

NUTRITIONAL EVALUATION OF α -CYCLODEXTRIN
USING CANINE, HAMSTER, AND *IN VITRO* MODELS

BY

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DISSERTATION

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ABSTRACT

The main objective of this research was to characterize the nutritional effects of α -cyclodextrin (ACD) using *in vitro*, canine, and hamster models. Study 1 evaluated the composition of ACD, validated select fat analysis techniques as related to ACD-fat complexes, and evaluated the gastrointestinal tolerance, total tract nutrient digestibility, and blood lipid profiles of dogs as affected by ACD supplementation. Hydrolysis of ACD showed that it is effectively composed only of glucose. The acid hydrolyzed fat (AHF) method proved to be valid for measuring fat bound to ACD. Intake of ACD was shown to be generally well tolerated by dogs and did not alter body weight (BW), body condition scores (BCS), or fecal scores. Intake of 6 or 12 g ACD daily decreased apparent nutrient digestibility and numerically reduced serum cholesterol concentrations in hypercholesterolemic dogs, but failed to reduce serum cholesterol concentrations in normocholesterolemic dogs. Study 2 evaluated *in vitro* fermentation characteristics of α -, β -, and γ -cyclodextrins, and *in vivo* total tract and ileal nutrient digestibilities, fecal microbiota concentrations, and blood lipid profiles of dogs as affected by ACD supplementation. Maximal *in vitro* production of total SCFA was lowest for ACD. However, the greatest maximal production of propionate also was noted for ACD. Average daily food intake, BW gain, BCS, and ileal nutrient digestibility were not significantly different among treatments. Total tract nutrient digestibility and fecal dry matter (DM) concentration decreased linearly for treatment groups receiving ACD. Fecal output expressed on an as-is basis, on a DM basis, and on an as-is per g DM intake basis increased linearly ($P < 0.05$) with increasing ACD supplementation. Bifidobacteria, *Clostridium perfringens*, and *E. coli* concentrations were not different among treatments. Serum cholesterol and triglyceride concentrations were

within normal ranges for dogs and were not different among treatments. Study 3 evaluated apparent nutrient digestibility, cecal SCFA concentrations, cecal microbiota concentrations, blood lipid profiles, bile acid excretion, and liver gene expression of hamsters as affected by ACD and cholesterol intakes. Fecal bile acid excretion was increased by cholesterol intake, but not by ACD intake. Cecum and cecal content weight were increased by ACD intake, whereas cholesterol intake tended to decrease ($P=0.08$) cecal content weight expressed on a DM basis. Intake of ACD alone resulted in higher propionate concentrations in cecal contents. Total CFU of lactobacilli and total microbes in cecal contents were greater for hamsters fed ACD than for those not fed ACD. Intake of diets containing ACD tended to increase ($P=0.10$) expression of 7 α -dehydroxylase (CYP7A1), whereas intake of diets containing cholesterol resulted in a reduction in expression of hydroxymethylglutaryl (HMG)-CoA reductase. α -Cyclodextrin intake resulted in reduced cholesterol concentrations in serum of normocholesterolemic hamsters, but failed to reduce cholesterol concentrations in dietary-induced hypercholesterolemic hamsters. In summary, ACD intake varied in its ability to affect cholesterol metabolism, nutrient digestibility, fecal characteristics, and hindgut microbiota and fermentation patterns. This variation in response is due to differences in the model used to evaluate it, inclusion level in diet, and matrix where it is supplied. The hypocholesterolemic effect of ACD appears to be a combination of factors including up-regulation of cholesterol degradation through CYP7A1, changes in hindgut fermentation profiles, and, perhaps, changes in hindgut microbiota. However, further research is necessary to define the mechanisms for such up-regulation and microbiota effects on bile acid degradation and excretion.

DEDICATION

To my wife and family, who have supported me every step of the way.

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CHAPTER 1

INTRODUCTION

Obesity is a disorder often associated with type 2 diabetes, dyslipidemia, and cardiovascular disease (CVD). Risk factors for all of these conditions include sedentary lifestyle, high intake of saturated fats and cholesterol and, in general, a greater caloric intake than caloric expenditure. In Western societies, these factors are common and, thus, the increase in prevalence of these diseases in the United States. It has been estimated that between 66 and 70% of the U.S. adult population is either obese or overweight (Flegal et al., 2010). The sciences of genetics, physiology, neuroendocrinology, microbiology, epidemiology, and nutrition have contributed to an understanding of the possible causes, prevalence, and prospective treatments of obesity and related disorders. These related disorders include hyperlipidemia and diabetes type II; however, obesity and hyperlipidemia cannot be treated as the same pathology (Ceska et al., 2011).

