56	Carnitine	250.58	1456.05	mg/kg
58	Alanine	0.51	2.96	%
59	Arginine	0.52	3.01	%
60	Aspartic Acid	0.84	4.90	%
61	Glutamic Acid	1.29	7.51	%
62	Glycine	0.49	2.86	%
63	Histidine	0.24	1.37	%
64	Isoleucine	0.38	2.22	%
65	Leucine	0.68	3.93	%
66	Lysine	0.72	4.21	%
67	Methionine	0.20	1.16	%
68	Met-Cystine	0.26	1.54	%
69	Phenylalanine	0.37	2.13	%
70	Phe-Tyrosine	0.66	3.83	%
71	Proline	0.38	2.21	%
72	Serine	0.37	2.14	%
73	Threonine	0.41	2.37	%
74	Tryptophan	0.11	0.63	%
75	Valine	0.40	2.34	%
76	Taurine	525.40	3052.95	mg/kg
81	FOS	0.11	0.65	%

Appendix F

Test diet processing and feeding

Test diet 1 – Retorting (canned wet) Food

Lacquer cans (13oz & 5.5 oz.) containing a raw mixture of a loaf-type commercial dog food were heated in a pressurized retorting system. The product is heated to a maximum temperature of approximately 253°F and the retort achieves a maximum temperature of approximately 254°F during the cooking cycle. After the retort process was complete, cans were stored until further use.

Test diet 2 – Extruded (dry kibble) Food

Food was produced in a single screw extruder (Wenger X-165 with DDC Preconditioner) in Pawnee City, NE. After extrusion, food was dried to less than 10% moisture in a multiple pass, forced air/convection oven with temperatures ranging from 215 to 265°F. After drying and while the product is still hot, the kibbles were coated with fat and palatant in a rotating drum coater. After coating, a vertical cooler is utilized to bring kibbles to within 15°F of ambient temperature. After cooling, product is transferred to holding bins and then are packaged in multi-walled, side gusseted bags and stored until further use.

Test diet 3 – Air-dried Food

Two components of this food were produced: one comprised of protein-containing ingredients while the other was comprised of carbohydrate-containing ingredients. The protein and carbohydrate components were made using similar methods. Each component's ingredients were mixed together and then spread onto a tray at a thickness of about 0.7 cm. The tray was dried at about 140°F for about 12 hours until about 12% moisture or less. Dried material was then cut into pieces about 1 cm x 1 cm x 0.5 cm. After pieces were cut into the appropriate size, the protein component was mixed with the carbohydrate component at a weight ratio of about 62:38, respectively.

Test diet 4 – Mildly cooked/raw Food

Two components of this food were made: Meat and Dry Mix. For the meat component, ingredients were mixed and then placed in 105°F for about 10 hours. The mixture was then vacuum packed into bags and stored frozen until being fed. For the dry mix, ingredients were mixed together and then vacuum packaged and stored frozen until being fed. When the diet was ready to be fed, the meat was thawed out in ambient conditions and mixed with the dry mix and water in the proportions of 56:8:36, respectively.

Additional details for test diet 3 (air-dried) and test diet 4 (raw)

Test diet	Component	Abbreviation	Storage Condition	Remarks
Mildly cooked/raw	Stabilized Meat	R-M	Store at 4◦C	Sent frozen to assure good quality When fed, was combined with either R-SP and R-D to make a complete diet OR combine with R-DSP to make a complete diet.
Mildly cooked/raw	Boiled Sweet Potatoes	R-SP	Store at 4◦C	Boiled carbohydrate source to enable digestion Sent frozen to assure good quality When fed, was combined with R-M and R-D to make a complete diet.
Mildly cooked/raw	Dry ingredients	R-D	Ambient	When fed, was combine with R-M and R-SP to make a complete diet.
Mildly cooked/raw	Dry ingredients and Pre-cooked Dehydrated Sweet Potato	R-DSP	Ambient	When fed, was combined with R-M to make a complete diet.
Air-Dried	Protein source	AD-P	Ambient	When fed, was combined with Air-Dried protein source for complete diet.
Air-Dried	Carbohydrate source	AD-CHO	Ambient	When fed, was combined with Air-Dried carbohydrate source for complete diet.

Appendix G

Feeding protocol in a Latin square experimental design

End Point	**1**	**2**	**3**	**4**
Dog No.	**Week 1 - 4**	**Week 4 - 8**	**Week 8 - 12**	**Week 12 - 16**
1	2	1	3	4
2	2	1	3	4
3	4	3	1	2
4	4	3	1	2
5	3	2	4	1
6	3	2	4	1
7	1	4	2	3
8	1	4	2	3

Test diet 1 – Canned wet

Test diet 2 – Dry kibble

Test diet 3 – Air-dried

Test diet 4 – Mildly cooked/raw

Appendix H

Weekly body weights

Test diet 1 (canned wet)

		Weekly body weight (kg)						
Dog No.	**Sex**	**Base**	**1**	**2**	**3**	**4**	**Overall Change**	**% Change**
1	F	8.31	8.12	8.40	8.20	8.16	-0.15	-1.81
2	F	9.14	9.02	8.73	8.48	8.38	-0.76	-8.32
3	M	10.09	10.02	9.83	9.80	10.18	0.09	0.89
4	M	9.53	9.45	9.38	9.18	9.38	-0.15	-1.57
5	F	8.85	8.72	8.87	8.91	9.02	0.17	1.92
6	M	12.82	12.79	12.64	12.71	13.03	0.21	1.64
7	M	14.68	14.15	13.53	12.59	12.50	-2.18	-14.85
8	F	10.09	10.11	9.99	10.00	9.90	-0.19	-1.88
	Mean	10.44	10.30	10.17	9.98	10.07	-0.37	-3.00
	SD	2.187	2.096	1.892	1.753	1.804	0.793	5.785

Test diet 2 (dry kibble)

		Weekly body weight (kg)						
Dog No.	**Sex**	**Base**	**1**	**2**	**3**	**4**	**Overall Change**	**% Change**
1	F	8.59	8.26	8.14	8.30	8.31	-0.28	-3.26
2	F	10.11	9.93	9.91	9.32	9.14	-0.97	-9.59
3	M	10.18	9.96	9.90	9.80	10.16	-0.02	-0.20
4	M	9.38	9.31	9.32	9.52	9.57	0.19	2.03
5	F	8.76	8.70	8.51	8.63	8.76	0.00	0.00
6	M	13.70	13.55	13.86	13.38	13.59	-0.11	-0.80
7	M	13.33	13.82	14.03	13.85	14.35	1.02	7.65
8	F	9.90	9.62	9.57	9.65	9.77	-0.13	-1.31
	Mean	10.49	10.39	10.41	10.31	10.46	-0.04	-0.69
	SD	1.956	2.115	2.273	2.108	2.253	0.550	4.840

Appendix H – continued

Test diet 3 (air-dried)

		Weekly body weight (kg)						
Dog No.	**Sex**	**Base**	**1**	**2**	**3**	**4**	**Overall Change**	**% Change**
1	F	8.16	8.49	8.78	8.37	8.44	0.28	3.43
2	F	8.38	8.78	9.09	9.31	9.78	1.40	16.71
3	M	9.80	9.30	9.90	10.04	10.09	0.29	2.96
4	M	9.51	9.00	9.38	9.50	9.53	0.02	0.21
5	F	9.16	9.05	8.76	8.56	8.76	-0.40	-4.37
6	M	14.53	14.41	13.68	13.54	13.70	-0.83	-5.71
7	M	14.35	14.26	14.10	14.16	14.43	0.08	0.56
8	F	9.77	9.84	9.74	10.01	9.93	0.16	1.64
	Mean	10.46	10.39	10.43	10.44	10.58	0.13	1.93
	SD	2.530	2.466	2.178	2.197	2.231	0.641	6.810

Test diet 4 (mildly cooked/raw)

		Weekly body weight (kg)						
Dog No.	**Sex**	**Base**	**1**	**2**	**3**	**4**	**Overall Change**	**% Change**
1	F	8.44	8.33	8.21	8.22	8.14	-0.30	-3.55
2	F	9.78	9.57	9.20	9.19	9.26	-0.52	-5.32
3	M	10.39	10.08	9.92	9.61	9.80	-0.59	-5.68
4	M	10.30	10.20	9.66	9.58	9.51	-0.79	-7.67
5	F	8.76	8.70	8.58	8.68	8.85	0.09	1.03
6	M	13.59	13.40	13.09	12.76	12.82	-0.77	-5.67
7	M	12.50	12.90	13.24	13.20	13.33	0.83	6.64
8	F	9.90	9.85	10.08	9.67	9.90	0.00	0.00
	Mean	10.46	10.38	10.25	10.11	10.20	-0.26	-2.53
	SD	1.762	1.834	1.910	1.842	1.865	0.547	4.753

Appendix I

Sample collection schedule

Sample	**Week**																
	Initial	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Blood	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Urine	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Feces	X				X				X				X				X
Diets	X					X				X				X			

Appendix J

Daily Food Consumption

Test diet 1 (canned wet)

	Average Weekly Food Consumption Weight (g)					
Dog No.	**Sex**	**1**	**2**	**3**	**4**	**Average**
1	F	817	1001	946	889	913
2	F	684	544	614	656	625
3	M	1001	1098	1300	1500	1225
4	M	960	1050	688	1250	987
5	F	881	980	979	977	954
6	M	1114	1082	1075	1252	1131
7	M	932	689	362	524	627
8	F	946	946	864	808	891
	Mean	917	924	854	982	919
	SD	127.8	200.1	292.5	331.2	212.9

Test diet 2 (dry kibble)

	Average Weekly Food Consumption Weight (g)					
Dog No.	**Sex**	**1**	**2**	**3**	**4**	**Average**
1	F	166	173	195	246	195
2	F	214	226	175	174	197
3	M	218	244	244	293	250
4	M	217	242	242	242	236
5	F	181	192	243	250	216
6	M	268	298	291	327	296
7	M	300	300	300	350	313
8	F	216	230	247	234	232
	Mean	223	238	242	264	242
	SD	43.4	44.7	42.2	56.3	43.0

Appendix J continued

Daily Food Consumption

Test diet 3 (air-dried)

		Average Weekly Food Consumption Weight (g)				
Dog No.	**Sex**	**1**	**2**	**3**	**4**	**Average**
1	F	330	304	270	218	281
2	F	332	311	315	290	312
3	M	216	350	350	350	317
4	M	216	350	350	350	317
5	F	117	187	223	204	182
6	M	266	247	316	345	294
7	M	280	317	335	326	314
8	F	182	193	220	216	202
	Mean	242	282	297	287	277
	SD	74.1	65.6	53.5	65.1	54.2

Test diet 4 (mildly cooked/raw)

		Average Weekly Food Consumption Weight (g)				
Dog ID No.	**Sex**	**1**	**2**	**3**	**4**	**Average**
1	F	409	661	653	723	611
2	F	608	495	588	765	614
3	M	651	772	872	1072	842
4	M	644	589	818	918	743
5	F	611	701	858	901	768
6	M	864	950	982	907	926
7	M	860	935	1001	1114	978
8	F	648	685	702	720	689
	Mean	662	724	809	890	771
	SD	146.4	158.0	150.2	149.9	136.0

Appendix K

Clinical parameter statistical analyses – Friedman's test

Parameter	**Chi-square**	**p-value**
Total Protein	2.614	0.455
Albumin	1.576	0.665
Globulin	3.689	0.297
Albumin/Globulin (A/G) Ratio	5.4	0.145
Aspartate Transaminase (AST)	9.385	0.025
Alanine Transferase (ALT)	8.392	0.039
Alkaline Phosphatase	10.05	0.018
Gamma-Glutamyl Transpeptidase (GGTP)	2.868	0.412
Total Bilirubin	0	< 0.0001
Urea Nitrogen	0.041	0.998
Creatinine	12.364	0.006
Blood Urea Nitrogen (BUN)/Creatinine Ratio	3.355	0.34
Phosphorus	9.269	0.026
Glucose	4.831	0.185
Calcium	1.913	0.591
Magnesium	4.765	0.19
Sodium	3.085	0.379
Potassium	4.279	0.233
Chloride	8.727	0.033
Cholesterol	12.9	0.005
Triglycerides	5.7	0.127
Creatinine Phosphokinase (CPK)	4.35	0.226
White Blood Cells (WBC)	0.45	0.93
Red Blood Cells (RBC)	7.423	0.06
Hemoglobin	7.95	0.047
Hematocrit	7.769	0.051
Mean Corpuscular Volume (MCV)	1.519	0.678
Mean Corpuscular Hemogobin (MCH)	2.377	0.498
Mean Corpuscular Hemoglobin Concentration (MCHC)	1.455	0.693
Platelets	2.85	0.415
Abs Polys (neutrophils)	0.6	0.896
Abs Lymphs (lymphocytes)	6.75	0.08
Abs Monos (monocytes)	0.45	0.93
Abs Eos (eosinophils)	7.65	0.054
Urine pH	11.866	0.008
Urine specific gravity	19.35	< 0.0001
Body weight	5.308	0.151
Food consumption	20.7	< 0.0001

Appendix K continued

Wilcoxon signed rank tests for multiple comparisons between diets for clinical parameters statistically significant after Friedman's tests.

Parameter	Diet 1	Diet 2	Z value	p - value	Based on (+) ranks	Based on (-) ranks
AST	DF	WF	-1.510[b]	0.131	b	c
	ADF	WF	-.140[b]	0.888		
	RF	WF	-1.829[c]	0.067		
	ADF	DF	-1.053[c]	0.292		
	RF	DF	-2.524[c]	0.012		
	RF	ADF	-2.178[c]	0.029		
ALT	DF	WF	-1.761[b]	0.078	b	c
	ADF	WF	-.140[b]	0.889		
	RF	WF	-1.823[c]	0.068		
	ADF	DF	-.676[c]	0.499		
	RF	DF	-2.033[c]	0.042		
	RF	ADF	-1.960[c]	0.05		
ALP	DF	WF	-1.823[b]	0.068	c	b
	ADF	WF	-.983[c]	0.326		
	RF	WF	-1.122[b]	0.262		
	ADF	DF	-2.240[c]	0.025		
	RF	DF	-.561[c]	0.575		
	RF	ADF	-2.313[b]	0.021		
Creatinine	DF	WF	-2.414[b]	0.016	b	c
	ADF	WF	-.108[b]	0.914		
	RF	WF	-.351[b]	0.726		
	ADF	DF	-2.428[c]	0.015		
	RF	DF	-2.232[c]	0.026		
	RF	ADF	-.272[b]	0.785		
Phosphorus	DF	WF	-2.197[b]	0.028	c	b
	ADF	WF	-.845[b]	0.398		
	RF	WF	-.070[b]	0.944		
	ADF	DF	-1.963[c]	0.05		
	RF	DF	-2.524[c]	0.012		
	RF	ADF	-.280[b]	0.779		
Chloride	DF	WF	-1.552[b]	0.121	b	c
	ADF	WF	-2.121[c]	0.034		
	RF	WF	-1.378[b]	0.168		
	ADF	DF	-2.207[c]	0.027		
	RF	DF	-.138[c]	0.89		
	RF	ADF	-2.047[b]	0.041		

Appendix K continued

Wilcoxon signed rank tests for multiple comparisons between diets for clinical parameters statistically significant after Friedman's tests (continued)

Parameter	Diet 1	Diet 2	Z value	p - value	Based on (+) ranks	Based on (-) ranks
Cholesterol	DF	WF	-.700[b]	0.484	b	c
	ADF	WF	-.561[b]	0.575		
	RF	WF	-2.521[c]	0.012		
	ADF	DF	.000[d]	1	d*	d*
	RF	DF	-2.524[c]	0.012	b	c
	RF	ADF	-2.383[c]	0.017		
Hemoglobin	DF	WF	-2.100[b]	0.036	b	c
	ADF	WF	-.631[b]	0.528		
	RF	WF	-.280[c]	0.779		
	ADF	DF	-1.820[c]	0.069		
	RF	DF	-2.243[c]	0.025		
	RF	ADF	-1.122[c]	0.262		
Urine pH	DF	WF	-2.401[b]	0.016	c	b
	ADF	WF	-2.187[b]	0.029		
	RF	WF	-1.866[b]	0.062		
	ADF	DF	-.743[c]	0.458		
	RF	DF	-2.111[c]	0.035		
	RF	ADF	-.966[c]	0.334		
Urine SG	DF	WF	-2.524[b]	0.012	c	b
	ADF	WF	-2.524[b]	0.012		
	RF	WF	-.841[c]	0.4		
	ADF	DF	-.563[b]	0.574		
	RF	DF	-2.533[c]	0.011		
	RF	ADF	-2.524[c]	0.012		
Food consumption	DF	WF	-2.521[b]	0.012	b	c
	ADF	WF	-2.521[b]	0.012		
	RF	WF	-1.540[b]	0.123		
	ADF	DF	-1.260[c]	0.208		
	RF	DF	-2.521[c]	0.012		
	RF	ADF	-2.521[c]	0.012		

Abbreviations – WF – canned wet; DF – dry kibble; ADF – air-dried; RF – mildly cooked/raw

Appendix L

sRAGE concentration (pg/ml) as determined by

enzyme-linked immunosorbent assay (ELISA)

Dog No.	**Canned Wet**			
	Week 1	**Week 2**	**Week 3**	**Week 4**
1	177.213	171.426	206.959	241.965
2	2601.531	2092.149	2947.553	3031.687
3	1972.999	2525.266	3006.746	2929.003
4	834.352	680.088	723.766	744.906
5	164504.816	111969.670	48005.958	35110.787
6	665.597	611.164	503.487	461.926
7	205.611	237.132	210.186	378.378
8	235.812	233.833	249.704	247.716

Dog No.	**Dry Kibble**			
	Week 1	**Week 2**	**Week 3**	**Week 4**
1	128.306	149.351	127.035	159.613
2	1050.139	1746.037	2004.464	2024.107
3	3261.940	3946.560	2765.366	3202.947
4	601.609	599.223	672.279	666.653
5	4446.187	3869.542	3439.536	3250.793
6	180.833	176.489	131.067	202.597
7	247.087	232.660	319.022	197.028
8	275.697	226.704	279.047	253.558

Appendix L continued

sRAGE concentration (pg/ml) as determined by

enzyme-linked immunosorbent assay (ELISA)

Dog No.	**Air Dried**			
	Week 1	**Week 2**	**Week 3**	**Week 4**
1	154.096	278.295	194.809	309.945
2	2483.109	2906.743	2666.002	3045.292
3	2856.096	1904.913	1784.461	2181.062
4	969.517	764.511	1005.315	939.542
5	118.793	1939.090	3596.036	4048.240
6	128.306	104.272	167.335	175.724
7	245.338	312.213	334.889	363.302
8	288.072	244.592	259.222	278.136

Dog No.	**Mildly cooked/Raw**			
	Week 1	**Week 2**	**Week 3**	**Week 4**
1	207.395	238.623	245.422	325.898
2	3956.988	3505.144	3941.336	4186.963
3	2449.327	2853.141	2173.333	2965.196
4	571.201	731.950	723.559	690.929
5	53221.294	318545.365	215696.167	174285.678
6	448.572	893.225	835.138	689.768
7	375.735	298.531	337.018	314.046
8	167.811	206.959	271.287	308.131

Appendix M

Statistical analysis of sRAGE concentrations – Friedman's test

Descriptive Statistics (sRAGE concentrations)

	Percentiles		
Diet	**25th**	**50th (Median)**	**75th**
Canned Wet	280.381	603.416	3006.016
Dry Kibble	198.420	460.106	2908.237
Air Dried	286.089	651.422	2829.235
Mildly cooked/Raw	317.009	690.348	3881.521

Friedman's Test

Ranks

Diet	**Mean Rank**
Canned Wet	2.5
Dry Kibble	1.63
Air Dried	2.5
Mildly cooked/Raw	3.38

Test Statistics	
N	8
Chi-Square	7.35
df	3
Asymp. Sig.	0.062

Appendix N

Means, standard deviations (SD) and standard error of the mean (SE) of the relative intensities of discriminatory serum metabolites for all diets over four weeks (FWER at 0.1).

Serum Metabolite	Canned Wet											
	1			2			3			4		
	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
Valine	0.475	0.063	0.022	0.467	0.063	0.022	0.534	0.064	0.022	0.468	0.068	0.026
Unknown1	0.289	0.032	0.011	0.317	0.025	0.009	0.338	0.030	0.011	0.333	0.034	0.013
Unknown2	0.797	0.065	0.023	0.842	0.046	0.016	0.908	0.051	0.018	0.888	0.108	0.041
Acetic acid	0.258	0.026	0.009	0.253	0.011	0.004	0.253	0.014	0.005	0.250	0.013	0.005
Acetylcarnitine	0.161	0.013	0.004	0.166	0.023	0.008	0.164	0.015	0.005	0.172	0.019	0.007
Betaine	1.045	0.299	0.106	1.069	0.332	0.117	1.234	0.382	0.135	1.194	0.382	0.144
Glycine	0.435	0.058	0.020	0.474	0.064	0.022	0.513	0.074	0.026	0.501	0.066	0.025
Creatine	0.136	0.025	0.009	0.141	0.017	0.006	0.157	0.027	0.010	0.141	0.028	0.011

Serum Metabolite	Dry Kibble											
	1			2			3			4		
	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
Valine	0.504	0.053	0.019	0.499	0.055	0.019	0.470	0.046	0.016	0.511	0.054	0.021
Unknown1	0.246	0.042	0.015	0.248	0.026	0.009	0.244	0.013	0.005	0.229	0.025	0.009
Unknown 2	0.836	0.074	0.026	0.776	0.062	0.022	0.748	0.061	0.022	0.773	0.054	0.020
Acetic acid	0.268	0.020	0.007	0.263	0.026	0.009	0.249	0.013	0.004	0.252	0.013	0.005
Acetylcarnitine	0.145	0.011	0.004	0.149	0.021	0.007	0.138	0.024	0.008	0.134	0.011	0.004
Betaine	0.952	0.142	0.050	0.933	0.197	0.070	0.893	0.141	0.050	0.993	0.188	0.071
Glycine	0.427	0.080	0.028	0.410	0.087	0.031	0.396	0.066	0.023	0.406	0.054	0.020
Creatine	0.121	0.023	0.008	0.107	0.026	0.009	0.105	0.023	0.008	0.122	0.039	0.015

Serum Metabolite	Air Dried											
	1			2			3			4		
	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
Valine	0.553	0.050	0.018	0.492	0.059	0.021	0.512	0.045	0.016	0.560	0.050	0.018
Unknown1	0.239	0.033	0.012	0.233	0.033	0.012	0.225	0.026	0.009	0.227	0.018	0.006
Unknown 2	0.875	0.059	0.021	0.841	0.071	0.025	0.842	0.043	0.015	0.824	0.062	0.022
Acetic acid	0.278	0.030	0.010	0.263	0.028	0.010	0.251	0.014	0.005	0.239	0.011	0.004
Acetylcarnitine	0.162	0.020	0.007	0.164	0.021	0.008	0.168	0.021	0.007	0.164	0.019	0.007
Betaine	1.027	0.140	0.050	1.086	0.211	0.074	1.148	0.218	0.077	1.219	0.252	0.089
Glycine	0.496	0.091	0.032	0.490	0.056	0.020	0.489	0.064	0.023	0.443	0.068	0.024
Creatine	0.155	0.021	0.007	0.165	0.046	0.016	0.171	0.034	0.012	0.177	0.037	0.013

Serum Metabolite	Mildly cooked/Raw											
	1			2			3			4		
	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
Valine	0.522	0.031	0.011	0.487	0.047	0.016	0.493	0.043	0.015	0.515	0.063	0.022
Unknown1	0.241	0.038	0.014	0.229	0.014	0.005	0.246	0.033	0.012	0.227	0.023	0.008
Unknown 2	0.833	0.061	0.022	0.798	0.079	0.028	0.770	0.074	0.026	0.782	0.065	0.023
Acetic acid	0.300	0.019	0.007	0.306	0.032	0.011	0.304	0.028	0.010	0.307	0.019	0.007
Acetyl-carnitine	0.172	0.024	0.008	0.168	0.018	0.006	0.169	0.010	0.003	0.167	0.015	0.005
Betaine	1.090	0.275	0.097	0.841	0.144	0.051	0.863	0.123	0.044	0.823	0.177	0.062
Glycine	0.456	0.057	0.020	0.419	0.073	0.026	0.415	0.057	0.020	0.422	0.045	0.016
Creatine	0.159	0.025	0.009	0.138	0.012	0.004	0.131	0.019	0.007	0.141	0.019	0.007

Appendix O

Tukey HSD post-hoc analyses for all discriminatory serum metabolites. Multiple comparisons performed per diet group for each week of feeding (FWER = 0.1).

Week 1							
Serum Metabolites	**ppm**	**Diet 1**	**Diet 2**	**Mean Difference**	**p-adj**	**95% Confidence Interval**	
						lower	**upper**
Valine	1.0387	WF	DF	0.0291	0.6484	-0.0402	0.0984
		WF	ADF	0.0783	0.0221	0.0091	0.1476
		WF	RF	0.0469	0.2727	-0.0224	0.1162
		DF	ADF	0.0492	0.2348	-0.02	0.1185
		DF	RF	0.0178	0.8925	-0.0515	0.0871
		ADF	RF	-0.0314	0.5981	-0.1007	0.0379
Unknown1	1.4168	WF	DF	-0.0426	0.1147	-0.0925	0.0073
		WF	ADF	-0.05	0.0491	-0.0999	-0.0001
		WF	RF	-0.0476	0.0654	-0.0975	0.0023
		DF	ADF	-0.0074	0.9	-0.0573	0.0425
		DF	RF	-0.005	0.9	-0.0549	0.0449
		ADF	RF	0.0024	0.9	-0.0475	0.0523
Acetic Acid	1.9798	WF	DF	0.0096	0.8391	-0.0231	0.0423
		WF	ADF	0.02	0.358	-0.0127	0.0527
		WF	RF	0.0419	0.0082	0.0092	0.0746
		DF	ADF	0.0104	0.8	-0.0223	0.0431
		DF	RF	0.0323	0.0538	-0.0004	0.065
		ADF	RF	0.0219	0.2829	-0.0108	0.0545
Creatine	3.918	WF	DF	-0.015	0.5834	-0.0474	0.0174
		WF	ADF	0.0196	0.3693	-0.0128	0.0519
		WF	RF	0.0235	0.2177	-0.0088	0.0559
		DF	ADF	0.0346	0.0331	0.0022	0.0669
		DF	RF	0.0385	0.015	0.0062	0.0709
		ADF	RF	0.004	0.9	-0.0284	0.0364

Appendix O continued

Week 2							
Serum Metabolite	**ppm**	**Diet 1**	**Diet 2**	**Mean Difference**	**p-adj**	**95% Confidence Interval**	
						lower	**upper**
Unknown1	1.4168	WF	DF	-0.0692	0.001	-0.1041	-0.0344
		WF	ADF	-0.0841	0.001	-0.119	-0.0493
		WF	RF	-0.0877	0.001	-0.1226	-0.0529
		DF	ADF	-0.0149	0.6377	-0.0497	0.02
		DF	RF	-0.0185	0.4816	-0.0534	0.0164
		ADF	RF	-0.0036	0.9	-0.0385	0.0313
Acetic Acid	1.9798	WF	DF	0.01	0.8518	-0.0252	0.0452
		WF	ADF	0.0104	0.836	-0.0248	0.0455
		WF	RF	0.0531	0.0016	0.0179	0.0882
		DF	ADF	0.0004	0.9	-0.0348	0.0355
		DF	RF	0.0431	0.0119	0.0079	0.0782
		ADF	RF	0.0427	0.0128	0.0075	0.0778

Week 3							
Serum Metabolite	**ppm**	**Diet 1**	**Diet 2**	**Mean Difference**	**p-adj**	**95% Confidence Interval**	
						lower	**upper**
Unknown1	1.4168	WF	DF	-0.0931	0.001	-0.1296	-0.0566
		WF	ADF	-0.1129	0.001	-0.1495	-0.0764
		WF	RF	-0.0911	0.001	-0.1276	-0.0546
		DF	ADF	-0.0198	0.4618	-0.0563	0.0167
		DF	RF	0.002	0.9	-0.0345	0.0385
		ADF	RF	0.0218	0.379	-0.0147	0.0583
Creatine	3.918	WF	DF	-0.0518	0.0029	-0.0881	-0.0156
		WF	ADF	0.0142	0.6905	-0.022	0.0504
		WF	RF	-0.0261	0.2253	-0.0623	0.0102
		DF	ADF	0.066	0.001	0.0298	0.1023
		DF	RF	0.0257	0.2355	-0.0105	0.062
		ADF	RF	-0.0403	0.025	-0.0765	-0.004
Unknown2	1.7189	WF	DF	-0.1597	0.001	-0.2395	-0.0799
		WF	ADF	-0.0659	0.1338	-0.1457	0.014
		WF	RF	-0.1377	0.001	-0.2175	-0.0579
		DF	ADF	0.0939	0.0165	0.0141	0.1737
		DF	RF	0.0221	0.8641	-0.0578	0.1019
		ADF	RF	-0.0718	0.0894	-0.1516	0.008
Acetylcarnitine	3.1852	WF	DF	-0.026	0.0372	-0.0508	-0.0012
		WF	ADF	0.0041	0.9	-0.0207	0.0289
		WF	RF	0.0048	0.9	-0.02	0.0296
		DF	ADF	0.0301	0.0129	0.0053	0.0549
		DF	RF	0.0308	0.0106	0.006	0.0556
		ADF	RF	0.0007	0.9	-0.0241	0.0255
Glycine	3.5495	WF	DF	-0.1169	0.0069	-0.2065	-0.0274
		WF	ADF	-0.024	0.877	-0.1135	0.0656
		WF	RF	-0.098	0.028	-0.1875	-0.0084
		DF	ADF	0.093	0.0397	0.0034	0.1825
		DF	RF	0.019	0.9	-0.0706	0.1085
		ADF	RF	-0.074	0.133	-0.1635	0.0156

Week 4							
Serum Metabolite	**ppm**	**Diet 1**	**Diet 2**	**Mean Difference**	**p-adj**	**95% Confidence Interval**	
						lower	**upper**
Valine	1.0387	WF	DF	0.0431	0.5275	-0.0434	0.1297
		WF	ADF	0.0924	0.0266	0.0086	0.1762
		WF	RF	0.0475	0.4222	-0.0363	0.1313
		DF	ADF	0.0493	0.3898	-0.0345	0.1331
		DF	RF	0.0044	0.9	-0.0794	0.0881
		ADF	RF	-0.0449	0.4405	-0.1259	0.036
Unknown1	1.4168	WF	DF	-0.1031	0.001	-0.1404	-0.0657
		WF	ADF	-0.1055	0.001	-0.1416	-0.0694
		WF	RF	-0.105	0.001	-0.1411	-0.0689
		DF	ADF	-0.0024	0.9	-0.0386	0.0337
		DF	RF	-0.0019	0.9	-0.0381	0.0342
		ADF	RF	0.0005	0.9	-0.0344	0.0354
Acetic Acid	1.9798	WF	DF	0.0027	0.9	-0.0185	0.0238
		WF	ADF	-0.0106	0.4952	-0.0311	0.0098
		WF	RF	0.0578	0.001	0.0373	0.0783
		DF	ADF	-0.0133	0.304	-0.0338	0.0072
		DF	RF	0.0552	0.001	0.0347	0.0756
		ADF	RF	0.0685	0.001	0.0487	0.0882
Creatine	3.918	WF	DF	-0.019	0.663	-0.0654	0.0275
		WF	ADF	0.036	0.1513	-0.009	0.0809
		WF	RF	-0.0002	0.9	-0.0451	0.0448
		DF	ADF	0.0549	0.0124	0.01	0.0999
		DF	RF	0.0188	0.6482	-0.0262	0.0638
		ADF	RF	-0.0361	0.1283	-0.0796	0.0073
Unknown2	1.7189	WF	DF	-0.1142	0.0378	-0.2233	-0.0051
		WF	ADF	-0.064	0.3639	-0.1697	0.0416
		WF	RF	-0.1057	0.0498	-0.2113	-0.0001
		DF	ADF	0.0502	0.5631	-0.0555	0.1558
		DF	RF	0.0085	0.9	-0.0972	0.1141
		ADF	RF	-0.0417	0.6631	-0.1438	0.0604

Appendix O continued

Week 4 continued							
Serum Metabolite	**ppm**	**Diet 1**	**Diet 2**	**Mean Difference**	**p-adj**	**95% Confidence Interval**	
						lower	**upper**
Acetylcarnitine	3.1852	WF	DF	-0.0373	0.0014	-0.0615	-0.0131
		WF	ADF	-0.0074	0.8052	-0.0308	0.0161
		WF	RF	-0.0051	0.9	-0.0285	0.0183
		DF	ADF	0.03	0.0084	0.0066	0.0534
		DF	RF	0.0322	0.0044	0.0088	0.0557
		ADF	RF	0.0023	0.9	-0.0204	0.0249
Glycine	3.5495	WF	DF	-0.0953	0.0265	-0.1816	-0.0089
		WF	ADF	-0.0574	0.26	-0.141	0.0262
		WF	RF	-0.0787	0.0704	-0.1623	0.0049
		DF	ADF	0.0379	0.5954	-0.0457	0.1215
		DF	RF	0.0165	0.9	-0.067	0.1001
		ADF	RF	-0.0214	0.8804	-0.1021	0.0594
Betaine	3.2562	WF	DF	-0.201	0.4813	-0.5812	0.1792
		WF	ADF	0.0257	0.9	-0.3424	0.3938
		WF	RF	-0.3702	0.0483	-0.7384	-0.0021
		DF	ADF	0.2267	0.3497	-0.1414	0.5948
		DF	RF	-0.1692	0.5859	-0.5374	0.1989
		ADF	RF	-0.3959	0.0249	-0.7516	-0.0403

Abbreviations – WF – canned wet; DF – dry kibble; ADF – air-dried; RF – mildly cooked/raw

Appendix P

Means, standard deviations (SD) and standard error of the mean (SE) of the relative intensities of discriminatory urine metabolites for all diets over four weeks (FWER at 0.1).