Hyperlipidemia, also known as dyslipidemia, is defined as the elevation of plasma cholesterol and (or) triglycerides (TG), or a low HDL-cholesterol (HDL-C) concentration that contributes to the development of atherosclerosis. Hyperlipidemia has been clearly linked to the risk of CVD. Furthermore, a meta-analysis conducted in the U.S. population demonstrated that a major decline (44%) in deaths from CVD could be attributed to changes in risk factors (Bruckert and Rosenbaum, 2011). This has led to the search for safe and practical ways to either lose or maintain body weight (BW) and prevent risks associated with overweight. The main goals in this search include the reduction of lipid absorption from the gastrointestinal tract and normalization of blood lipid profiles. In order to reach these goals, drugs and nutritional interventions have been developed and studied.

Cholesterol-lowering medications have been very successful and are widely used. However, nutritional interventions provide unique opportunities to decrease CVD risk throughout life.

Among the nutritional interventions, plant sterols, polyunsaturated fatty acids (PUFA), and dietary fiber (DF) stand out as having sufficient scientific research to back their hypolipidemic effects. Plant sterols, also known as phytosterols, are plant compounds that have chemical structures similar to cholesterol. They interfere with the micellar solubilization of cholesterol in the intestine and reduce the efficiency of cholesterol absorption (Ostlund and Lin, 2006). However, dietary intake of plant sterols is usually too low to efficiently decrease LDL-C (Demonty et al., 2009). Intake of PUFA in place of saturated fatty acids has been shown to be an efficient dietary intervention for decreasing plasma concentrations of cholesterol (Clarke et al., 1997; Hodson et al., 2001; Czernichow et al., 2010). Brown et al. (1999), in a meta-analysis of the cholesterol-lowering effects of DF, reported that intakes of 2-10 g/d of soluble fiber were associated with small but significant decreases in total cholesterol (-0.045 mmol/L/g soluble fiber) and LDL-C (-0.057 mmol/L/g soluble fiber). The authors concluded that various soluble fibers reduced total cholesterol and LDL-C by similar amounts, but the effect was small within the practical range of fiber intakes. Therefore, the major limitation of some of the nutritional interventions is the high intake needed to significantly affect cholesterolemia. This limitation could be overcome by using nutritional supplements with higher concentrations of specific components.

A nutritional supplement, α -cyclodextrin (ACD), is being commercialized as a DF with the unique ability to bind nine times its weight in fat (Artiss et al., 2006).

Cyclodextrins are cyclic oligosaccharides comprised of glucopyranose units. The family of

cyclodextrins is very large, including several rare minor cyclic oligosaccharides, and the three major cyclodextrins, ACD with 6 glucose units, β -cyclodextrin (BCD) with 7, and γ -cyclodextrin (GCD) with 8 (Szejtli, 1998). Cyclodextrins are conical cylinders with hydrophilic sites on the exterior and hydrophobic sites on the interior of the cylinder. This structure makes them both soluble in water and capable of including other apolar molecules of the appropriate size, also called guest molecules, in the axial open cavity forming inclusion complexes (Biwer et al., 2002; Szejtli, 2004). Because complexation changes the physical and chemical properties of the hydrophobic molecule, cyclodextrins are currently used by many industries, including the pharmaceutical, cosmetic, household and toiletry, and food industries.

It has been reported that ACD binds fats, forming stable emulsions. This emulsion, or complex, is hypothesized to render fats indigestible and decrease weight gain and blood cholesterol concentrations (Artiss et al., 2006; Grunberger et al., 2007; Wagner et al., 2008; Comerford et al., 2011). However, feeding cyclodextrins to different experimental animal models has resulted in inconsistent hypolipidemic effects. Therefore, assessing the impact of ACD intake in proper practical settings is a pre-condition to accurate data interpretation and potential use of ACD in food products.

The major objectives of this research were to evaluate ACD capacity for binding fats, validate fat analysis techniques for assaying ACD-fat complexes, and quantify the effect of ACD intake on fecal characteristics, nutrient digestibilities, fermentation patterns, intestinal microbiota concentrations, cholesterol metabolism-related gene expression, and blood lipid profiles.

To accomplish these objectives, Study 1 was designed to evaluate the composition of ACD, validate select fat analysis techniques as they relate to ACD-fat complexes, and quantify gastrointestinal tolerance, total tract nutrient digestibility, and serum lipid profiles of dogs as affected by ACD supplementation. Once fat analysis techniques were validated, and doses of ACD that were well tolerated by dogs were defined, Study 2 was designed to evaluate *in vivo* total tract and ileal nutrient digestibilities, fecal microbiota concentrations, and serum lipid profiles of dogs as affected by ACD supplementation. Another objective was to evaluate *in vitro* fermentation characteristics of cyclodextrins. Based on observations from previous experiments and reports of the effect of ACD on blood cholesterol concentrations, Study 3 was designed to evaluate total tract nutrient digestibility, cecal SCFA concentrations, cecal microbiota concentrations, serum lipid profiles, and liver gene expression of hamsters as affected by ACD and cholesterol supplementation.

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CHAPTER 2

LITERATURE REVIEW

Carbohydrates are known as economical energy sources in animal and human nutrition. However, there is great interest in the relationship between carbohydrates and health because of the overwhelming evidence, from epidemiological and experimental studies, of the health benefits obtained from dietary fibers (DF), whole grains, and novel carbohydrates (Mann and Cummings, 2009).