Urine Metabolite	**Canned Wet**											
	1			**2**			**3**			**4**		
	Mean	**SD**	**SE**	**Mean**	**SD**	**SE**	**Mean**	**SD**	**SE**	**Mean**	**SD**	**SE**
Acetic acid	0.603	0.086	0.030	0.584	0.107	0.038	0.649	0.126	0.048	0.565	0.124	0.044
Carnitine	0.809	0.080	0.028	0.809	0.164	0.058	0.910	0.174	0.066	0.852	0.157	0.055
Dimethylamine	0.324	0.028	0.010	0.339	0.065	0.023	0.305	0.032	0.012	0.338	0.093	0.033
Dimethyl-sulfone	0.357	0.019	0.007	0.365	0.038	0.014	0.364	0.036	0.014	0.369	0.028	0.010
Creatinine	2.924	0.467	0.165	2.947	0.628	0.222	2.828	0.501	0.189	3.072	0.707	0.250
Methylnicotinamide	0.265	0.026	0.009	0.267	0.031	0.011	0.262	0.046	0.017	0.282	0.048	0.017

Urine Metabolite	**Dry Kibble**											
	1			**2**			**3**			**4**		
	Mean	**SD**	**SE**	**Mean**	**SD**	**SE**	**Mean**	**SD**	**SE**	**Mean**	**SD**	**SE**
Acetic acid	0.516	0.080	0.028	0.536	0.069	0.024	0.515	0.094	0.033	0.490	0.058	0.020
Carnitine	0.432	0.051	0.018	0.431	0.072	0.026	0.444	0.079	0.028	0.403	0.073	0.026
Dimethylamine	0.275	0.026	0.009	0.278	0.047	0.016	0.289	0.059	0.021	0.313	0.072	0.025
Dimethyl-sulfone	0.268	0.044	0.015	0.258	0.040	0.014	0.242	0.057	0.020	0.258	0.074	0.026
Creatinine	2.104	0.186	0.066	2.113	0.220	0.078	2.274	0.705	0.249	2.310	0.651	0.230
Methylnicotinamide	0.164	0.034	0.012	0.145	0.010	0.004	0.173	0.047	0.017	0.179	0.071	0.025

Urine Metabolite	Air Dried											
	1			2			3			4		
	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
Acetic acid	0.673	0.091	0.032	0.635	0.112	0.040	0.672	0.147	0.052	0.643	0.138	0.049
Carnitine	1.112	0.169	0.060	1.107	0.305	0.108	1.038	0.225	0.080	1.005	0.151	0.053
Dimethylamine	0.417	0.041	0.014	0.402	0.057	0.020	0.403	0.060	0.021	0.398	0.030	0.011
Dimethyl-sulfone	0.415	0.028	0.010	0.381	0.032	0.011	0.392	0.066	0.023	0.393	0.053	0.019
Creatinine	3.645	0.288	0.102	3.592	0.349	0.123	3.606	0.376	0.133	3.545	0.440	0.156
Methylnicotinamide	0.298	0.058	0.020	0.252	0.082	0.029	0.283	0.041	0.015	0.248	0.062	0.022

Urine Metabolite	Mildly cooked/Raw											
	1			2			3			4		
	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
Acetic acid	0.408	0.041	0.014	0.480	0.078	0.028	0.542	0.096	0.034	0.513	0.083	0.031
Carnitine	1.162	0.097	0.034	1.073	0.222	0.078	1.308	0.243	0.086	1.312	0.222	0.084
Dimethylamine	0.346	0.040	0.014	0.342	0.070	0.025	0.324	0.043	0.015	0.311	0.031	0.012
Dimethyl-sulfone	0.335	0.038	0.013	0.351	0.060	0.021	0.334	0.025	0.009	0.307	0.013	0.005
Creatinine	2.885	0.363	0.128	2.945	0.563	0.199	2.662	0.256	0.090	2.358	0.219	0.083
Methylnicotinamide	0.361	0.078	0.028	0.309	0.069	0.024	0.348	0.094	0.033	0.354	0.099	0.038

Appendix Q

Tukey HSD post-hoc analyses for all discriminatory urine metabolites. Multiple comparisons performed per diet group for each week of feeding (FWER = 0.1).

Week 1							
Urine Metabolite	**ppm**	**Diet 1**	**Diet 2**	**Mean Difference**	**p-adj**	**95% Confidence Interval**	
						lower	**upper**
Acetic Acid	1.9189	WF	DF	-0.0866	0.1332	-0.1789	0.0056
		WF	ADF	0.0705	0.2783	-0.0217	0.1628
		WF	RF	-0.1949	0.001	-0.2872	-0.1027
		DF	ADF	0.1572	0.0018	0.0649	0.2494
		DF	RF	-0.1083	0.0411	-0.2005	-0.016
		ADF	RF	-0.2654	0.001	-0.3577	-0.1732
Dimethylamine	2.7205	WF	DF	-0.0491	0.0382	-0.0905	-0.0077
		WF	ADF	0.0929	0.001	0.0515	0.1343
		WF	RF	0.0219	0.5806	-0.0195	0.0632
		DF	ADF	0.142	0.001	0.1007	0.1834
		DF	RF	0.071	0.0016	0.0296	0.1124
		ADF	RF	-0.071	0.0016	-0.1124	-0.0297
Dimethyl sulfone	3.1483	WF	DF	-0.0894	0.001	-0.1296	-0.0493
		WF	ADF	0.0581	0.0087	0.0179	0.0982
		WF	RF	-0.022	0.5554	-0.0622	0.0182
		DF	ADF	0.1475	0.001	0.1074	0.1877
		DF	RF	0.0674	0.0021	0.0273	0.1076
		ADF	RF	-0.0801	0.001	-0.1202	-0.0399
Creatinine	4.0454	WF	DF	-0.8194	0.001	-1.2297	-0.4091
		WF	ADF	0.7217	0.0012	0.3114	1.132
		WF	RF	-0.0391	0.9	-0.4493	0.3712
		DF	ADF	1.5411	0.001	1.1308	1.9514
		DF	RF	0.7803	0.001	0.3701	1.1906
		ADF	RF	-0.7607	0.001	-1.171	-0.3504

Week 2							
Urine Metabolite	**ppm**	**Diet 1**	**Diet 2**	**Mean Difference**	**p-adj**	**95% Confidence Interval**	
						lower	**upper**
Acetic Acid	1.9189	WF	DF	-0.0486	0.7059	-0.1607	0.0634
		WF	ADF	0.0505	0.6833	-0.0615	0.1626
		WF	RF	-0.1043	0.1384	-0.2164	0.0078
		DF	ADF	0.0992	0.1701	-0.0129	0.2112
		DF	RF	-0.0557	0.6226	-0.1678	0.0564
		ADF	RF	-0.1548	0.0127	-0.2669	-0.0428
Dimethylamine	2.7205	WF	DF	-0.0613	0.2018	-0.1338	0.0113
		WF	ADF	0.0624	0.1886	-0.0101	0.1349
		WF	RF	0.0026	0.9	-0.0699	0.0751
		DF	ADF	0.1237	0.0017	0.0512	0.1962
		DF	RF	0.0639	0.1731	-0.0086	0.1364
		ADF	RF	-0.0598	0.2194	-0.1323	0.0127
Dimethyl sulfone	3.1483	WF	DF	-0.1068	0.001	-0.1595	-0.0542
		WF	ADF	0.0166	0.8635	-0.0361	0.0692
		WF	RF	-0.0132	0.9	-0.0659	0.0394
		DF	ADF	0.1234	0.001	0.0708	0.176
		DF	RF	0.0936	0.0011	0.041	0.1462
		ADF	RF	-0.0298	0.5309	-0.0824	0.0228

Week 3							
Urine Metabolite	**ppm**	**Diet 1**	**Diet 2**	**Mean Difference**	**p-adj**	**95% Confidence Interval**	
						lower	**upper**
Acetic Acid	1.9189	WF	DF	-0.134	0.1467	-0.2801	0.012
		WF	ADF	0.0225	0.9	-0.1236	0.1685
		WF	RF	-0.1073	0.3101	-0.2534	0.0387
		DF	ADF	0.1565	0.058	0.0154	0.2976
		DF	RF	0.0267	0.9	-0.1144	0.1678
		ADF	RF	-0.1298	0.1451	-0.2709	0.0113
Dimethylamine	2.7205	WF	DF	-0.0168	0.9	-0.0793	0.0457
		WF	ADF	0.0973	0.0045	0.0348	0.1597
		WF	RF	0.0187	0.8838	-0.0438	0.0811
		DF	ADF	0.1141	0.001	0.0537	0.1744
		DF	RF	0.0354	0.502	-0.0249	0.0958
		ADF	RF	-0.0786	0.0203	-0.139	-0.0182
Dimethyl sulfone	3.1483	WF	DF	-0.1226	0.001	-0.1838	-0.0614
		WF	ADF	0.0278	0.6774	-0.0334	0.089
		WF	RF	-0.0302	0.6271	-0.0913	0.031
		DF	ADF	0.1504	0.001	-0.0913	0.2095
		DF	RF	0.0925	0.0043	0.0333	0.1516
		ADF	RF	-0.058	0.1097	-0.1171	0.0011
Creatinine	4.0454	WF	DF	-0.5544	0.1506	-1.1623	0.0536
		WF	ADF	0.7777	0.0231	0.1698	1.3856
		WF	RF	-0.1666	0.9	-0.7745	0.4413
		DF	ADF	1.332	0.001	0.7448	1.9193
		DF	RF	0.3877	0.403	-0.1996	0.975
		ADF	RF	-0.9443	0.0033	-1.5316	-0.357
Carnitine	2.4362	WF	DF	-0.4652	0.001	-0.7042	-0.2262
		WF	ADF	0.1282	0.5695	-0.1108	0.3671
		WF	RF	0.3981	0.0023	0.1591	0.6371
		DF	ADF	0.5933	0.001	0.3625	0.8242
		DF	RF	0.8633	0.001	0.6324	1.0941
		ADF	RF	0.2699	0.0423	0.039	0.5008
Methylnicotinamide	4.4725	WF	DF	-0.089	0.0425	-0.1651	-0.0128
		WF	ADF	0.0207	0.9	-0.0554	0.0969
		WF	RF	0.0857	0.0533	0.0095	0.1619
		DF	ADF	0.1097	0.0067	0.0361	0.1833
		DF	RF	0.1747	0.001	0.1011	0.2483
		ADF	RF	0.065	0.1711	-0.0086	0.1386

Week 4							
Urine Metabolite	**ppm**	**Diet 1**	**Diet 2**	**Mean Difference**	**p-adj**	**95% Confidence Interval**	
						lower	**upper**
Acetic Acid	1.9189	WF	DF	-0.075	0.504	-0.2206	0.0706
		WF	ADF	0.0785	0.467	-0.0671	0.224
		WF	RF	-0.0516	0.7641	-0.2023	0.0991
		DF	ADF	0.1534	0.036	0.0079	0.299
		DF	RF	0.0234	0.9	-0.1273	0.1741
		ADF	RF	-0.13	0.1093	-0.2807	0.0207
Dimethylamine	2.7205	WF	DF	-0.025	0.8453	-0.1116	0.0617
		WF	ADF	0.0602	0.2508	-0.0264	0.1469
		WF	RF	-0.0266	0.8324	-0.1163	0.0631
		DF	ADF	0.0852	0.0552	-0.0014	0.1719
		DF	RF	-0.0016	0.9	-0.0913	0.088
		ADF	RF	-0.0869	0.0603	-0.1765	0.0028
Dimethyl sulfone	3.1483	WF	DF	-0.1114	0.001	-0.1784	-0.0443
		WF	ADF	0.0235	0.7511	-0.0435	0.0906
		WF	RF	-0.0625	0.0889	-0.1319	0.0069
		DF	ADF	0.1349	0.001	0.0678	0.202
		DF	RF	0.0489	0.2406	-0.0205	0.1183
		ADF	RF	-0.086	0.0109	-0.1554	-0.0166
Creatinine	4.0454	WF	DF	-0.7622	0.0452	-1.512	-0.0125
		WF	ADF	0.4732	0.3298	-0.2765	1.223
		WF	RF	-0.7142	0.0796	-1.4903	0.0618
		DF	ADF	1.2355	0.001	0.4857	1.9852
		DF	RF	0.048	0.9	-0.728	0.8241
		ADF	RF	-1.1875	0.0014	-1.9635	-0.4114
Carnitine	2.4362	WF	DF	-0.4494	0.001	-0.6639	-0.2348
		WF	ADF	0.1534	0.2296	-0.0612	0.3679
		WF	RF	0.4595	0.001	0.2374	0.6816
		DF	ADF	0.6027	0.001	0.3882	0.8172
		DF	RF	0.9089	0.001	0.6868	1.1309
		ADF	RF	0.3062	0.0042	0.0841	0.5282
Methylnicotinamide	4.4725	WF	DF	-0.1025	0.037	-0.2002	-0.0048
		WF	ADF	-0.0332	0.7667	-0.1309	0.0644
		WF	RF	0.0723	0.229	-0.0288	0.1734
		DF	ADF	0.0693	0.2356	-0.0284	0.1669
		DF	RF	0.1748	0.001	0.0737	0.2759
		ADF	RF	0.1056	0.0383	0.0045	0.2066

Abbreviations – WF – canned wet; DF – dry kibble; ADF – air-dried; RF – mildly cooked/raw

Appendix R

Relative intensities for all discriminatory serum metabolites across all four weeks for all diets

Week 1

Valine	Acetic acid	Acetylcarnitine	Betaine	Glycine	Creatine	Unknown 1	Unknown 2	food type
0.55872	0.26111	0.15006	0.76104	0.33613	0.11399	0.34744	0.75966	WF
0.50443	0.27642	0.14354	0.80619	0.38524	0.136	0.30056	0.73217	WF
0.35066	0.2228	0.17462	1.0988	0.49391	0.11353	0.3009	0.73261	WF
0.47534	0.28539	0.17845	1.4477	0.52015	0.16865	0.24367	0.87558	WF
0.50629	0.29127	0.16155	0.77807	0.42409	0.12905	0.2779	0.84866	WF
0.43964	0.24864	0.17043	1.5299	0.44991	0.15899	0.3041	0.83477	WF
0.44648	0.25267	0.1521	0.92734	0.43411	0.10191	0.27665	0.86616	WF
0.51662	0.22526	0.15449	1.0145	0.43945	0.16266	0.25788	0.72909	WF
0.52381	0.2545	0.12656	0.82484	0.40497	0.10636	0.24105	0.74846	DF
0.48225	0.2589	0.15682	1.2451	0.48285	0.1389	0.32883	0.84213	DF
0.47791	0.26104	0.14665	0.96654	0.28472	0.082594	0.24572	0.79912	DF
0.60422	0.25898	0.13882	0.84739	0.45571	0.11686	0.20898	0.86136	DF
0.42142	0.29586	0.15898	0.94175	0.55173	0.13496	0.28591	0.99092	DF
0.52656	0.30287	0.14431	0.82015	0.38785	0.12116	0.23095	0.77658	DF
0.47771	0.25342	0.14853	0.91902	0.46041	0.15687	0.22104	0.85701	DF
0.51707	0.2546	0.13818	1.0532	0.38598	0.10709	0.20556	0.81375	DF
0.54014	0.26547	0.15876	1.178	0.48283	0.13216	0.20457	0.93008	ADF
0.57781	0.34692	0.17986	0.99812	0.54719	0.17135	0.19149	0.90815	ADF
0.53463	0.26092	0.13863	0.83058	0.42578	0.18374	0.28653	0.87863	ADF
0.49207	0.27981	0.15624	1.0508	0.67084	0.15864	0.24141	0.96633	ADF
0.52724	0.27252	0.1545	1.2696	0.51593	0.15391	0.24024	0.78559	ADF
0.66208	0.28256	0.18871	0.91357	0.49286	0.15951	0.2476	0.86661	ADF
0.54261	0.26151	0.18415	0.9772	0.36089	0.11843	0.2196	0.84242	ADF
0.54837	0.25388	0.13627	0.99492	0.47486	0.16355	0.27733	0.82577	ADF
0.53708	0.2974	0.20381	1.4985	0.56246	0.18858	0.21582	0.85847	RF
0.52491	0.32152	0.15446	1.0046	0.40223	0.15373	0.21094	0.90815	RF
0.50895	0.29855	0.14255	0.78044	0.40193	0.18059	0.31971	0.74015	RF
0.50251	0.30366	0.16797	0.88133	0.48635	0.12267	0.25673	0.90186	RF
0.57468	0.29834	0.18987	1.3885	0.44514	0.17689	0.20314	0.87059	RF
0.52693	0.3277	0.18812	1.2998	0.49751	0.14757	0.25441	0.80433	RF
0.53177	0.28259	0.14186	0.80451	0.40094	0.17646	0.21755	0.77253	RF
0.4668	0.26872	0.19013	1.0617	0.45374	0.12664	0.24977	0.80451	RF