Physiological outcomes of carbohydrate ingestion are critical. The definition of DF adopted by the American Association of Cereal Chemists (2001) indicates: "...Dietary fibers promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation". The Institute of Medicine of the National Academies specifies that functional fibers "...consist of isolated, nondigestible carbohydrates that have beneficial physiological effects...". Most recently, the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO), through the Codex Alimentarius (2010), adopted a definition for DF that includes, for two of the three categories, the stipulation: "shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities...".

The connection between carbohydrates, and more specifically DF, and health has fueled the interest of researchers and companies to develop novel carbohydrates using microbial enzymes, chemicals, and heat treatments. Cyclodextrins and V-amylose are examples of such carbohydrates.

V-Amylose

Amylose is the unbranched or linear portion of starch that adopts a double helix structure. Amylose can be complexed with lipids, which makes it less susceptible to enzymatic attack. This complex is generally referred to as V-complex or V-amylose because it exhibits a V-type X-ray diffraction pattern (Rappenecker and Zugenmaier, 1981). Furthermore, X-ray diffraction studies indicate that the V-complex assumes a single-stranded helical conformation and that the diameter of the helix is controlled by the size of the complexing agent. When amylose is complexed with linear alcohols and fatty acids, it forms helices having 6 glucosyl residues per turn (Biliaderis and Galloway, 1989). The glucosyl hydroxyl groups are located at the outer surface of the helix, whereas the inner surface is lined with methylene groups and glycosidic oxygens resulting in a more hydrophobic cavity similar to that of cyclodextrins (Immel and Lichtenthaler, 2000). This close analogy allows for the consideration of V-amylose as a tubular analog of cyclodextrins.

The importance of V-amylose is reflected in numerous applications, such as the use of emulsifiers to retard bread staling, wrapping of carbon nanotubes in nanotechnology, and artificial chaperoning of proteins in biotechnology (Gelders et al., 2004). V-complex also has been incorporated into enteral formulas for the purpose of controlling digestion of dietary carbohydrates and subsequent absorption rate. Murray et al. (1998) evaluated apparent digestibility by ileal-cannulated dogs of enteral diets containing a debranched amylopectin-lipid complex. Dogs received a control diet and ones containing V-complex or resistant starch (RS). Authors reported a reduction ($P < 0.05$) in total tract and ileal digestibility of dry matter (DM) (12%), organic matter (OM) (17%), and carbohydrates

(15%) when dogs received the V-complex diet compared to the control. The RS diet had the lowest ileal digestibility of DM (60.2%), OM (61.8%), and carbohydrates (47.6%), as well as the lowest total tract digestibility of DM (63.7%), OM (64.4%), and carbohydrates (42.6%). Interestingly enough, fat digestibility was not changed by the RS diet, but dogs fed the V-complex diet had lower ileal (81.2%) and total tract (81.8%) fat digestibility. Authors attributed this reduction to the possible entrapment of fats within a highly crystalline and poorly digested portion of the V-complex.

Cyclodextrins

Cyclodextrins are non-reducing cyclic oligosaccharides comprised of α -1,4-glycosidic-linked glucopyranose units. The family of cyclodextrins is very large, including the three major cyclodextrins, α -cyclodextrin (ACD) with 6 glucose units, β -cyclodextrin (BCD) with 7, and γ -cyclodextrin (GCD) with 8 (Szejtli, 1998) and several rare minor cyclic oligosaccharides with 9, 10, 11, or 12 glucose units. α -Cyclodextrin is also known as Schardinger's α -dextrin, cyclohexomaltose, cyclohexaglucon, cyclohexaamylose, and C6A. However, the most standardized nomenclature is α -cyclodextrin or ACD (Szejtli, 1996).

Cyclodextrins are conical cylinders with hydrophilic sites on the exterior of the cylinder and the edge of the cylinder base and hydrophobic sites on the interior and the top edge of the cylinder (Szejtli, 1998). This structure of cyclodextrins makes them both soluble in water and capable of including other apolar molecules of the appropriate size, also called guest molecules, in the axial open cavity forming inclusion complexes (Biber et al., 2002; Szejtli, 2004). The volume of the hydrophobic cavity of cyclodextrins varies due to the number of glucose units, with ACD having a volume of 174 \AA^3 , BCD, 262 \AA^3 , and GCD, 427 \AA^3 (Szejtli, 2004). For the formation of such complexes, the presence of water is an

important factor. Water is not only a medium for dissolution of cyclodextrins, but also is required as a driving force for the hydrophobic interaction of the guest molecule with the cavity of the cyclodextrin (Hedges, 1998).

Synthesis

Cyclodextrins are derived from starch via enzymatic processes. The cyclodextrin glucosyl transferase enzymes (CGT-ases) are amylolytic enzymes produced naturally by a large number of microorganisms like *Bacillus macerans*, *Klebsiella oxytoca*, *Bacillus circulans*, and *Alkalophylic bacillus*. The CGT-ases act on pre-hydrolyzed starch by catalyzing several transglycosylation steps (Biwer et al., 2002). Cyclodextrin glucosyl transferases cleave helical amylose molecules at regular intervals of 6, 7, or 8 glucose units and, at the same time, form a ring by an intermolecular glucosyltransferase reaction (Lina and Bär, 1998). Following the transglycosylation steps, intramolecular cyclisation in the absence of water leads to cyclodextrins (Szejtli, 1996). After these reactions, a mixture α -, β -, and γ -cyclodextrin are synthesized.

In general and for thermodynamic reasons, BCD is the favored product. However, the type and amount of cyclodextrin formed depends on substrate, bacterial origin of the CGT-ases, complexing agent, and reaction conditions (Blackwood and Bucke, 2000). If 1-decanol is added to the mixture, the conversion is shifted toward formation of ACD (Szejtli, 1998). This effect can also be obtained by addition of acetonitrile, ethanol, or tetrahydrofuran (Blackwood and Bucke, 2000).

Digestion and absorption

Because cyclodextrins are linked by α -1,4 bonds, α -amylases are capable of hydrolyzing them. However, α -amylases act slowly on cyclodextrins (Szejtli, 1996). The rate and extent of digestion by amylases depend on the type of cyclodextrin and the isoform of α -amylases. Ingested GCD appears to be more readily digested by salivary and pancreatic amylases. In contrast, BCD is resistant to the action of these enzymes (Koutsou et al., 1999). While ACD is hydrolyzed by α -amylases of fungal and bacterial origin (Saha and Zeikus, 1992), human salivary and pancreatic amylases cannot hydrolyze ACD to a significant extent (Kondo et al., 1990). For this reason, most ACD is not digested and absorbed in the small intestine, with up to 98% reaching the large bowel (Andersen et al., 1963) where it is metabolized by resident microbiota, and the primary metabolites (acyclic maltodextrin, maltose, glucose) are further metabolized (Szejtli, 1996). Approximately 2% of ACD is absorbed intact from the small intestine and is rapidly excreted in urine (De Bie et al., 1998).

Toxicity and safety

An initial report of cyclodextrin toxicity in rats (French, 1957) was followed by intense research on this topic. This study has been praised as very complete, but the toxicity assertion has been criticized because of the lack of information on dosage of cyclodextrin fed and its content of organic solvent and other impurities (Szejtli, 2004). Acute oral toxicity has not been determined for cyclodextrins because, even at the highest doses tested, no mortality or toxic effects were observed (Matsuda et al., 1983).

Til and Bär (1998) reported results of a subchronic oral toxicity study in Beagle dogs. Dogs received GCD in the diet at concentrations of 0, 5, 10, or 20% (n=4/treatment). No treatment-related changes were noted in behavior or appearance of the dogs and no

mortalities occurred. Transient diarrhea occurred in some dogs on the 5 and 10% dosage groups and in all dogs at the 20% dosage. This diarrhea was attributed to the well-known physiological responses to large amounts of undigested fermentable carbohydrates. However, all dogs remained in good health, gained weight, and food intakes and food efficiencies were not different among groups. No treatment-related differences were observed with respect to ophthalmoscopic examinations, hematological characteristics, clinicochemical analyses of plasma, and semiquantitative urine analyses. No organ abnormalities were noted at necropsy that could be attributed to treatment. Cecum weights were numerically higher in dogs consuming the 10 and 20% treatments compared to the control group, indicative of a fiber effect on intestinal morphology. Authors concluded that daily GCD consumption of up to 7.7 g/kg body weight (BW) can be tolerated by dogs without any toxic effect.

Lina and Bär (2004) did a similar subchronic oral toxicity study feeding ACD to dogs, finding comparable results to the GCD study. However, in this study, diarrhea occurred in all groups consuming ACD. The authors concluded that the “no observed adverse effect level” (NOAEL) for dogs is 9.8 g ACD/kg BW/d. They also concluded that the diarrhea and enlargement of the cecum were transient and reversible and associated with large amounts of undigested fermentable carbohydrates in the lower gut.

A longer 52 week toxicity study was conducted by Bellringer et al. (1995) using rats and beagle dogs. Rats were fed 0, 1.25, 2.5, and 5% BCD and dogs were fed 0, 0.62, 1.25, and 5% BCD. At 2.5 and 5%, rats had higher plasma liver transaminase and reduced plasma triglyceride concentrations. There was no pathological evidence of toxicity in dogs. However, there was a higher incidence of liquid feces in dogs fed BCD. Authors concluded

that the changes observed were not of toxicological importance, and the NOAEL for BCD for rats was 654 mg/kg BW/d and 1181 mg/kg BW/d for dogs.

Dietary ACD, BCD, and GCD appear to be absorbed unchanged in very small quantities from the gastrointestinal tract, not metabolized to a significant extent, and rapidly excreted in urine (De Bie et al., 1998). Cyclodextrins can be hydrolyzed to a different extent to absorbable sugars in the gastrointestinal tract, absorbed, and metabolized to carbon dioxide and water. From available research data, it appears that cyclodextrins are not toxic when included in the diet.