Appendix R continued

Week 2

Valine	Acetic acid	Acetylcarnitine	Betaine	Glycine	Creatine	Unknown 1	Unknown 2	food type
0.47391	0.2498	0.19585	1.6804	0.58609	0.16607	0.35999	0.88377	WF
0.54134	0.26361	0.15615	0.88887	0.40375	0.14417	0.27119	0.89948	WF
0.41998	0.25111	0.15678	0.97344	0.39491	0.12677	0.32407	0.81117	WF
0.4873	0.25032	0.15545	0.78992	0.46031	0.13203	0.31268	0.80625	WF
0.50028	0.26902	0.14287	0.81691	0.43943	0.14991	0.30719	0.79752	WF
0.50661	0.24709	0.18197	1.4642	0.49942	0.15994	0.30469	0.79238	WF
0.33601	0.25871	0.19937	1.1042	0.48497	0.118	0.32801	0.89628	WF
0.46976	0.23449	0.14335	0.83341	0.52322	0.13369	0.32823	0.85149	WF
0.49001	0.29778	0.1362	0.74603	0.3931	0.10458	0.223	0.78993	DF
0.46309	0.26454	0.13122	0.7677	0.35234	0.078387	0.25553	0.75379	DF
0.43584	0.24773	0.16325	0.9878	0.57246	0.10769	0.29011	0.82063	DF
0.52933	0.22072	0.13208	1.012	0.35574	0.091219	0.24209	0.66157	DF
0.50992	0.2486	0.13332	0.89639	0.33109	0.087654	0.24718	0.72866	DF
0.47597	0.2744	0.14236	0.67716	0.36028	0.095686	0.21212	0.86594	DF
0.61468	0.29772	0.17231	1.233	0.39701	0.1546	0.2354	0.79931	DF
0.46932	0.25267	0.18398	1.144	0.51624	0.13489	0.27678	0.78646	DF
0.44019	0.25693	0.17484	1.3341	0.54651	0.22251	0.21098	0.81946	ADF
0.52621	0.26649	0.15747	1.4773	0.49759	0.25051	0.16982	0.81921	ADF
0.43974	0.24542	0.16102	0.93762	0.52212	0.15195	0.25514	0.83478	ADF
0.52348	0.24744	0.13949	0.97799	0.41359	0.14183	0.26986	0.79911	ADF
0.40618	0.27395	0.17417	0.99526	0.51565	0.12326	0.25338	0.84277	ADF
0.48633	0.24395	0.14639	0.89546	0.45437	0.12983	0.23442	0.7628	ADF
0.52617	0.24558	0.14927	1.1111	0.41536	0.15111	0.25526	0.8457	ADF
0.58399	0.32736	0.20617	0.95684	0.5579	0.14918	0.21427	1.0038	ADF
0.50147	0.34212	0.17662	1.0263	0.44643	0.1574	0.2159	0.80259	RF
0.4833	0.34951	0.19776	0.89192	0.56998	0.145	0.22553	0.83577	RF
0.45121	0.29421	0.15543	0.70955	0.35913	0.123	0.22356	0.78607	RF
0.47704	0.261	0.13873	0.82879	0.40159	0.13731	0.23867	0.84607	RF
0.5029	0.29345	0.15613	0.64535	0.33806	0.12977	0.22648	0.62622	RF
0.48883	0.33027	0.17092	0.72794	0.45187	0.15009	0.21505	0.88956	RF
0.57522	0.30739	0.16788	1.041	0.37118	0.13319	0.23031	0.83315	RF
0.41277	0.27071	0.17876	0.85847	0.41549	0.13058	0.25877	0.76583	RF

Appendix R continued

Week 3

Valine	Acetic acid	Acetylcarnitine	Betaine	Glycine	Creatine	Unknown 1	Unknown 2	food type
0.47898	0.24675	0.16347	1.0218	0.484	0.13205	0.36173	0.85379	WF
0.45589	0.23375	0.1691	1.2896	0.64456	0.18021	0.30087	0.91886	WF
0.50593	0.27146	0.19154	2.0315	0.5608	0.15694	0.39148	1.0039	WF
0.58018	0.2523	0.15768	1.0704	0.4864	0.14903	0.33428	0.93126	WF
0.51412	0.27304	0.14167	0.8716	0.49569	0.1608	0.33435	0.91099	WF
0.62995	0.24365	0.17214	1.0356	0.3835	0.13482	0.32954	0.85416	WF
0.5017	0.24598	0.15426	1.0126	0.52075	0.13345	0.34786	0.92413	WF
0.60915	0.25702	0.16493	1.5395	0.52977	0.21009	0.3005	0.86313	WF
0.53464	0.25287	0.13502	0.93757	0.34909	0.092448	0.247	0.76429	DF
0.45072	0.23672	0.10836	0.82599	0.36719	0.096806	0.26041	0.71834	DF
0.45831	0.23444	0.1167	0.68712	0.31157	0.0877	0.23699	0.68393	DF
0.42215	0.26083	0.16534	1.1312	0.43325	0.10794	0.2521	0.76278	DF
0.48763	0.25417	0.12931	0.86051	0.40129	0.1242	0.25603	0.79263	DF
0.46771	0.25174	0.13832	0.88884	0.5036	0.15375	0.24928	0.74096	DF
0.52952	0.23302	0.13301	0.7823	0.34089	0.083989	0.22157	0.66415	DF
0.40731	0.26699	0.18067	1.0332	0.46303	0.096015	0.23231	0.85533	DF
0.47139	0.24524	0.15029	0.95214	0.41773	0.14545	0.26301	0.8199	ADF
0.48448	0.27248	0.18368	1.2381	0.59601	0.21383	0.2131	0.8956	ADF
0.46462	0.2677	0.16873	1.4317	0.52086	0.20679	0.17079	0.82871	ADF
0.59881	0.23329	0.20167	1.482	0.43926	0.21611	0.23616	0.82365	ADF
0.48799	0.25442	0.18989	1.0309	0.52572	0.14297	0.22941	0.83848	ADF
0.53285	0.25266	0.14787	0.9119	0.41096	0.14219	0.22286	0.79716	ADF
0.50902	0.23573	0.14721	0.99102	0.52252	0.14677	0.23904	0.81011	ADF
0.54469	0.24595	0.15803	1.1485	0.48059	0.1569	0.22266	0.9198	ADF
0.44029	0.26733	0.16422	0.84456	0.4783	0.11698	0.28841	0.75969	RF
0.49052	0.27769	0.17119	1.0788	0.36468	0.11941	0.27447	0.71652	RF
0.50223	0.29021	0.16228	0.85799	0.36882	0.11529	0.29108	0.70577	RF
0.47609	0.32851	0.17753	0.89168	0.38836	0.12739	0.21309	0.89336	RF
0.49727	0.28699	0.16858	0.68258	0.34257	0.14818	0.23149	0.66711	RF
0.46759	0.33047	0.18522	0.76355	0.48471	0.11779	0.21623	0.81439	RF
0.48359	0.30231	0.15383	0.80798	0.42421	0.13312	0.22106	0.83205	RF
0.58764	0.3469	0.17043	0.97602	0.47009	0.17056	0.23572	0.76997	RF

Appendix R

Week 4

Valine	Acetic acid	Acetylcarnitine	Betaine	Glycine	Creatine	Unknown 1	Unknown 2	food type
0.46103	0.24561	0.16364	1.011	0.49176	0.14236	0.31999	0.86247	WF
0.49768	0.2507	0.15232	0.87125	0.3785	0.11885	0.31441	0.84226	WF
0.49151	0.23349	0.15236	1.0126	0.49531	0.13736	0.38386	0.842	WF
0.53156	0.27126	0.19992	1.8992	0.58835	0.16658	0.36635	1.0824	WF
0.33021	0.24266	0.18668	1.0138	0.51757	0.13455	0.30601	0.8295	WF
0.51689	0.26326	0.16076	0.98969	0.55482	0.18562	0.34697	0.98625	WF
0.44539	0.23993	0.1858	1.5575	0.47949	0.10022	0.28991	0.7681	WF
0.41546	0.27416	0.15205	1.2085	0.50075	0.11813	0.21448	0.86728	WF
0.53554	0.25088	0.13878	0.95478	0.38566	0.093288	0.20167	0.80274	DF
0.58886	0.24331	0.12998	0.85319	0.37293	0.114	0.24774	0.75388	DF
0.5132	0.25134	0.13621	1.2792	0.38002	0.12626	0.27211	0.76807	DF
0.51019	0.26442	0.12203	0.8596	0.4047	0.10261	0.23313	0.74517	DF
0.53788	0.23906	0.13943	1.0087	0.45201	0.20621	0.22954	0.78333	DF
0.47503	0.2424	0.12164	0.784	0.34289	0.092226	0.20741	0.69335	DF
0.52261	0.24897	0.14669	0.858	0.35079	0.12547	0.20974	0.82289	DF
0.59291	0.23625	0.16427	1.3592	0.44586	0.17266	0.22851	0.78372	DF
0.52632	0.23452	0.1632	1.4031	0.46449	0.16779	0.22799	0.70596	ADF
0.56423	0.21879	0.1453	1.0983	0.39434	0.15417	0.23261	0.79942	ADF
0.48445	0.23765	0.15443	1.0584	0.57293	0.18197	0.22973	0.86612	ADF
0.60527	0.23294	0.19282	1.1435	0.40823	0.17766	0.26161	0.83342	ADF
0.54867	0.25528	0.15334	1.156	0.41957	0.17935	0.22733	0.90423	ADF
0.6368	0.24693	0.19419	1.6777	0.49151	0.25507	0.19852	0.87274	ADF
0.50557	0.30948	0.16113	0.70048	0.36992	0.12206	0.26574	0.75713	ADF
0.41926	0.27798	0.16873	0.69636	0.49116	0.12486	0.2316	0.76103	ADF
0.50623	0.28929	0.14821	0.72862	0.39207	0.12498	0.21032	0.84338	RF
0.56299	0.29538	0.16341	0.95504	0.39812	0.13458	0.249	0.65943	RF
0.63247	0.30947	0.18	1.1929	0.46897	0.17018	0.21144	0.86095	RF
0.52694	0.32906	0.14744	0.72439	0.43111	0.16728	0.19215	0.82646	RF
0.49869	0.31826	0.17075	0.87421	0.37591	0.14707	0.23639	0.79981	RF
0.46978	0.3301	0.19273	0.71463	0.44966	0.13404	0.22332	0.74676	RF

Abbreviations – WF – canned wet; DF – dry kibble; ADF – air-dried; RF – mildly cooked/raw

Appendix S

Relative intensities for all discriminatory urine metabolites across all four weeks for all diets.

Week 1

Acetic acid	Carnitine	Dimethylamine	Dimethyl-sulfone	Creatinine	Methylnicotinamide	food type
0.49676	0.6878	0.36184	0.3603	3.7904	0.27517	WF
0.71418	0.79849	0.32916	0.37104	2.6076	0.29877	WF
0.5916	0.81561	0.30002	0.33321	2.7074	0.28823	WF
0.4717	0.72694	0.34114	0.33985	3.0102	0.24902	WF
0.58981	0.80932	0.34391	0.36044	3.3404	0.26353	WF
0.60796	0.87596	0.27407	0.38116	2.768	0.21615	WF
0.68113	0.94717	0.33385	0.37651	2.8902	0.25025	WF
0.66727	0.81026	0.30569	0.33336	2.2762	0.27821	WF
0.65375	0.44346	0.28075	0.23479	2.1283	0.16206	DF
0.52428	0.43143	0.28091	0.28409	2.0666	0.17899	DF
0.62398	0.50961	0.23995	0.27375	2.1606	0.18839	DF
0.45062	0.4712	0.29876	0.26885	1.8923	0.13937	DF
0.47316	0.44804	0.26792	0.25783	1.9917	0.15284	DF
0.478	0.40795	0.28284	0.2074	2.1216	0.17196	DF
0.44711	0.3346	0.23567	0.25586	1.9692	0.10323	DF
0.47648	0.41276	0.30997	0.35782	2.5049	0.2172	DF
0.80462	1.1108	0.40985	0.38464	3.7193	0.23614	ADF
0.60313	1.3792	0.37674	0.38302	3.7402	0.23914	ADF
0.50459	1.0487	0.44827	0.40692	3.8002	0.34127	ADF
0.63545	0.9041	0.41867	0.39541	3.3594	0.40571	ADF
0.69716	0.98572	0.37907	0.41848	3.1051	0.32655	ADF
0.70901	1.0369	0.4865	0.42885	3.7746	0.29993	ADF
0.70939	1.3535	0.44191	0.45856	4.036	0.27816	ADF
0.72137	1.0744	0.37201	0.44467	3.629	0.25929	ADF
0.4301	1.1146	0.34296	0.3875	2.8907	0.40195	RF
0.37564	1.266	0.41758	0.39931	3.3995	0.49079	RF
0.43014	1.2601	0.29731	0.30717	2.3776	0.26616	RF
0.43797	1.2664	0.32919	0.31504	2.9217	0.42722	RF
0.31946	1.1828	0.3062	0.33104	3.0075	0.27242	RF
0.40937	1.0304	0.36617	0.32192	2.7293	0.37503	RF
0.42369	1.0478	0.32604	0.29571	2.4524	0.31361	RF
0.43476	1.1307	0.37919	0.32219	3.2993	0.33851	RF

Appendix S continued

Week 2

Acetic acid	Carnitine	Dimethylamine	Dimethyl-sulfone	Creatinine	Methylnicotinamide	food type
0.39354	0.63083	0.46405	0.3693	3.4048	0.288	WF
0.49379	0.70658	0.32927	0.33064	2.6393	0.29541	WF
0.71094	1.0019	0.27381	0.36428	2.7292	0.25907	WF
0.60088	0.89718	0.30434	0.30872	2.6452	0.22658	WF
0.66465	0.9692	0.31579	0.40491	3.5856	0.21752	WF
0.5615	0.95318	0.41524	0.42225	3.9683	0.30103	WF
0.69201	0.59376	0.31464	0.38046	2.171	0.2858	WF
0.55747	0.72303	0.29858	0.33678	2.4312	0.26431	WF
0.59467	0.50403	0.29205	0.28125	1.9977	0.15153	DF
0.47334	0.40888	0.27372	0.26931	2.1	0.14601	DF
0.58054	0.39766	0.24738	0.23471	1.9639	0.14247	DF
0.54359	0.44249	0.3652	0.31555	2.4014	0.13276	DF
0.64324	0.52508	0.22938	0.22916	1.9705	0.15042	DF
0.48675	0.36851	0.29404	0.26053	1.9267	0.1574	DF
0.52726	0.3138	0.22139	0.18735	2.0298	0.12727	DF
0.4364	0.48518	0.30249	0.28469	2.5107	0.152	DF
0.63539	1.1044	0.40595	0.34425	3.1677	0.32614	ADF
0.69293	1.1972	0.47898	0.42102	4.125	0.19077	ADF
0.5808	0.66205	0.27765	0.33752	3.3153	0.12112	ADF
0.43631	0.74113	0.41632	0.36351	3.3386	0.38257	ADF
0.74948	1.486	0.421	0.39131	3.7899	0.22509	ADF
0.78805	1.4034	0.39139	0.39508	4.0021	0.22795	ADF
0.56371	0.9312	0.40373	0.42322	3.5986	0.30147	ADF
0.6324	1.329	0.41998	0.37388	3.3948	0.24182	ADF
0.58256	1.3966	0.35986	0.33459	2.5159	0.22187	RF
0.46301	0.84962	0.27518	0.34962	2.4802	0.36741	RF
0.59707	1.3997	0.36816	0.33003	2.5148	0.22124	RF
0.46936	1.12	0.27634	0.2902	2.3885	0.27484	RF
0.40753	1.0185	0.43732	0.38072	3.6695	0.35897	RF
0.4139	0.8276	0.26272	0.32341	3.0361	0.29685	RF
0.39294	1.0217	0.4374	0.48515	3.8095	0.41328	RF
0.51395	0.95245	0.31961	0.31768	3.1452	0.31979	RF

Appendix S continued

Week 3

Acetic acid	Carnitine	Dimethylamine	Dimethyl-sulfone	Creatinine	Methylnicotinamide	food type
0.73952	1.0395	0.29396	0.4001	2.8386	0.29196	WF
0.46378	0.85905	0.3666	0.37672	3.6235	0.30992	WF
0.69466	0.58592	0.3304	0.38567	2.1779	0.32352	WF
0.85318	1.055	0.27735	0.39529	2.7242	0.22801	WF
0.61776	0.90849	0.29838	0.30491	2.32	0.24903	WF
0.60225	1.0863	0.28386	0.32859	3.2515	0.22603	WF
0.57206	0.83303	0.28696	0.35941	2.8625	0.20725	WF
0.44377	0.59888	0.3953	0.13006	3.9242	0.22032	DF
0.65426	0.44067	0.35682	0.33325	2.4084	0.25877	DF
0.63656	0.48073	0.23524	0.23026	1.9361	0.18164	DF
0.43794	0.47114	0.27818	0.25912	2.2572	0.14904	DF
0.44442	0.32491	0.24019	0.23502	1.9413	0.10962	DF
0.44144	0.43899	0.28852	0.2389	2.051	0.14865	DF
0.58187	0.40647	0.23713	0.22964	1.6335	0.16239	DF
0.47973	0.39373	0.27721	0.27794	2.04	0.15571	DF
0.73918	1.1574	0.51954	0.3551	4.2334	0.29187	ADF
0.60502	0.80324	0.37391	0.36234	3.3456	0.24856	ADF
0.70203	0.99245	0.38206	0.42203	3.1153	0.32163	ADF
0.39967	0.73253	0.34646	0.28172	3.5486	0.24178	ADF
0.72598	1.2997	0.45821	0.47172	3.6828	0.32639	ADF
0.80123	1.374	0.38572	0.38912	3.8354	0.2254	ADF
0.84984	0.9927	0.41107	0.48271	3.8752	0.32925	ADF
0.5491	0.95021	0.34406	0.37295	3.2118	0.27891	ADF
0.5694	1.1348	0.27985	0.32778	2.4866	0.29665	RF
0.3664	1.2331	0.33146	0.33456	2.7356	0.51404	RF
0.52228	1.3187	0.32203	0.33316	2.8722	0.23534	RF
0.71834	1.4038	0.34732	0.32783	2.6466	0.39761	RF
0.51469	1.6393	0.40258	0.36447	3.1602	0.31078	RF
0.5329	0.87572	0.26394	0.35993	2.4156	0.42151	RF
0.564	1.2838	0.30794	0.28213	2.5514	0.35534	RF
0.54544	1.5724	0.33707	0.344	2.4254	0.25237	RF