Industrial uses and applications

The ability of cyclodextrins to form complexes with apolar substances was first described by Freudenberg et al. (1953). In that patent, they demonstrated protection of easily oxidizable substances against oxidation, the enhancement of solubility of poorly soluble drugs, and the reduction of the loss of highly volatile substances by cyclodextrin complexation. By the end of the 1960's, cyclodextrins were considered as interesting and promising molecules, but very expensive and with very limited availability. Once production and use increased, the price decreased to levels where they became acceptable for most industrial purposes (Szejtli, 2004).

Because complexation changes the physical and chemical properties of the hydrophobic molecule, cyclodextrins are currently used by many industries, including the pharmaceutical, cosmetic, household and toiletry, and food industries. Probably the most common use of cyclodextrins is in the pharmaceutical industry. When complexed drugs are consumed, the physiological conditions of the gastrointestinal tract promote a rapid dissociation of the substance from the cyclodextrins, thus allowing the absorption of the

drug (Szejtli, 1996). Other benefits of complexed drugs are the reduction of irritation to tissues and the masking of bitterness or undesirable odors of the uncomplexed drugs (Hedges, 1998).

In the food industry, cyclodextrins have been used to stabilize flavors by decreasing their volatility (Szejtli, 2004). Cyclodextrins are able to bind the cholesterol molecule and form a complex. This feature is being used in the dairy industry to produce low-cholesterol milk and butter. After pasteurization, BCD is added to milk, mixed, and then left static for 6 h to allow time for binding cholesterol. The treated whole milk then is passed through a centrifugal separator where the insoluble cholesterol-BCD complex is removed. This process allows for the removal of up to 95% of cholesterol from milk without affecting milk fatty acid composition (Alonso et al., 2009). Another use in the food industry is as vitamin carriers, enhancing bioavailability and protecting the vitamins from light, temperature, and oxygen degradation.

Cyclodextrins also are used by the household industry where their deodorizing capacity is utilized (Szejtli, 2004), and by the agroindustry to control plant growth and to improve pesticide potency (Biwer et al., 2002). In environmental sciences, cyclodextrins play an important role immobilizing organic contaminants and heavy metals from soil and water. Cyclodextrins also are used in water treatment to encapsulate and adsorb contaminants (Del Valle, 2004).

Cholesterol

Cholesterol is a member of a family of polycyclic compounds known as sterols. It was first recognized as a component of gallstones in 1769 and was isolated from animal fats in 1815 (Olson, 1998). Cholesterol is an essential component of all mammalian plasma

membranes, playing vital roles in membrane organization, fluidity, and integrity. At the same time, cholesterol is the starting material for a variety of important molecules including oxysterols, vitamin D, bile acids, and steroid hormones.

Cholesterol occurs in the free form, esterified to long-chain fatty acids (cholesterol esters), and in other covalent and non-covalent linkages including plasma lipoproteins. Seventy to eighty percent of serum cholesterol is esterified with fatty acids, mostly with polyunsaturated fatty acids (Ikonen, 2008).

Animal cells can obtain cholesterol either from the diet or by endogenous synthesis. The cellular concentration of cholesterol is controlled by three different mechanisms: regulation of synthesis by adjusting hydroxymethylglutaryl (HMG)-CoA reductase expression and activity (Miller et al., 1989; Ness and Gertz, 2004), regulation of excess intracellular free cholesterol through the activity of acyl-CoA:cholesterol acyltransferase (ACAT) (Chang et al., 2009), and regulation of plasma cholesterol concentrations via low-density lipoproteins (LDL) receptor-mediated uptake and high-density lipoproteins (HDL)-mediated reverse transport (Brown and Goldstein, 1976).

Synthesis and metabolism

Cholesterol biosynthesis from acetate involves a highly complex series of enzymatic reactions catalyzed by more than 25 different enzymes (Bloch, 1965). The first steps involve the synthesis of mevalonic acid from acetyl-CoA and acetoacetyl-CoA, both derived from acetate, in two reactions. The first reaction is catalyzed by HMG-CoA synthase. The second reaction, catalyzed by HMG-CoA reductase, is considered the rate-limiting step. For this reason, this enzyme is the main target of a group of hypocholesterolemic drugs known as “statins”. Physiologically, the activity of HMG-CoA reductase is regulated at different levels

including synthesis, degradation, and phosphorylation of the enzyme (Brown and Goldstein, 1976; Reena et al., 2011).

The short-term regulation of HMG-CoA reductase is controlled by reversible phosphorylation. The enzyme becomes inactive when it is in the phosphorylated form (Parker et al., 1986; Miller et al., 1989). The phosphorylation of HMG-CoA reductase is catalyzed by AMP-activated protein kinase (AMPK), whereas the dephosphorylation and, consequently, restoration of its activity is catalyzed by different phosphatases. Low energy status, signaled by higher concentrations of AMP and glucagon, will increase the activity of AMPK, inducing phosphorylation of HMG-CoA reductase, thus reducing cholesterol synthesis (Carling et al., 1987). At the same time, the phosphorylated form of HMG-CoA reductase is more rapidly degraded (Miller et al., 1989).