Appendix S continued

Week 4

Acetic acid	Carnitine	Dimethylamine	Dimethyl-sulfone	Creatinine	Methylnicotinamide	food type
0.56641	0.71444	0.32638	0.36048	3.09	0.35495	WF
0.42259	0.85942	0.36391	0.36117	4.0425	0.32168	WF
0.6779	1.0073	0.31972	0.3715	2.776	0.28971	WF
0.65058	0.95932	0.27342	0.37216	2.649	0.25801	WF
0.61552	1.012	0.28036	0.32644	2.3274	0.24203	WF
0.6006	0.94821	0.29851	0.34776	2.897	0.20083	WF
0.65576	0.71572	0.28527	0.39217	2.533	0.28454	WF
0.3296	0.59984	0.55474	0.421	4.2616	0.30065	WF
0.50249	0.44097	0.40144	0.35683	2.764	0.29358	DF
0.45558	0.42221	0.24914	0.20298	1.9633	0.14445	DF
0.48996	0.36623	0.27277	0.25234	2.0176	0.14452	DF
0.5476	0.35094	0.25767	0.23731	1.8932	0.17681	DF
0.45732	0.30857	0.23055	0.25254	1.8149	0.065187	DF
0.5577	0.3839	0.34216	0.13484	2.1991	0.23875	DF
0.52669	0.39665	0.32668	0.26824	2.0791	0.14531	DF
0.38173	0.55198	0.42211	0.3566	3.7474	0.22382	DF
0.70797	1.133	0.44818	0.39848	3.8563	0.2738	ADF
0.87377	0.97328	0.37842	0.44108	3.6099	0.24499	ADF
0.54511	1.0141	0.396	0.31374	3.2758	0.20268	ADF
0.62755	1.1817	0.39548	0.386	3.1501	0.18995	ADF
0.64888	1.0011	0.36003	0.38045	2.9012	0.18986	ADF
0.76674	1.0487	0.37421	0.37746	3.5152	0.22542	ADF
0.52285	0.67647	0.43424	0.35402	3.7559	0.28625	ADF
0.45373	1.0147	0.39762	0.48967	4.2981	0.37348	ADF
0.63602	1.4282	0.2845	0.29515	2.537	0.2392	RF
0.5718	1.5678	0.35922	0.31543	2.159	0.35334	RF
0.58958	1.3559	0.30938	0.31703	2.6079	0.22562	RF
0.44527	0.94993	0.27417	0.31052	2.2207	0.4576	RF
0.4362	1.2107	0.34068	0.30311	2.0326	0.34384	RF
0.44347	1.5251	0.31963	0.3197	2.4394	0.4896	RF
0.47077	1.1432	0.29062	0.28524	2.5083	0.36778	RF

Abbreviations – WF – canned wet; DF – dry kibble; ADF – air-dried; RF – mildly cooked/raw

Appendix T

Relative abundance (%) of fecal microbiome – phyla

Diet	Actinobacteria	Bacteroidetes	Proteobacteria	Firmicutes	Fusobacteria	Others
Canned Wet	0.0157	0.1015	0.1153	98.2680	0.3916	0.0370
Canned Wet	0.0279	21.4153	0.9648	72.4157	2.9023	0.0182
Canned Wet	3.7090	16.5104	1.5587	72.0336	5.3196	0.0493
Canned Wet	1.6993	25.9551	4.0271	56.8991	10.7960	0.0684
Canned Wet	2.3605	31.8135	2.8722	49.2691	12.1677	0.1886
Canned Wet	0.1890	28.8678	2.4658	48.2020	18.4134	0.0121
Canned Wet	0.0655	4.2672	3.4985	85.0833	6.2307	0.0315
Canned Wet	1.3432	0.1687	1.5055	85.3591	8.4608	0.0411
Dry Kibble	0.0488	29.7188	7.1511	38.9165	19.5594	0.0074
Dry Kibble	0.7671	0.0269	0.8822	88.1785	9.7318	0.0288
Dry Kibble	1.0883	11.4999	0.4180	82.7135	1.8746	0.0014
Dry Kibble	2.1902	17.9772	2.0792	64.2878	12.4316	0.2269
Dry Kibble	0.6789	7.7364	0.2902	80.2599	10.2111	0.0160
Dry Kibble	1.0380	15.4910	11.4478	54.3560	15.8013	0.0036
Dry Kibble	0.5706	14.3153	1.4556	57.9610	24.5564	0.0068
Dry Kibble	0.4883	18.9029	0.9528	75.5964	2.8495	0.1820
Air Dried	0.0199	7.9928	0.1202	87.5439	1.7723	0.0081
Air Dried	3.4402	8.3274	2.0288	78.0116	6.7922	0.0095
Air Dried	1.1328	26.6073	1.3478	53.2916	17.0503	0.0223
Air Dried	0.2420	30.1610	3.6889	59.9026	2.0036	0.0124
Air Dried	2.6491	11.2539	0.5781	77.9706	6.7267	0.0126
Air Dried	0.9439	24.5992	4.1416	57.3801	11.2485	0.1199
Air Dried	0.1040	20.9533	3.5986	61.9173	12.2427	0.1169
Air Dried	0.6530	18.1732	2.6242	70.9918	6.6099	0.0421
Mildly cooked/Raw	0.5778	14.9483	2.4109	67.3003	13.5595	0.0400
Mildly cooked/Raw	1.3913	27.7181	5.5493	52.5723	11.9265	0.0644
Mildly cooked/Raw	0.2246	16.9719	1.0469	67.9167	12.4951	0.0443
Mildly cooked/Raw	1.0339	16.9578	0.8025	63.9977	16.2566	0.0539
Mildly cooked/Raw	0.0110	14.8696	8.0216	58.0245	17.2013	0.0136
Mildly cooked/Raw	0.1091	3.9650	0.1203	90.3959	2.9188	0.0086
Mildly cooked/Raw	0.0471	21.2819	5.5632	52.2997	17.2046	0.0310

Appendix U

Relative abundance (%) of fecal microbiome – genera

Group	Megamonas	Bacteroides	Prevotella	Fusobacterium	Blautia
Canned Wet	2.5817	10.9685	9.9090	13.5651	17.9973
Canned Wet	1.7415	10.8616	12.7712	8.1118	4.5218
Canned Wet	0.1984	20.0641	18.3399	29.4019	2.2429
Canned Wet	16.8980	12.2536	4.8158	11.4482	13.6075
Canned Wet	8.6782	18.9890	3.5801	18.4734	5.0838
Canned Wet	0.3303	23.3333	6.7117	23.3033	3.6637
Canned Wet	2.0874	27.1517	0.8168	25.7147	2.5110
Canned Wet	1.7815	32.8658	8.2620	16.9870	7.8693
Dry Kibble	54.6788	6.8036	22.7258	2.8366	1.4346
Dry Kibble	53.7622	4.8875	19.4135	3.2621	3.6031
Dry Kibble	58.3344	6.0602	3.5908	2.1545	8.6557
Dry Kibble	57.6637	9.6602	0.6696	8.3581	5.5184
Dry Kibble	66.0928	2.7499	10.3061	2.1999	6.5399
Dry Kibble	60.2563	8.5767	10.3198	7.6186	4.5943
Dry Kibble	41.3657	3.0422	16.3274	3.1079	3.8739
Dry Kibble	45.9705	4.8123	0.0806	7.1609	0.3339
Air Dried	3.3823	25.3540	3.0939	14.6827	3.9984
Air Dried	3.4420	0.0684	0.1368	0.8434	3.6699
Air Dried	6.4185	13.5842	20.5765	22.7248	11.8895
Air Dried	2.4944	0.0344	0.0172	16.5319	13.5558
Air Dried	4.6530	18.5801	0.1279	19.8273	19.3796
Air Dried	36.9656	19.3257	1.0115	21.1868	1.1598
Air Dried	30.8534	17.7559	0.0675	33.0408	2.7950
Air Dried	2.4922	10.5771	0.0445	25.3523	3.7977
Mildly cooked/Raw	7.7007	14.3359	0.5691	16.3942	9.8196
Mildly cooked/Raw	3.3823	25.3540	3.0939	14.6827	3.9984
Mildly cooked/Raw	29.8615	4.7898	23.1882	4.6651	3.6547
Mildly cooked/Raw	14.0569	21.0721	0.5295	21.4692	11.1052
Mildly cooked/Raw	0.2025	4.1115	1.6508	4.3140	18.6887
Mildly cooked/Raw	4.8194	30.5933	2.1341	21.5760	3.3185
Mildly cooked/Raw	3.0901	0.1780	0.0971	13.2665	0.6795

Group	Streptococcus	Phascolarctobacterium	Turicibacter	Ruminococcus	Clostridium
Canned Wet	2.0892	13.8487	3.2085	4.0292	2.9846
Canned Wet	3.2844	27.8032	10.8921	0.5958	7.4397
Canned Wet	8.4529	0.0000	2.9753	0.5798	2.4107
Canned Wet	0.0857	14.5159	4.6444	2.7592	1.5424
Canned Wet	10.3967	6.1292	3.2508	6.7879	6.3869
Canned Wet	7.1171	6.4264	3.1982	0.4655	10.9760
Canned Wet	1.7244	17.5011	4.0085	1.8757	4.5984
Canned Wet	0.4629	10.0996	1.2204	0.7014	5.0779
Dry Kibble	0.0000	3.8909	0.0652	1.1629	0.1413
Dry Kibble	2.0459	1.5231	5.2057	0.6024	0.1478
Dry Kibble	0.3780	0.4536	0.1386	4.7877	0.6678
Dry Kibble	0.0496	0.6944	0.0000	6.2376	0.5332
Dry Kibble	0.0239	1.5901	5.3324	0.8847	0.0956
Dry Kibble	0.1616	0.2193	0.1616	2.1355	0.0923
Dry Kibble	3.5019	0.9192	0.4377	3.1079	0.2189
Dry Kibble	26.2952	0.0000	0.2303	7.0228	0.8865
Air Dried	1.5994	2.6875	0.5244	2.7268	0.1966
Air Dried	0.3647	0.1824	10.2120	2.3934	3.1001
Air Dried	6.7788	5.9915	2.0016	1.3611	0.1868
Air Dried	13.2978	0.0000	0.2064	1.3246	1.1698
Air Dried	0.1439	5.8043	0.1119	4.6690	1.0074
Air Dried	0.4181	0.0135	5.1247	1.2272	4.5853
Air Dried	4.7394	0.0000	1.4313	2.6195	0.4186
Air Dried	11.5116	0.2670	23.6018	1.9730	4.8806
Mildly cooked/Raw	0.5691	2.5911	0.0726	0.8476	0.4238
Mildly cooked/Raw	1.5994	2.6875	0.5244	2.7268	0.1966
Mildly cooked/Raw	0.0748	0.0125	0.1871	1.9209	2.7816
Mildly cooked/Raw	0.0132	2.8458	0.0265	1.0324	0.2912
Mildly cooked/Raw	0.2336	0.5451	0.1713	4.5943	1.4951
Mildly cooked/Raw	0.0235	3.8813	0.0235	0.3283	0.1290
Mildly cooked/Raw	18.7187	0.0162	1.2943	2.7342	3.1063

Group	Lactobacillus	Allobaculum	Slackia	Faecalibacterium	Anaerobiospirillum
Canned Wet	0.0298	1.7311	1.0446	0.4775	0.0448
Canned Wet	0.0000	0.1222	0.7027	0.3666	0.1680
Canned Wet	0.0305	0.6713	0.0305	0.5798	0.0000
Canned Wet	0.2399	2.3136	0.8055	0.3942	0.2228
Canned Wet	0.0143	0.6731	0.7876	0.2005	0.0859
Canned Wet	0.0150	0.3003	0.3153	0.4354	0.0000
Canned Wet	0.0000	0.4084	0.0454	0.5445	0.0303
Canned Wet	0.0000	0.7434	0.7715	0.2385	0.0000
Dry Kibble	0.0217	0.0326	0.0109	0.0217	3.0214
Dry Kibble	2.3528	0.1023	0.0000	0.2273	0.0341
Dry Kibble	12.1582	0.2520	0.0000	0.7308	0.0000
Dry Kibble	0.0372	0.2232	0.4836	0.0744	0.0000
Dry Kibble	0.5619	0.2511	0.0120	0.1554	0.0000
Dry Kibble	0.0231	0.0115	0.1039	0.1154	0.7157
Dry Kibble	17.1701	0.0219	0.0438	0.0438	0.0219
Dry Kibble	0.3914	0.0000	0.0000	0.0000	0.0576
Air Dried	0.0262	0.4064	0.5244	0.0393	0.0000
Air Dried	0.1140	0.3875	0.0000	0.7978	0.0000
Air Dried	0.0267	0.6539	0.4404	0.5338	0.0000
Air Dried	0.0000	0.0000	0.0000	0.0000	0.0000
Air Dried	0.0320	4.4292	1.4391	0.5117	0.0799
Air Dried	0.7148	0.8631	0.2023	0.0270	0.0809
Air Dried	0.0540	0.0270	0.0000	0.3916	0.0000
Air Dried	1.1868	1.0829	0.0148	0.0890	10.2655
Mildly cooked/Raw	0.1090	0.4601	0.1453	0.3753	2.1068
Mildly cooked/Raw	0.0262	0.4064	0.5244	0.0393	0.0000
Mildly cooked/Raw	1.0727	0.4865	0.0624	0.7235	0.0000
Mildly cooked/Raw	0.0397	0.4236	0.4500	0.1985	0.0000
Mildly cooked/Raw	1.6820	1.7287	0.0467	0.5918	0.0000
Mildly cooked/Raw	0.1642	0.1642	0.1407	0.2932	0.5277
Mildly cooked/Raw	2.8636	0.0162	0.0000	0.0000	0.0000

Group	Peptococcus	Pediococcus	Coprococcus	Oscillospira	Enterococcus
Canned Wet	1.5819	0.0000	2.5519	0.2835	0.0000
Canned Wet	0.6416	0.0764	0.6263	0.9624	0.0000
Canned Wet	0.1221	0.0458	0.4425	0.5798	0.0305
Canned Wet	2.3136	0.0171	0.5998	0.1542	0.0000
Canned Wet	0.0000	0.1146	0.4153	0.0859	0.0000
Canned Wet	1.3814	0.0450	0.6607	0.4054	0.0000
Canned Wet	0.0000	0.0151	0.3328	0.0908	0.0000
Canned Wet	1.1502	0.0140	0.9679	0.4348	0.0000
Dry Kibble	0.0000	0.0217	0.0000	0.0326	0.0000
Dry Kibble	0.0000	0.0455	0.0114	0.0114	0.0000
Dry Kibble	0.0756	0.0756	0.0126	0.0252	0.0000
Dry Kibble	0.0000	0.0000	0.0124	0.0124	0.0000
Dry Kibble	0.0000	0.0359	0.0000	0.0000	0.0000
Dry Kibble	0.0000	0.0115	0.0231	0.0693	0.0000
Dry Kibble	0.0875	0.2079	0.0219	0.0219	0.0219
Dry Kibble	0.0000	0.0115	0.0000	0.0000	0.0000
Air Dried	0.1704	35.4090	0.0393	0.0524	0.0262
Air Dried	0.0228	64.2124	0.0000	0.0000	0.3647
Air Dried	0.6272	0.0133	0.2135	0.0267	0.0000
Air Dried	0.0000	0.0172	0.0000	0.0000	0.0000
Air Dried	0.0000	0.0160	0.1279	0.0160	0.0640
Air Dried	0.0000	0.0539	0.0135	0.0000	3.1827
Air Dried	0.0000	0.0810	0.1620	0.0135	0.0000
Air Dried	0.0593	0.1335	0.0148	0.0000	0.0445
Mildly cooked/Raw	0.6780	39.2784	0.0121	0.0484	0.0000
Mildly cooked/Raw	0.1704	35.4090	0.0393	0.0524	0.0262
Mildly cooked/Raw	0.1372	18.3859	0.0499	0.2120	0.1372
Mildly cooked/Raw	0.0000	22.5017	0.0265	0.0529	0.0529
Mildly cooked/Raw	0.0000	49.8053	0.0623	0.0000	0.7164
Mildly cooked/Raw	0.0938	27.5915	0.0235	0.0235	0.0352
Mildly cooked/Raw	0.0000	44.6368	0.0000	0.0000	2.0385

Group	Roseburia	Mucispirillum	Bifidobacterium	Epulopiscium	Vagococcus
Canned Wet	0.0000	0.0000	0.0000	0.0000	0.0000
Canned Wet	0.0153	0.0000	0.0000	0.0000	0.0000
Canned Wet	0.0610	0.0000	0.0000	0.0458	0.0305
Canned Wet	0.0000	0.0000	0.0000	0.0000	0.0000
Canned Wet	0.0143	0.0000	0.0000	0.0286	0.0000
Canned Wet	0.0000	0.0450	0.0000	0.1502	0.0000
Canned Wet	0.0000	0.0151	0.0000	0.0151	0.0000
Canned Wet	0.0000	0.2104	0.0000	0.0000	0.0000
Dry Kibble	0.0000	0.0652	0.0109	0.0000	0.0000
Dry Kibble	0.0000	0.0114	0.0114	0.0000	0.0000
Dry Kibble	0.0000	0.0126	0.0000	0.0000	0.0000
Dry Kibble	0.0124	0.0000	0.0000	0.0000	0.0000
Dry Kibble	0.0000	0.0000	1.2912	0.0000	0.0000
Dry Kibble	0.0346	0.0000	0.0000	0.0000	0.0000
Dry Kibble	0.0000	0.1204	0.1094	0.0000	0.0000
Dry Kibble	0.0000	0.0000	0.0000	0.0115	0.0000
Air Dried	0.0262	0.0000	0.0000	0.0000	0.0131
Air Dried	0.0000	0.0000	0.0000	0.0000	0.4103
Air Dried	0.0000	0.0000	0.0000	0.0000	0.0000
Air Dried	0.0688	0.0000	0.0000	0.6537	0.0000
Air Dried	0.0000	0.0000	0.0000	0.0000	0.0160
Air Dried	0.0000	0.0000	0.9440	0.4990	0.4585
Air Dried	0.0000	0.0000	0.0000	0.0000	0.0000
Air Dried	0.0000	0.0000	0.0000	0.0297	0.1632
Mildly cooked/Raw	0.0000	0.0000	0.0000	0.0000	0.0121
Mildly cooked/Raw	0.0262	0.0000	0.0000	0.0000	0.0131
Mildly cooked/Raw	0.0125	0.0000	0.0374	0.0125	0.2245
Mildly cooked/Raw	0.0132	0.0529	0.0000	0.0000	0.0132
Mildly cooked/Raw	0.0000	0.0000	0.0156	0.0156	1.0902
Mildly cooked/Raw	0.0000	0.0000	0.0000	0.0000	0.0821
Mildly cooked/Raw	0.0000	0.0000	2.1518	0.6471	1.9900

Diet	Dorea	Sutterella	Collinsella	Eubacterium	Catenibacterium
Canned Wet	3.7905	2.2086	2.8802	2.1042	0.0746
Canned Wet	1.3749	0.8555	4.8579	0.8708	0.3055
Canned Wet	1.8004	10.8178	0.0153	0.0305	0.0000
Canned Wet	3.5647	0.7369	3.6332	2.1422	0.2571
Canned Wet	3.8236	3.3796	1.5180	1.0884	0.0000
Canned Wet	1.7868	7.9429	0.6156	0.3754	0.0000
Canned Wet	2.1026	7.9262	0.0151	0.4235	0.0303
Canned Wet	2.5389	3.8154	2.4688	1.3045	0.0000
Dry Kibble	0.2282	1.6737	0.4239	0.3913	0.3043
Dry Kibble	0.2387	0.9889	0.0114	0.9207	0.5228
Dry Kibble	0.5670	0.1512	0.0000	0.4536	0.2520
Dry Kibble	0.9177	1.8725	3.6334	1.6741	1.5625
Dry Kibble	0.6098	0.3587	0.0000	0.2630	0.6456
Dry Kibble	0.6233	1.4198	0.5772	1.4545	0.6349
Dry Kibble	0.6675	0.8317	0.2955	2.6921	1.6415
Dry Kibble	4.4439	2.1299	0.0691	0.0691	0.0230
Air Dried	2.4384	1.6911	0.7079	0.0524	0.0524
Air Dried	8.2744	0.1368	0.0000	0.4787	0.3647
Air Dried	2.1084	1.7614	1.0675	0.3870	0.6138
Air Dried	44.5037	0.8945	1.2386	3.8878	0.0000
Air Dried	10.4093	3.1500	2.0467	2.8462	0.5117
Air Dried	0.5260	0.7687	0.2428	0.4046	0.0000
Air Dried	2.8760	1.8904	0.7291	0.0270	0.0000
Air Dried	1.6615	0.4895	0.0000	0.2670	0.0000
Mildly cooked/Raw	2.0705	0.7870	0.5449	0.0363	0.0121
Mildly cooked/Raw	2.4384	1.6911	0.7079	0.0524	0.0524
Mildly cooked/Raw	1.5467	0.2744	0.0374	2.4323	2.8190
Mildly cooked/Raw	1.7340	1.0192	0.9001	0.0265	0.0132
Mildly cooked/Raw	7.0394	0.1713	0.0467	0.4828	0.4516
Mildly cooked/Raw	0.9381	2.1693	0.0704	0.5277	0.3283
Mildly cooked/Raw	1.1810	1.2458	0.0000	0.0162	0.0162

Group	Corynebacterium	Actinomyces	Helicobacter
Canned Wet	0.0149	0.0000	0.0000
Canned Wet	0.0000	0.0153	0.0153
Canned Wet	0.0000	0.0000	0.0000
Canned Wet	0.0000	0.0343	0.0000
Canned Wet	0.0000	0.0143	0.0000
Canned Wet	0.0000	0.0000	0.0000
Canned Wet	0.0000	0.0000	0.0151
Canned Wet	0.0000	0.0140	0.0000
Dry Kibble	0.0000	0.0000	0.0000
Dry Kibble	0.0000	0.0000	0.0568
Dry Kibble	0.0126	0.0000	0.0000
Dry Kibble	0.0496	0.0496	0.0000
Dry Kibble	0.0000	0.0000	0.0000
Dry Kibble	0.0346	0.0000	0.0115
Dry Kibble	0.0328	0.0328	0.0109
Dry Kibble	0.0000	0.0000	0.0000
Air Dried	0.0000	0.0000	0.0787
Air Dried	0.0228	0.0000	0.0000
Air Dried	0.0000	0.0000	0.0133
Air Dried	0.0172	0.0344	0.0516
Air Dried	0.0000	0.0000	0.0000
Air Dried	0.0000	0.0000	0.0000
Air Dried	0.0135	0.0000	0.0135
Air Dried	0.0000	0.0000	0.0000
Mildly cooked/Raw	0.0000	0.0000	0.0000
Mildly cooked/Raw	0.0000	0.0000	0.0787
Mildly cooked/Raw	0.0000	0.0000	0.1996
Mildly cooked/Raw	0.0132	0.0132	0.0132
Mildly cooked/Raw	0.0311	0.0156	0.0000
Mildly cooked/Raw	0.0000	0.0000	0.0000
Mildly cooked/Raw	0.0000	0.0000	0.0162

Appendix V

Shannon and Simpson indices as a measure of alpha diversity for the fecal microbiome

Diet	Dog No.	Shannon Index	Simpson Index
Canned Wet	1	3.05	0.93
Canned Wet	2	3.04	0.93
Canned Wet	3	2.87	0.91
Canned Wet	4	3.02	0.93
Canned Wet	5	3.06	0.93
Canned Wet	6	3.13	0.94
Canned Wet	7	2.84	0.91
Canned Wet	8	2.90	0.91
Dry Kibble	1	2.16	0.75
Dry Kibble	2	2.11	0.75
Dry Kibble	3	2.04	0.71
Dry Kibble	4	2.33	0.81
Dry Kibble	5	1.91	0.71
Dry Kibble	6	2.19	0.76
Dry Kibble	7	1.98	0.68
Dry Kibble	8	2.03	0.78
Air Dried	1	2.28	0.83
Air Dried	2	2.80	0.91
Air Dried	3	2.78	0.91
Air Dried	4	2.50	0.86
Air Dried	5	2.63	0.87
Air Dried	6	2.32	0.85
Air Dried	7	2.37	0.85
Air Dried	8	2.79	0.91
Mildly cooked/Raw	1	2.62	0.88
Mildly cooked/Raw	2	2.57	0.85
Mildly cooked/Raw	3	2.30	0.84
Mildly cooked/Raw	4	2.41	0.85
Mildly cooked/Raw	5	2.63	0.87
Mildly cooked/Raw	6	2.50	0.88
Mildly cooked/Raw	7	2.70	0.88

Appendix W

Principal component analysis (PCA) loadings and scores at genus taxa level for fecal microbiome for all diet groups

PCA loading

Bacteria	**PC1**	**PC2**	**PC3**	**PC4**
Megamonas	-0.4090	0.3444	0.6050	-0.1076
Pediococcus	-0.0736	-0.8139	0.3010	0.1915
Fusobacterium	-0.4404	-0.1467	-0.2493	-0.1776
Bacteroides	-0.4315	-0.0407	-0.1973	0.1898
Prevotella	-0.2117	0.3104	0.0526	0.4352
Blautia	-0.2721	-0.0644	-0.0132	0.1879
Streptococcus	-0.0843	0.0304	-0.2151	-0.5713
Phascolarctobacterium	-0.1301	0.0514	-0.3310	0.4276
Turicibacter	-0.0502	0.1238	-0.2142	-0.0911
Clostridium	-0.0362	0.0062	-0.2660	-0.0812
Ruminococcus	-0.1014	0.0066	0.0983	-0.1470
Dorea	-0.0980	-0.1656	-0.1155	-0.1800
Sutterella	-0.0714	0.0348	-0.2075	-0.0111
Eubacterium	0.0326	0.1033	0.0219	0.0637
Collinsella	0.0312	0.0651	-0.1168	0.1320
Lactobacillus	0.0590	0.1096	0.2208	-0.0633
Allobaculum	0.0679	-0.0078	-0.0698	0.0806
Slackia	0.0993	0.0190	-0.0594	0.0877
Catenibacterium	0.0901	0.0712	0.1050	0.0847
Faecalibacterium	0.1017	0.0143	-0.0099	0.0593
Coprococcus	0.1118	0.0467	-0.0910	0.0795
Peptococcus	0.1032	0.0347	-0.0830	0.1109
Anaerobiospirillum	0.0943	0.0284	-0.0039	-0.0034
Oscillospira	0.1269	0.0347	-0.0444	0.0581
Enterococcus	0.1246	-0.0094	0.0189	-0.0321
Vagococcus	0.1320	-0.0272	0.0151	-0.0089
Lactococcus	0.1284	-0.0637	0.0189	0.0218
Roseburia	0.1424	0.0263	-0.0060	0.0318
Helicobacter	0.1407	0.0240	-0.0005	0.0357
Epulopiscium	0.1369	0.0175	-0.0075	-0.0061
Corynebacterium	0.1425	0.0275	-0.0032	0.0325

Bacteria	PC5	PC6	PC7	PC8
Actinomyces	0.1426	0.0277	-0.0049	0.0328
Coprobacillus	0.1417	0.0277	-0.0114	0.0345
Mucispirillum	0.1407	0.0305	-0.0059	0.0381
Megamonas	-0.1282	-0.1923	-0.1380	-0.0206
Pediococcus	0.2855	0.0095	-0.0190	0.0645
Fusobacterium	-0.0499	-0.1606	0.1230	-0.0110
Bacteroides	-0.0576	-0.4257	0.1310	0.2268
Prevotella	0.5525	0.3028	0.2944	-0.2001
Blautia	-0.2720	0.4610	-0.1448	0.0207
Streptococcus	0.3067	0.2116	0.2933	0.0755
Phascolarctobacterium	-0.0744	0.0481	-0.1085	0.2346
Turicibacter	0.3373	-0.0013	-0.6916	-0.0636
Clostridium	0.1795	0.0221	-0.2467	0.3017
Ruminococcus	-0.1943	0.1269	0.0694	0.3918
Dorea	-0.2902	0.4194	0.0144	-0.2418
Sutterella	0.0218	-0.1820	0.3642	-0.0373
Eubacterium	-0.1473	0.2306	0.0631	0.0463
Collinsella	-0.2320	0.0634	-0.0057	0.0487
Lactobacillus	0.1799	0.1906	0.0423	0.6794
Allobaculum	-0.1466	0.0304	-0.0606	0.1159
Slackia	-0.1045	-0.0257	0.0460	0.0759
Catenibacterium	0.0031	0.0401	0.0903	0.0438
Faecalibacterium	-0.0124	-0.0141	0.0316	0.0321
Coprococcus	-0.0273	0.0050	0.0465	0.0599
Peptococcus	-0.0278	0.0072	0.0211	0.0486
Anaerobiospirillum	0.0289	-0.2009	-0.1182	-0.1195
Oscillospira	0.0107	-0.0439	0.0580	0.0062
Enterococcus	0.0269	-0.1196	-0.0408	0.1059
Vagococcus	0.0287	-0.0349	0.0193	0.0795
Lactococcus	-0.0006	0.0091	0.0425	0.0858
Roseburia	-0.0267	-0.0602	0.0588	0.0015
Helicobacter	-0.0198	-0.0570	0.0593	0.0049
Epulopiscium	-0.0179	-0.0516	0.0446	0.0017
Corynebacterium	-0.0295	-0.0600	0.0555	0.0104
Actinomyces	-0.0297	-0.0593	0.0547	0.0113
Coprobacillus	-0.0274	-0.0623	0.0520	0.0150
Mucispirillum	-0.0245	-0.0599	0.0613	0.0185

Bacteria	PC9	PC10	PC11	PC12
Megamonas	-0.2944	0.0642	-0.1081	0.1154
Pediococcus	-0.1925	0.0238	-0.1481	0.0075
Fusobacterium	0.2046	-0.0791	-0.2284	0.4353
Bacteroides	0.2184	-0.0453	0.0056	-0.1990
Prevotella	0.0839	0.0805	-0.1656	-0.1082
Blautia	0.3002	-0.2659	0.0107	0.0838
Streptococcus	-0.3282	-0.2798	-0.1524	0.0489
Phascolarctobacterium	-0.6037	-0.2018	0.1659	0.0812
Turicibacter	0.0095	-0.0420	0.0826	-0.0516
Clostridium	0.0699	0.5420	-0.3173	-0.0511
Ruminococcus	-0.1534	0.1294	-0.1333	-0.5686
Dorea	-0.0196	0.1185	0.0481	-0.1588
Sutterella	-0.0053	0.1788	0.2669	-0.1611
Eubacterium	0.0737	0.1531	-0.3081	0.1564
Collinsella	-0.2413	0.0596	-0.3436	0.1870
Lactobacillus	0.2464	-0.2475	0.2120	0.1577
Allobaculum	0.1735	-0.0277	-0.1072	-0.1343
Slackia	-0.0676	-0.0047	-0.1133	0.0189
Catenibacterium	0.0459	0.0964	-0.2212	0.0244
Faecalibacterium	0.0650	-0.0020	0.0374	-0.0276
Coprococcus	-0.0393	0.0180	-0.0790	0.0142
Peptococcus	-0.0622	-0.0673	-0.1303	0.1152
Anaerobiospirillum	0.1039	-0.5444	-0.5057	-0.3638
Oscillospira	-0.0131	0.0096	-0.0592	0.0389
Enterococcus	0.0694	0.1414	-0.0857	0.2592
Vagococcus	0.0107	0.0257	-0.0678	0.0947
Lactococcus	0.0333	-0.0223	0.0182	-0.0017
Roseburia	-0.0084	-0.0450	-0.0325	0.0213
Helicobacter	-0.0111	-0.0344	-0.0485	0.0226
Epulopiscium	0.0008	-0.0049	-0.0495	0.1404
Corynebacterium	-0.0093	-0.0458	-0.0319	0.0201
Actinomyces	-0.0127	-0.0462	-0.0336	0.0246
Coprobacillus	-0.0119	-0.0442	-0.0322	0.0165
Mucispirillum	-0.0090	-0.0477	-0.0280	0.0269

Appendix W continued

Bacteria	PC13	PC14	PC15	PC16
Megamonas	-0.0163	-0.0331	-0.0747	-0.1956
Pediococcus	0.0496	0.1326	-0.0176	-0.0335
Fusobacterium	0.0134	-0.0106	-0.2238	0.1572
Bacteroides	0.1958	0.1681	0.1626	-0.3161
Prevotella	0.1167	-0.1421	-0.1035	-0.0505
Blautia	0.1066	-0.1868	0.1835	0.1081
Streptococcus	0.1580	0.0203	0.0421	-0.1230
Phascolarctobacterium	-0.2723	-0.0268	-0.2517	-0.0111
Turicibacter	0.3428	0.3223	-0.1121	0.0855
Clostridium	-0.3585	-0.2119	0.1938	-0.1646
Ruminococcus	0.2739	-0.0835	-0.1146	0.2428
Dorea	-0.1147	0.1029	-0.1526	-0.4594
Sutterella	-0.1479	0.1504	-0.1090	0.2981
Eubacterium	-0.2082	0.4783	-0.2270	0.0850
Collinsella	0.2489	0.0226	0.4052	0.2813
Lactobacillus	-0.1826	0.1486	0.0464	-0.0610
Allobaculum	0.2215	-0.0214	-0.5038	-0.1550
Slackia	0.1963	0.0755	-0.0550	-0.0681
Catenibacterium	0.0259	0.4312	-0.0495	0.1524
Faecalibacterium	0.0559	0.0524	0.0535	-0.1819
Coprococcus	0.1315	-0.0509	0.0429	-0.1388
Peptococcus	0.0987	-0.0654	0.1208	-0.3163
Anaerobiospirillum	-0.3927	-0.0755	-0.0520	0.0607
Oscillospira	0.0599	0.0400	0.1480	-0.1032
Enterococcus	0.1174	-0.3040	-0.3634	-0.0451
Vagococcus	0.0671	-0.2017	-0.1938	0.1057
Lactococcus	0.1305	-0.3014	-0.1114	0.1366
Roseburia	0.0745	0.0561	0.0021	-0.0996
Helicobacter	0.0752	0.0830	-0.0123	-0.1291
Epulopiscium	0.0266	-0.0418	-0.0789	-0.0810
Corynebacterium	0.0734	0.0553	0.0049	-0.0883
Actinomyces	0.0716	0.0586	0.0029	-0.0935
Coprobacillus	0.0632	0.0514	0.0001	-0.1063
Mucispirillum	0.0452	0.0515	0.0103	-0.1085