In the long term, cholesterol biosynthesis is regulated mainly by the sterol regulatory element-binding protein (SREBP) 2. The inactive precursor of SREBP-2 is found in the endoplasmic reticulum (ER) with an escort protein. This protein is termed SREBP cleavage-activating protein (SCAP). When SCAP senses low cholesterol concentrations in the ER, it escorts SREBP-2 to the Golgi apparatus, where two specific proteases cleave the SREBP-2, liberating the active regulatory domain, which enters the nucleus, activating sterol-regulatory elements (SRE) in the promoter of target genes. These target genes include the HMG-CoA reductase and LDL receptor (Wong et al., 2006).

Synthesis of cholesterol occurs mainly in the liver. However, nearly all mammalian cells are capable of synthesizing cholesterol. Therefore, it seems that extrahepatic cholesterol synthesis can be as large as or even larger than hepatic synthesis, depending on the species and conditions that alter net sterol balance (e.g., change in cholesterol intake).

Furthermore, changes in net sterol balance in the body will influence more markedly cholesterol pools in the intestine and liver. Thus, cholesterol synthesis in these two organs will be influenced more by changes in net sterol balance than is the case for other extrahepatic organs (Dietschy et al., 1993).

Cholesterol esters and lipoproteins

Cholesterol can be esterified with fatty acids to form cholesterol esters, which appear to be the preferred form of transport and a biologically inert storage (only free cholesterol can be sensed by cells) because they are less polar than free cholesterol. Cholesterol esters are synthesized in plasma by the enzyme, lecithin cholesterol acyl transferase (LCAT) (Borggreve et al., 2003), whereas in tissues, they are synthesized by ACAT (Chang et al., 2009).

Lipoproteins are complex aggregates of proteins and lipids that make the lipids compatible with the aqueous conditions of the blood and other body fluids, making them efficient fat transporters (Olson, 1998). Lipoproteins can be classified based on the relative densities as chylomicrons (CM), very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). In general, lipoproteins have a core of triglycerides and cholesterol esters surrounded by a surface monolayer of phospholipids and free cholesterol. Chylomicrons carry dietary triglycerides, whereas VLDL contain triglycerides made primarily in the liver. Catabolism of VLDL results in LDL and, later, HDL, which contains most of the plasma cholesterol (Smith et al., 1978). Furthermore, HDL is responsible for the reverse transport of cholesterol from peripheral tissues to the liver, which can play a vital role in the prevention of atherosclerosis and cardiac diseases

(Assmann and Gotto, 2004), and LDL, through its receptor, helps in intracellular cholesterol homeostasis (Ramjiganesh et al., 2002).

Cholesterol catabolism and bile salts

Cholesterol is not easily oxidized; therefore, it is not an energy source for animal tissues. The only organ that can degrade cholesterol is the liver and the greatest proportion of cholesterol is degraded through bile acid synthesis. The rate-limiting step in bile acid synthesis is catalyzed by the enzyme, cholesterol 7 α -hydroxylase also known as cytochrome P450 7A1 (CYP7A1) whose activity is regulated by the bile acids reabsorbed from the portal blood as a negative feedback mechanism (Leuschner, 2010).

Bile acids are secreted into the intestinal lumen where they facilitate absorption of dietary lipids, cholesterol, and fat-soluble vitamins. They are later reabsorbed, transferred by portal venous blood to the liver, taken up, metabolized, and then secreted again in a process called enterohepatic circulation that recovers more than 90% of the secreted bile acids (Hofmann, 2009). In addition to their function as surfactants, bile acids are potent signaling molecules in both the liver and intestine (Maillette de Buy Wenniger and Beuers, 2010).

Cholesterol and disease

Even though cholesterol is vital in cell growth and survival, dysregulation of cholesterol metabolism can be detrimental and even lethal as is the case for cholelithiasis (Van Erpecum, 2011), Niemann-Pick disease type C (Karten et al., 2009), and atherosclerosis (Brown and Goldstein, 1976).

Hyperlipidemia, also known as dyslipidemia, is defined as the elevation of plasma cholesterol and/or triglycerides (TGs), or a low HDL-cholesterol (HDL-C) concentration that contributes to the development of atherosclerosis. Even though hyperlipidemia is related

to obesity, and in some cases to diabetes type II, obesity and hyperlipidemia cannot be treated as the same pathology (Ceska et al., 2011). Hyperlipidemia can be induced by a variable contribution of primary and secondary causes. The most common secondary causes of hyperlipidemia in developed countries are sedentary lifestyle with elevated intake of saturated fats, cholesterol, and trans-fats (Beers et al., 2006).