Appendix W continued

Bacteria	PC17	PC18	PC19	PC20
Megamonas	0.1990	-0.0059	0.0534	-0.1500
Pediococcus	0.0863	0.0817	0.0181	-0.1170
Fusobacterium	0.0551	0.0344	-0.1673	0.2570
Bacteroides	-0.2286	-0.1423	-0.0483	-0.0834
Prevotella	-0.1264	0.0261	-0.0321	0.0161
Blautia	0.3839	-0.1469	-0.0039	-0.2876
Streptococcus	0.1009	-0.1348	0.1273	-0.1457
Phascolarctobacterium	0.0020	-0.1554	-0.0392	0.0758
Turicibacter	0.0384	0.0682	-0.1077	-0.0639
Clostridium	0.1844	-0.1412	0.0809	-0.1273
Ruminococcus	0.0023	0.0637	-0.2720	0.1886
Dorea	-0.2469	-0.0121	-0.0421	0.0318
Sutterella	0.3597	0.2598	0.1592	-0.3168
Eubacterium	-0.0681	0.1247	-0.2586	-0.1931
Collinsella	-0.3216	0.1585	0.2630	-0.0404
Lactobacillus	-0.1730	0.1645	0.0567	0.0001
Allobaculum	0.1009	0.1139	0.5547	-0.0212
Slackia	-0.0225	0.0172	0.1462	-0.3766
Catenibacterium	0.2152	-0.4377	0.1481	0.3446
Faecalibacterium	0.2815	-0.1739	0.0437	0.2635
Coprococcus	0.2542	0.3231	-0.4791	-0.0637
Peptococcus	0.3010	0.4699	0.0730	0.3934
Anaerobiospirillum	-0.0282	0.0729	-0.0019	-0.0605
Oscillospira	-0.0160	-0.0894	0.0306	-0.0277
Enterococcus	-0.2150	0.0275	-0.0334	-0.1123
Vagococcus	0.0439	-0.0884	-0.0142	0.0420
Lactococcus	0.0001	-0.3175	-0.1582	-0.0470
Roseburia	0.0502	-0.0973	-0.1001	-0.0987
Helicobacter	0.0628	-0.1263	-0.1046	-0.0687
Epulopiscium	-0.0115	-0.0419	-0.1097	-0.1339
Corynebacterium	0.0610	-0.0965	-0.1044	-0.0858
Actinomyces	0.0551	-0.0989	-0.0873	-0.0865
Coprobacillus	0.0696	-0.0934	-0.1261	-0.1109
Mucispirillum	0.0385	-0.0732	-0.0828	-0.1222

Bacteria	PC21	PC22	PC23	PC24
Megamonas	0.0853	0.1151	0.0353	-0.0648
Pediococcus	0.0046	0.0248	0.0130	-0.0699
Fusobacterium	-0.1725	0.1458	-0.1098	0.0915
Bacteroides	0.1986	-0.0680	0.0530	-0.0718
Prevotella	-0.0564	0.0162	-0.0366	-0.0279
Blautia	-0.0087	-0.2008	0.0515	-0.0697
Streptococcus	0.0505	-0.0397	0.0349	-0.0350
Phascolarctobacterium	-0.0425	-0.0170	0.0186	0.0062
Turicibacter	0.1030	0.0099	0.0096	-0.1272
Clostridium	-0.0133	0.0128	-0.0474	0.0593
Ruminococcus	-0.1549	-0.1577	-0.1391	-0.0317
Dorea	0.1398	0.0687	0.2128	-0.1257
Sutterella	0.1640	-0.0240	0.1413	-0.2542
Eubacterium	0.1597	0.0411	-0.2828	-0.1601
Collinsella	0.0552	0.1677	0.1409	-0.2534
Lactobacillus	0.0134	0.1049	0.0945	-0.0369
Allobaculum	-0.0385	0.2681	-0.0417	0.1496
Slackia	-0.2257	-0.0682	-0.0963	0.2995
Catenibacterium	0.1222	-0.2061	0.4348	0.1988
Faecalibacterium	-0.3515	0.2603	-0.0352	-0.6541
Coprococcus	-0.0986	0.3495	0.5259	0.2230
Peptococcus	0.3943	-0.3190	-0.1986	-0.0091
Anaerobiospirillum	0.0227	-0.0006	0.1265	-0.1190
Oscillospira	-0.0932	0.2587	-0.0463	0.0474
Enterococcus	-0.0499	-0.4193	0.4107	-0.3237
Vagococcus	0.1239	0.1383	-0.1005	-0.1184
Lactococcus	0.6364	0.3350	-0.0583	-0.0237
Roseburia	-0.0446	-0.0440	-0.1218	-0.0476
Helicobacter	-0.0583	-0.0255	-0.1404	-0.0077
Epulopiscium	-0.0975	-0.2295	-0.1316	-0.0315
Corynebacterium	-0.0122	-0.0497	-0.0659	-0.0592
Actinomyces	-0.0033	-0.0513	-0.0715	-0.0569
Coprobacillus	-0.0195	-0.0237	-0.0587	-0.0144
Mucispirillum	0.0483	-0.0287	0.0106	0.0544

Bacteria	PC25	PC26	PC27	PC28
Megamonas	-0.0958	0.0168	-0.0188	0.0063
Pediococcus	-0.0467	0.0354	0.0009	0.0089
Fusobacterium	-0.3072	0.0109	0.0406	-0.1136
Bacteroides	0.2158	-0.0523	-0.0616	0.1126
Prevotella	-0.1359	-0.0230	0.0123	-0.0092
Blautia	0.0391	0.0721	-0.0994	0.0219
Streptococcus	0.2322	0.0212	0.0122	0.0273
Phascolarctobacterium	0.0045	0.0397	-0.0079	-0.0028
Turicibacter	-0.1436	0.0060	-0.0424	-0.0032
Clostridium	-0.0888	-0.0166	0.0435	0.0048
Ruminococcus	-0.1108	0.0911	-0.0420	-0.0505
Dorea	-0.3243	-0.0723	-0.0974	0.0208
Sutterella	-0.2356	0.0553	-0.0534	0.0145
Eubacterium	0.3919	0.0418	0.0086	-0.0569
Collinsella	-0.1961	-0.0389	0.0738	0.1138
Lactobacillus	-0.2343	-0.0296	0.0018	0.0146
Allobaculum	0.0522	0.1549	0.1103	0.1232
Slackia	-0.0005	-0.3987	0.0526	-0.3056
Catenibacterium	-0.0514	-0.0205	-0.0244	0.0535
Faecalibacterium	0.1328	-0.1763	0.1800	-0.1225
Coprococcus	0.1537	-0.0415	0.0598	0.1451
Peptococcus	-0.0156	-0.0003	-0.0345	-0.0950
Anaerobiospirillum	-0.0796	-0.0098	-0.0108	-0.0148
Oscillospira	-0.0099	0.6691	-0.4895	-0.1669
Enterococcus	0.1222	0.1583	-0.0276	-0.2024
Vagococcus	0.0480	-0.4298	-0.6889	0.3189
Lactococcus	-0.0355	0.0415	0.3217	-0.1426
Roseburia	-0.1596	0.1348	0.0141	-0.0203
Helicobacter	-0.1889	0.0278	0.1094	0.3290
Epulopiscium	-0.1526	0.1328	0.2106	0.6052
Corynebacterium	-0.1290	0.0671	0.0275	-0.1199
Actinomyces	-0.1749	0.0734	0.0424	-0.0164
Coprobacillus	-0.1992	-0.0841	-0.1511	-0.0662
Mucispirillum	-0.2866	-0.1945	-0.0862	-0.3344

Bacteria	PC29	PC30	PC31
Megamonas	0.0338	-0.0222	0.0048
Pediococcus	-0.0145	-0.0027	-0.0064
Fusobacterium	0.0462	-0.0195	0.0098
Bacteroides	-0.0261	-0.0188	-0.0082
Prevotella	-0.0017	-0.0170	-0.0039
Blautia	-0.0100	0.0350	-0.0058
Streptococcus	-0.0712	0.0347	-0.0145
Phascolarctobacterium	-0.0080	0.0045	-0.0039
Turicibacter	0.0737	-0.0318	0.0116
Clostridium	-0.0415	-0.0016	-0.0117
Ruminococcus	0.0226	0.0042	0.0010
Dorea	0.1374	-0.0835	0.0243
Sutterella	0.1050	-0.0105	0.0184
Eubacterium	-0.0544	0.0471	-0.0054
Collinsella	-0.0837	0.0273	-0.0258
Lactobacillus	0.0707	-0.0367	0.0149
Allobaculum	-0.2492	0.0608	-0.0711
Slackia	0.4838	-0.1838	0.1368
Catenibacterium	0.0703	-0.0635	0.0267
Faecalibacterium	0.1140	-0.0466	0.0404
Coprococcus	-0.0370	-0.0313	-0.0002
Peptococcus	0.1232	-0.0155	0.0368
Anaerobiospirillum	0.0990	-0.0267	0.0241
Oscillospira	0.2790	-0.0283	0.0341
Enterococcus	0.0563	0.1193	0.0358
Vagococcus	0.0412	-0.1148	-0.0184
Lactococcus	0.1734	-0.0391	0.0606
Roseburia	-0.1325	-0.1658	0.3665
Helicobacter	0.3552	0.6867	-0.0170
Epulopiscium	0.0731	-0.3122	-0.0541
Corynebacterium	-0.0195	-0.2716	-0.8192
Actinomyces	-0.2668	-0.3346	0.3587
Coprobacillus	-0.4376	0.2817	0.0711
Mucispirillum	-0.2697	0.2160	-0.1432

Appendix W continued

PCA scores

Diet/Dog No.	PC1	PC2	PC3	PC4
WF1	-6.9412	1.1691	-3.4571	1.9463
WF2	-6.0916	1.6302	-4.2713	2.0443
WF3	-6.4326	0.4245	-3.7191	-1.2101
WF6	-6.6157	0.3073	-5.3387	-0.1069
WF4	-7.1029	1.6523	-1.0292	2.0679
WF5	-8.1520	1.0472	-2.8251	-1.5428
WF7	-6.9975	-0.0481	-4.2549	-0.0877
WF8	-7.2077	0.7261	-3.8561	2.6144
DF2	-7.3071	3.7739	2.4980	0.3390
DF4	-7.2560	1.7141	2.3774	-0.4242
DF6	-7.8220	2.6958	3.0199	0.6141
DF7	-6.6864	3.5888	3.8794	-0.4808
DF1	-7.1627	3.4313	2.5316	2.3290
DF3	-6.4725	2.5822	4.4547	-0.2893
DF5	-6.7362	3.2357	3.1129	1.0247
DF8	-6.8077	1.1138	1.3612	-5.9135
ADF2	-2.2523	-4.5954	2.1277	-0.8510
ADF3	-8.3780	1.6406	-1.8671	0.7526
ADF5	-6.8941	-0.5287	-1.8492	0.4564
ADF6	-6.9838	1.4343	0.7505	-1.8607
ADF1	-7.5044	-4.4280	0.2979	1.1359
ADF4	-4.4376	-1.0461	-1.1362	-4.3730
ADF7	-8.0077	0.4607	0.3772	-3.5314
ADF8	-5.7168	0.0229	-2.7133	-4.1072
RF3	-6.7421	-1.0895	4.3642	1.8152
RF5	-4.0516	-5.8259	1.2710	0.8203
RF6	-7.8104	-4.1085	0.6275	2.3071
RF1	-7.4346	-4.8304	1.3117	1.1502
RF2	-7.5044	-4.4280	0.2979	1.1359
RF4	-8.0329	-3.9804	1.5522	1.3151
RF7	-3.3023	-4.6350	1.4000	-4.0509

Appendix W continued

Diet/Dog No.	PC5	PC6	PC7	PC8
WF1	-0.8335	2.3206	-0.3302	0.5865
WF2	1.8574	1.0903	-1.9066	0.2751
WF3	3.0341	-0.3402	2.4399	-1.4447
WF6	1.9848	-0.2224	0.8250	0.2918
WF4	-1.9012	0.9538	-1.9792	0.1876
WF5	-0.0815	0.5659	0.0556	1.1363
WF7	-0.3007	-1.7078	-0.5488	0.8392
WF8	-0.5051	0.1021	0.4445	0.2831
DF2	2.5191	0.5830	-0.8182	-0.4393
DF4	-3.4813	-1.0565	0.1857	0.2737
DF6	-0.6185	-0.6679	0.9087	-1.4607
DF7	1.6366	2.4316	1.5492	2.2742
DF1	0.7573	-1.4564	0.7501	-1.5852
DF3	-0.5706	0.8737	-0.2921	2.4135
DF5	0.8427	0.5468	-2.3010	-1.5683
DF8	-0.7383	-0.6223	1.5903	0.0651
ADF2	1.2159	1.6196	-3.4050	-1.4214
ADF3	0.8566	1.4398	1.2257	-0.8889
ADF5	-4.6804	0.8956	0.1081	0.4683
ADF6	0.1616	-3.1889	-2.2668	0.5516
ADF1	0.5963	-0.5259	1.0669	0.0784
ADF4	-2.4893	4.2394	0.5603	-2.3904
ADF7	-1.5655	-1.7985	0.2926	-0.6004
ADF8	1.4621	-0.9381	-3.1628	0.2661
RF3	2.0481	1.2432	0.4768	-0.1770
RF5	-0.2394	2.7805	-0.3224	0.8214
RF6	0.3693	-2.0575	0.9473	-0.2108
RF1	-0.7567	-0.9683	-0.4112	-0.2587
RF2	0.5963	-0.5259	1.0669	0.0784
RF4	-1.5238	-1.2746	-0.2460	0.0020
RF7	2.9499	0.4635	0.2431	1.2448

Diet/Dog No.	PC9	PC10	PC11	PC12
WF1	-0.4447	0.0369	-0.6416	-0.2625
WF2	-1.7207	0.2496	-0.4592	0.5959
WF3	2.3025	0.5778	0.6860	-0.4697
WF6	0.4102	0.8095	0.3401	0.1493
WF4	-0.7834	-0.2351	-0.2678	0.4155
WF5	-1.3061	0.7440	-0.7285	-0.9235
WF7	-0.4808	0.5484	1.7470	-0.3212
WF8	0.6018	0.8748	-0.0794	0.3384
DF2	-0.1329	-0.6693	0.9188	0.5257
DF4	-0.3000	1.1500	-0.7610	-0.0460
DF6	0.7933	0.3795	-0.6845	-0.2471
DF7	0.2648	-0.7689	0.0783	0.4678
DF1	-1.1086	-0.6510	-0.4354	-1.2336
DF3	1.2260	-0.5340	1.3232	-0.3994
DF5	-0.1611	-0.1163	1.2102	0.0953
DF8	-2.1838	0.3700	0.2214	-1.1231
ADF2	-0.7005	1.4428	1.0719	-1.0272
ADF3	-0.2897	-1.4068	0.0043	0.5730
ADF5	0.9308	0.1411	0.3228	-0.4313
ADF6	1.4949	1.7832	-0.3237	1.2144
ADF1	-0.6360	-0.3035	0.1231	-0.5202
ADF4	0.3796	-0.1216	-0.0396	1.2391
ADF7	0.1655	-0.1673	0.3811	0.4451
ADF8	1.7683	-2.3735	-1.6064	-1.0676
RF3	0.7315	2.1777	-1.9328	-0.1066
RF5	1.7228	0.2370	0.3868	-1.0459
RF6	0.0899	-0.6114	0.3648	0.6699
RF1	-0.1658	-1.7100	-0.7577	0.2795
RF2	-0.6360	-0.3035	0.1231	-0.5202
RF4	-0.1342	-0.5471	0.3380	1.0389
RF7	-1.3079	0.3238	-0.3370	1.3584

Diet/Dog No.	PC13	PC14	PC15	PC16
WF1	0.6970	-0.3478	-0.2916	0.0262
WF2	-0.0330	-0.0217	0.9092	0.2215
WF3	0.6632	0.0719	0.1697	0.3812
WF6	-0.7823	-0.2484	0.6065	-0.2401
WF4	0.4540	0.0102	-0.4236	-0.3192
WF5	0.2917	-0.0508	-0.1718	-0.1608
WF7	-1.3198	0.4759	-0.6399	0.4308
WF8	-0.6283	-0.3010	0.5966	-0.3039
DF2	0.4306	0.5690	-0.3171	-0.0761
DF4	0.4544	0.4371	0.8713	1.5241
DF6	-0.0203	-0.2946	0.1075	0.5575
DF7	-0.5735	1.0437	-0.1667	0.2231
DF1	-1.5866	-1.0870	-0.4710	0.2777
DF3	-0.4059	-0.7184	0.9845	-0.4097
DF5	0.5251	-0.2491	-0.2026	0.1406
DF8	0.1076	-0.1525	0.0115	-0.8902
ADF2	-0.2405	0.5857	0.3716	0.1136
ADF3	1.2007	-0.3448	-0.3864	-0.0289
ADF5	-0.2002	0.4494	-1.0896	0.1518
ADF6	0.3941	-0.5824	-0.7268	-0.5708
ADF1	0.8972	0.3458	0.0271	-0.1139
ADF4	-1.2066	0.0378	0.1374	-0.1897
ADF7	0.8424	0.1576	0.4956	-0.2642
ADF8	-0.2902	0.4583	0.0616	0.2694
RF3	-0.4308	0.6777	-0.2109	-0.6966
RF5	0.3823	-1.1051	-0.2400	0.2692
RF6	-0.7766	0.7349	-0.2685	0.0002
RF1	-0.7325	-0.1798	0.4259	-0.4853
RF2	0.8972	0.3458	0.0271	-0.1139
RF4	0.1617	-0.2657	0.4304	-0.0569
RF7	-0.2472	-0.9105	-0.5923	1.0600