Nutritional Interventions in Hyperlipidemia

Hyperlipidemia has been clearly linked to the risk of cardiovascular disease (CVD). More specifically, high LDL-cholesterol (LDL-C) serum concentrations may account for a large proportion of CVD in western cultures. Both a decrease in LDL-C serum concentrations and an increase in HDL-C serum concentrations have been associated with a decrease in the incidence of CVD. Research done in men showed that even a decrease of only 10% (0.6 mmol/L) of LDL-C concentrations was associated with an average decrease in incidence of ischemic heart disease between 27 and 38% (Law et al., 1994). Furthermore, a meta-analysis conducted in the U.S. population demonstrated that a major decline (44%) in deaths from CVD could be attributed to changes in risk factors. More specifically, a 6% reduction in total cholesterol was associated with a 24% decrease in CVD (Bruckert and Rosenbaum, 2011). Cholesterol-lowering medications are widely used for this reason; however, nutritional interventions provide unique opportunities to decrease CVD risk throughout a lifetime.

Plant sterols

Plant sterols, also known as phytosterols, are plant compounds that have chemical structures similar to cholesterol. The most common and studied plant sterols are sitosterol, campesterol, and stigmasterol. The dietary intake of plant sterols is usually too low to

efficiently decrease LDL-C. Demonty et al. (2009) performed a meta-analysis of randomized controlled trials in adults to predict LDL-C-lowering capacity of different phytosterol doses. Eighty-four trials including 141 trial arms were analyzed. The pooled LDL-C reduction was 0.34 mmol/L or 8.8% for a mean daily phytosterol dose of 2.15 g. Higher baseline LDL-C concentrations were associated with greater absolute LDL-C reductions. No significant differences were found between the dose–response curves established for plant sterols vs. stanols, fat-based vs. nonfat-based food formats, and dairy vs. non-dairy foods. There was a strong tendency ($P=0.054$) toward a slightly lower efficacy of single vs. multiple daily intakes of phytosterols.

Polyunsaturated fatty acids

Intake of polyunsaturated fatty acids (PUFA) in place of saturated fatty acids is efficient in decreasing plasma concentrations of cholesterol (Czernichow et al., 2010). Clarke et al. (1997) conducted a meta-analysis of 72 ward studies in healthy volunteers. It was estimated that replacement of 5% calories as saturated fatty acids by PUFA would lead to a decrease in total blood cholesterol of 0.39 mmol/L. Similarly, replacing saturated fatty acids with n-6 PUFA and keeping the total fat content at 30-33% of energy led to a decrease in plasma total cholesterol of 0.93 mmol/L and a reduction in LDL-C of 0.63 mmol/L (Hodson et al., 2001).

In a follow-up of the Nurse's Health Study (Oh et al., 2005), PUFA intake was inversely associated with CVD risk (relative risk (RR) = 0.75), whereas trans-fat intake was positively associated with risk of CVD (RR=1.33).

Dietary fiber (DF)

Increased DF intakes and consumption of whole grains are associated with lower prevalence of CVD in prospective studies (Anderson et al., 2009). However, Brown et al. (1999), in a meta-analysis of the cholesterol-lowering effects of DF, reported that intakes of 2-10 g/d of soluble fiber were associated with small but significant decreases in total cholesterol (-0.045 mmol/L/g soluble fiber) and LDL-C (-0.057 mmol/L/g soluble fiber). The authors concluded that various soluble fibers reduced total cholesterol and LDL-C by similar amounts, but the effect was small within the practical range of fiber intakes. Therefore, increasing soluble fiber intake can make only a small contribution to a dietary intervention to lower blood cholesterol concentration.

Cyclodextrins, Fat Absorption, and Blood Lipid Profiles

It has been reported that ACD binds fats, forming stable emulsions. This emulsion or complex is hypothesized to render fats indigestible and decrease weight gain and blood cholesterol concentrations. Artiss et al. (2006) investigated the effect of ACD on weight reduction and blood metabolites in male Wistar rats. Rats were divided into 4 groups and fed ad libitum for a period of 6 weeks a low fat (LF; 4% fat) diet, LF+ACD (0.4%), high fat (HF; 40% fat), and HF+ACD (4%). They reported a 7.4% reduction in BW gain and 30% reduction in plasma TG concentrations in rats fed the HF+ACD diet relative to rats fed the HF diet. Based on the difference in weight gain of rats, it was calculated that 1 g of ACD prevented the absorption of 9 g of fat. However, these authors failed to detect any difference in net fecal lipid excretion among treatments. Rats fed LF and LF+ACD excreted 0.16 g/fat, whereas rats fed both HF treatments excreted 0.29 g/fat. Conversely, they reported an

increased percentage of fecal lipids in rats fed the HF+ACD diet (9.5%) compared to rats fed the HF treatment (7.9%).

Other studies have shown that ACD selectively binds saturated TG, increasing their fecal excretion 7-fold compared to cellulose as assessed using radiolabeled triolein and tripalmitin (Gallaher et al., 2007). These authors also hypothesized that ACD binds TG with such affinity that traditional methods of measuring fat excretion are unable to extract fat from the ACD-fat complex. They also hypothesized that this might be the explanation as to why animal feeding trials failed to detect an increase in fat excretion when ACD was fed.