Diet/Dog No.	PC17	PC18	PC19	PC20
WF1	0.5664	0.8280	-0.5678	-0.0416
WF2	-0.8859	-0.5653	0.1578	0.1315
WF3	-0.0518	0.3982	0.3179	0.0095
WF6	0.6549	0.0582	0.2523	-0.0225
WF4	-0.0135	0.6257	0.2263	0.5063
WF5	0.2196	-0.4248	-0.1636	-0.6515
WF7	0.3475	-0.4128	-0.4264	0.3475
WF8	-0.2619	0.3288	0.0127	-0.1786
DF2	0.1299	-0.0007	0.0520	-0.4865
DF4	0.1684	-0.0489	0.1645	0.0774
DF6	0.0590	0.1051	-0.3984	-0.0936
DF7	-0.3777	0.2212	-0.0347	-0.0064
DF1	-0.5221	0.1291	0.0968	0.0303
DF3	-0.0252	0.1151	0.0182	0.1288
DF5	0.5066	-0.2869	0.0176	-0.1009
DF8	0.0433	-0.0034	0.1825	0.1213
ADF2	-0.0279	0.4947	0.1282	0.0047
ADF3	0.2381	-0.5581	0.1897	0.3789
ADF5	-0.1542	-0.0715	0.6719	-0.2044
ADF6	-0.6648	0.1193	-0.0636	-0.0928
ADF1	-0.5825	0.1528	-0.1308	-0.0474
ADF4	-0.4265	-0.0660	-0.2714	-0.0933
ADF7	0.0477	-0.0574	-0.2549	0.3669
ADF8	-0.0204	-0.0806	-0.0459	0.0303
RF3	0.4998	-0.4625	0.0505	0.3054
RF5	-0.2114	-0.5744	-0.1479	0.1369
RF6	-0.0803	0.0078	-0.2083	0.0808
RF1	0.5943	0.2597	0.2561	-0.0771
RF2	-0.5825	0.1528	-0.1308	-0.0474
RF4	0.3013	-0.3616	0.0334	-0.3994
RF7	0.2225	0.3430	0.1831	0.0429

Diet/Dog No.	PC21	PC22	PC23	PC24
WF1	-0.2183	0.1394	0.2491	0.0671
WF2	-0.1585	0.2172	0.0321	-0.0126
WF3	-0.1621	0.2217	0.0136	-0.0641
WF6	0.3090	-0.3206	-0.0803	0.0815
WF4	0.3512	0.0547	-0.3326	-0.0567
WF5	-0.1157	-0.0421	-0.1370	-0.1895
WF7	-0.0791	-0.0342	-0.0182	-0.0463
WF8	0.2520	0.0739	0.0627	0.1016
DF2	0.2875	0.1753	-0.0530	-0.1856
DF4	0.1291	-0.1984	0.1195	-0.0437
DF6	0.0195	-0.0519	-0.3016	-0.0150
DF7	0.2294	-0.0922	0.1855	0.0377
DF1	-0.0152	0.0806	0.1085	-0.0218
DF3	-0.4943	-0.0204	-0.1588	-0.0251
DF5	-0.0097	-0.1128	0.0452	0.2664
DF8	0.2360	0.0665	0.0818	0.1159
ADF2	-0.1074	-0.0778	0.0921	-0.0864
ADF3	-0.2082	-0.3211	0.1043	-0.0821
ADF5	-0.2477	0.1348	-0.0070	0.0635
ADF6	-0.0447	-0.2595	0.1533	-0.0535
ADF1	-0.0741	-0.1967	-0.0942	0.0895
ADF4	-0.0702	-0.1259	-0.0532	0.0001
ADF7	0.0472	0.3678	0.0787	-0.0951
ADF8	0.0370	0.0155	-0.0410	0.1325
RF3	-0.1756	0.1487	-0.0330	0.0527
RF5	0.5191	0.0772	0.0794	-0.0841
RF6	0.0088	0.0733	-0.0029	-0.0152
RF1	-0.0474	-0.1423	0.1472	-0.2226
RF2	-0.0741	-0.1967	-0.0942	0.0895
RF4	0.0414	0.2619	0.0061	0.1719
RF7	-0.1274	0.1209	-0.1119	0.0464

Diet/Dog No.	PC25	PC26	PC27	PC28
WF1	0.1191	0.0383	0.0305	0.0180
WF2	0.1215	0.0972	-0.0305	-0.0086
WF3	-0.0938	0.1194	0.0008	-0.0023
WF6	0.1023	-0.0136	0.0625	0.0124
WF4	-0.1056	0.0472	0.0281	-0.0029
WF5	-0.1412	-0.0339	-0.0093	-0.0123
WF7	-0.0997	0.0614	-0.0061	-0.0044
WF8	-0.0965	-0.1764	-0.0921	-0.0104
DF2	0.1234	-0.0339	0.0231	0.0675
DF4	0.0084	0.0025	0.0238	0.0559
DF6	0.1643	0.0362	-0.0544	-0.0963
DF7	-0.1123	0.0396	-0.0018	-0.0688
DF1	-0.0899	-0.0413	0.0648	0.0205
DF3	0.0502	-0.0249	0.0152	0.0284
DF5	-0.1042	0.0413	-0.0884	0.0336
DF8	0.1623	0.0637	-0.0251	-0.0127
ADF2	0.0288	-0.0840	0.0293	-0.0684
ADF3	0.0420	-0.1200	0.0228	-0.0515
ADF5	0.0954	-0.0281	-0.0234	-0.0069
ADF6	0.0292	0.0433	0.0178	-0.0068
ADF1	-0.0594	0.0040	0.0028	0.0326
ADF4	-0.0383	0.0310	0.0226	0.0416
ADF7	-0.1119	-0.1123	-0.0121	0.0016
ADF8	0.0149	-0.0395	0.0127	0.0052
RF3	-0.0468	-0.0096	0.0130	0.0284
RF5	0.0468	0.0270	-0.0106	0.0101
RF6	0.2489	-0.0527	-0.0170	0.0215
RF1	-0.0624	0.1145	-0.0815	0.0000
RF2	-0.0594	0.0040	0.0028	0.0326
RF4	-0.0806	0.0478	0.1124	-0.0672
RF7	-0.0114	-0.0545	-0.0327	0.0105

Appendix W continued

Diet/Dog No.	PC29	PC30
WF1	0.0050	0.0009
WF2	0.0121	-0.0028
WF3	-0.0233	-0.0046
WF6	0.0372	-0.0176
WF4	-0.0183	-0.0087
WF5	-0.0164	-0.0282
WF7	-0.0006	0.0262
WF8	-0.0220	0.0234
DF2	0.0082	0.0290
DF4	-0.0161	0.0042
DF6	0.0180	0.0090
DF7	0.0088	-0.0129
DF1	0.0077	-0.0066
DF3	-0.0101	0.0008
DF5	-0.0017	-0.0224
DF8	-0.0206	0.0115
ADF2	-0.0018	-0.0016
ADF3	-0.0159	0.0110
ADF5	0.0269	-0.0012
ADF6	-0.0021	-0.0003
ADF1	0.0140	0.0082
ADF4	-0.0061	0.0008
ADF7	0.0415	-0.0186
ADF8	-0.0081	0.0059
RF3	0.0018	0.0096
RF5	0.0006	-0.0042
RF6	-0.0352	-0.0376
RF1	0.0114	0.0073
RF2	0.0140	0.0082
RF4	-0.0124	0.0092
RF7	0.0041	0.0037

Abbreviations – WF – canned wet; DF – dry kibble; ADF – air-dried; RF – mildly cooked/raw

Appendix X

Principal component analysis (PCA) loadings and scores at

genus taxa level for air-dried and mildly cooked/raw diets

PCA Loadings

Bacteria	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**
Pediococcus	-0.7164	0.0879	-0.0565	-0.0227	-0.1959	0.0173	-0.0266
Pseudomonas	-0.0922	-0.5626	0.3639	0.2170	-0.2341	0.1070	-0.0277
Delftia	-0.0951	-0.4810	-0.3750	0.0777	0.3355	-0.3845	0.3243
Carnobacterium	-0.0117	-0.3222	0.0753	0.0035	0.0462	-0.1554	-0.1187
Lactococcus	-0.0668	-0.2719	0.0886	-0.6531	0.3705	0.4881	-0.2613
Macrococcus	0.0058	-0.2583	-0.4236	0.0223	-0.2942	0.2090	-0.0755
Acinetobacter	0.0261	-0.2204	0.0247	0.0714	-0.1470	0.0589	0.0792
Psychrobacter	0.0374	-0.1860	-0.2029	-0.0112	0.0973	-0.0858	-0.1692
Brochothrix	0.0358	-0.2257	0.4171	0.0918	0.0840	-0.1129	0.0127
Geobacillus	0.0619	-0.1187	-0.2728	0.0047	-0.1955	0.1520	-0.0172
Vagococcus	0.0563	-0.0715	0.0407	-0.2996	-0.4338	-0.2329	-0.1704
Bacillus	0.0681	-0.1025	-0.2651	0.0068	-0.2487	0.1914	0.0183
Leuconostoc	0.0691	-0.0804	0.0683	-0.1768	-0.2515	-0.0347	0.1253
Streptococcus	0.0746	-0.1011	0.0981	0.0704	-0.0450	0.0085	-0.1177
Serratia	0.0748	-0.1145	0.2083	0.0687	0.0155	-0.0067	0.0305
Lactobacillus	0.0776	0.0022	0.0591	-0.5670	-0.2245	-0.5022	0.1288
Corynebacterium	0.0866	-0.0526	-0.2923	-0.0497	0.1601	-0.0941	-0.1702
Clostridium	0.0958	0.0272	-0.0310	0.2122	-0.0488	-0.2461	-0.7766
Myroides	0.1048	-0.0246	0.0429	0.0369	-0.1669	0.1399	0.1075
Cloacibacterium	0.1073	-0.0122	-0.0424	-0.0054	0.1177	-0.0648	-0.0175
Enterococcus	0.1002	-0.0128	-0.0701	-0.0245	-0.0423	-0.0470	-0.1360
Erwinia	0.1092	-0.0119	0.0241	0.0157	-0.0135	0.0321	0.0314
Kocuria	0.1120	-0.0011	-0.0319	0.0022	-0.0133	0.0333	0.0185
Peptoniphilus	0.1104	-0.0088	0.0205	0.0076	0.0578	-0.0182	-0.0034
Janthinobacterium	0.1111	-0.0072	0.0247	0.0090	0.0461	-0.0097	0.0043
Chryseobacterium	0.1128	0.0006	0.0070	-0.0048	-0.0064	0.0130	0.0275
Staphylococcus	0.1143	0.0035	-0.0042	0.0078	-0.0299	0.0454	0.0373
Providencia	0.1120	-0.0049	0.0269	0.0105	0.0284	0.0031	0.0147
Salinicoccus	0.1107	-0.0019	-0.0714	-0.0062	-0.0007	0.0244	-0.0003
Enterobacter	0.1137	0.0011	0.0053	0.0057	0.0185	0.0107	0.0154

Appendix X continued

Bacteria	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Wautersiella	0.1153	0.0056	0.0013	0.0071	-0.0177	0.0369	0.0339
Rummeliibacillus	0.1140	0.0013	0.0136	0.0072	0.0189	0.0104	0.0179
Prevotella	0.1132	0.0002	0.0028	0.0177	-0.1131	0.1042	0.0791
Weissella	0.1147	0.0124	-0.0005	-0.0475	-0.0221	-0.0207	0.0364
Shewanella	0.1141	0.0028	-0.0003	0.0145	-0.0925	0.0898	0.0690
Peptostreptococcus	0.1106	-0.0102	0.0476	0.0064	0.1299	-0.0696	-0.0311
Microbacterium	0.1159	0.0079	-0.0066	0.0042	-0.0091	0.0311	0.0282
Sphingomonas	0.1142	0.0020	0.0115	0.0033	0.0548	-0.0149	-0.0001
Photobacterium	0.1164	0.0090	-0.0055	0.0050	-0.0181	0.0375	0.0334
Oribacterium	0.1162	0.0083	-0.0035	0.0050	-0.0119	0.0331	0.0308
Megamonas	0.1144	0.0105	-0.0084	-0.0014	-0.0374	0.0598	0.0426
Flavobacterium	0.1164	0.0111	-0.0070	-0.0087	-0.0222	0.0254	0.0354
Enhydrobacter	0.1166	0.0099	-0.0111	0.0031	-0.0124	0.0336	0.0293
Brachybacterium	0.1162	0.0087	-0.0104	0.0053	-0.0303	0.0462	0.0378
Bacteroides	0.1157	0.0070	-0.0028	0.0032	0.0121	0.0159	0.0186
Fusobacterium	0.1159	0.0101	-0.0100	-0.0031	-0.0197	0.0475	0.0336
Rhodococcus	0.1168	0.0106	-0.0114	0.0021	-0.0034	0.0273	0.0250
Luteococcus	0.1168	0.0116	-0.0130	-0.0043	-0.0130	0.0266	0.0295
Leucobacter	0.1166	0.0098	-0.0094	0.0021	0.0027	0.0229	0.0223
Anoxybacillus	0.1161	0.0093	-0.0225	0.0009	-0.0099	0.0319	0.0243

PCA scores

Diet Sample	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Week 1 Air dried	-7.6266	-5.9381	2.0555	0.0033	0.5233	-0.2259	-0.0145
Week 3 Air-dried	-8.1723	-5.0698	0.3553	0.2303	-0.7674	0.3224	0.0230
Week 4 Air-dried	-8.3766	-3.6171	-3.1253	-0.1600	0.2434	-0.1049	-0.0138
Week 3 Mildly cooked/Raw	-8.8025	3.6046	0.1292	0.7497	-0.1552	-0.1935	-0.1943
Week 2 Mildly cooked/Raw	-8.9584	3.2425	0.2631	-0.2255	0.4438	0.6486	-0.0095
Week 4 Mildly cooked/Raw	-8.8772	3.4681	0.0077	0.7558	0.0278	-0.2083	0.1972
Week 1 Mildly cooked/Raw	-8.7026	2.9248	0.4639	-1.3623	-0.3005	-0.2632	0.0095

Appendix Y

Log2 fold change between air-dried (ADF) and mildly cooked/raw (RF) diets

Bacteria	**Fold Change**	**log2(FC)**
Pseudomonas	836	9.7074
Serratia	289.33	8.1766
Psychrobacter	192	7.585
Brochothrix	111.24	6.7975
Carnobacterium	85.867	6.424
Delftia	82.213	6.3613
Cloacibacterium	57.333	5.8413
Acinetobacter	57	5.8329
Streptococcus	37.524	5.2297
Chryseobacterium	12	3.585
Lactococcus	6.1994	2.6321
Leuconostoc	6.0333	2.593
Enterococcus	4.75	2.2479
Vagococcus	3.187	1.6722
Fusobacterium	2.6667	1.415
Pediococcus	0.44098	-1.1812
Clostridium	0.46465	-1.1058

Appendix Z

Firmicutes to *Bacteroidetes* ratio for fecal microbiota samples

Diet	***Firmicutes***	***Bacteroidetes***	**Ratio**
Canned Wet	71.09	14.85	4.79
	72.03	16.51	4.36
	38.92	29.72	1.31
	77.97	11.25	6.93
	63.55	17.82	3.57
	51.93	21.39	2.43
	52.3	21.28	2.46
	49.27	31.81	1.55
Dry Kibble	64.12	27.56	2.33
	72.42	21.42	3.38
	87.54	7.99	10.96
	78.01	8.33	9.36
	82.71	11.5	7.19
	70.99	18.17	3.91
	75.6	18.9	4.00
	85.08	4.27	19.93
Air Dried	57.38	24.6	2.33
	98.27	0.1	982.70
	53.29	26.61	2.00
	88.18	0.03	2939.33
	64.29	17.98	3.58
	54.36	15.49	3.51
	57.96	14.32	4.05
	58.02	14.87	3.90
Mildly cooked/Raw	67.3	14.95	4.50
	57.38	24.6	2.33
	66.5	22.44	2.96
	64	16.96	3.77
	90.4	3.96	22.83
	48.2	28.87	1.67
	85.36	0.17	502.12

Appendix AA

Bacterial metabolic degradation of substrates across diets based on phylogenetic classification.

Diet	Sample No.	Utilize Carbohydrates	Xylan Degrader	Amino acid Degrader	Saccharolytic	Cellulose Degrader	Chitin Degradation
Canned Wet	1	0	9.46	0	0	5.33	2
	2	0	8.16	0	0	1.15	4.87
	3	0	13.45	0	0	2.47	1.6
	4	0	8.88	0	0	3.39	0.9
	5	0	14.58	0	0	6.41	4.47
	6	0	16.08	0	0	2.35	7.32
	7	0	18.32	0	0	2.51	3.05
	8	0	25.02	0	0	2.21	3.62
Dry Kibble	1	0	4.25	0	0	6.42	0.77
	2	0	6.68	0	0	2.69	0.13
	3	0	5.17	0	0	1.38	0.13
	4	0	5.2	0	0	6.67	0.54
	5	0	9.52	0	0	6.67	0.43
	6	0	3.62	0	0	1.67	0.09
	7	0	8.82	0	0	3.08	0.09
	8	0	5.41	0	0	3.7	0.24
Air Dried	1	0	20	0	0	5	0.34
	2	0	0.42	0	0	1.86	1.71
	3	0	10.82	0	0	6.39	0.15
	4	0	2.3	0	0	3.95	0.7
	5	0	14.31	0	0	4.95	0.67
	6	0	15.48	0	0	2.59	5.77
	7	0	13.19	0	0	7.44	0.32
	8	0	7.36	0	0	2.2	3.33
Mildly cooked/ Raw	1	0	12.02	0	0	2.39	0.37
	2	0	20	0	0	5	0.34
	3	0	5.89	0	0	2.98	2.39
	4	0	16.72	0	0	2.06	0.69
	5	0	4.92	0	0	5.28	3.37
	6	0	26.76	0	0	2.12	0.2
	7	0	2.34	0	0	2.3	4.09