It also has been reported that because of the effect of ACD on fat absorption, plasma lipid profiles are altered. Wagner et al (2008) studied the effect of ACD intake using LDL-receptor knockout mice fed a high-fat (21%) diet. Authors reported that even though total plasma TGs were not significantly different ($P=0.08$), the proportion of some individual fatty acids (C 17:1 and C 22:0) were lower in the ACD group. As expected, plasma total cholesterol (TC) concentrations increased from baseline in both control and ACD groups when switched to the high-fat diet, but this increase was significantly lower (15%) in the group fed 2.1% ACD. In this case, authors hypothesized that differences in plasma TGs due to ACD intake could be observed only when high fat diets were fed.

Grunberger et al. (2007) tested ACD supplementation of obese patients with type 2 diabetes in a double-blind study. Participants were instructed to take two 1-g tablets of ACD or placebo per each fat-containing meal (total of 6 tablets per day). Data revealed no difference in BW change between groups. However, subjects receiving ACD treatment maintained their BW despite an increase in caloric intake, whereas the placebo group increased BW (1.3%). It was concluded that fat-ACD complexes would be unavailable for

absorption in the small intestine and not fermentable in the large intestine, therefore contributing to prevention of BW gain. Similarly, Comerford et al. (2011) investigated the effect of ACD on blood lipids and weight loss in healthy overweight, but not obese, nondiabetic individuals (8 males and 20 females) in a double-blinded, crossover study. When the active phase was compared to the control phase, there were significant decreases in BW (-0.4 kg), serum TC (-0.30 mmol/L), and LDL-C (-0.23 mmol/L).

Other cyclodextrins also have been reported to affect fat digestibility. Spears et al. (2005b) evaluated pullulan and GCD effects on nutrient digestibility. Total tract and ileal digestibility of fat by dogs was reduced two percentage units by GCD at a 25% inclusion rate. However, when 1 or 2 g/d of GCD were supplemented, neither ileal digestibility nor total tract digestibility of fat were affected (Spears et al., 2005a).

Feeding cyclodextrins to different experimental animal models has resulted in inconsistent hypolipidemic effects. Suzuki and Sato (1985) reported that feeding a mixture of maltodextrin, ACD, BCD, and GCD lowered plasma TG in rats. However, they fed unusually high and impractical concentrations of cyclodextrins (24% ACD, 12% BCD, and 4% GCD). Riottot et al. (1993) evaluated the consumption of 1, 5, 10, and 20% BCD in rats and hamsters. Over the 8 wk treatment period, a decrease in plasma TG (67%) and TC (35%) concentrations was observed in both hamsters and rats when receiving 20% BCD in their diets. Additionally, hamsters had decreased blood TG concentrations (25%) when fed 1 and 5% BCD. Conversely, Favier et al. (1995) reported a decrease in plasma concentrations of both TC (-20%) and TG (-44%) when rats were fed 5% BCD. This hypolipidemic effect was slightly lower when rats were fed 2.5% BCD (14% decrease in TC and 32% decrease in serum TG concentrations). Similarly, Garcia-Mediavilla et al. (2003) examined the effects

of BCD on cholesterol and bile metabolism using rats divided into 4 groups: control, 2% cholesterol (A), A+2.5% BCD (B), and A+5.0% BCD (C). It was reported that BCD lowered plasma phospholipid concentration (B: -21%; C: -29%), enhanced CYP7A1 activity (B: 50%; C: 100%) and mRNA expression (B: 14%, C: 29%), and reduced plasma TG concentration (C: -38%). However, serum TC concentrations were not different among groups receiving cholesterol (A, B, C). Intake of BCD also resulted in a high incidence of hepatocyte necrosis and portal inflammatory cell infiltration, which could indicate hepatotoxic effects.

Kaewprasert et al. (2001) evaluated the effect of dietary cyclodextrins on liver and serum lipids, and cecal short-chain fatty acid (SCFA) concentrations in male Wistar rats. Animals were divided into 4 groups and fed a basal diet and diets containing 5% ACD, BCD, or GCD. In this study, there was no difference in BW gain among groups. Compared to the control group, serum TG concentrations were not different in rats fed ACD or GCD, but were lower when fed BCD (-38%). A 15% decrease in serum TC concentration was observed in ACD-fed and BCD-fed rats. Dietary ACD and BCD treatments exhibited an approximate 60% increase in the weight of cecal tissues and over a two-fold increase in cecal content weight. Both treatments also exhibited over a 3-fold increase in total SCFA, acetate, and propionate, and a 2-fold increase in butyrate concentrations, in the cecum.

Conclusion

Cyclodextrins are oligosaccharides used by many industries for a variety of applications because of their unique structure and ability to bind and encapsulate non-polar substances. α -Cyclodextrin has been shown to possess dietary fat-binding capacity and, thus the ability to reduce body weight gain and serum TC concentrations. These capabilities

could potentially open up new avenues for ACD utilization in clinical nutrition with potential incorporation into food products such as enteral formulas for overweight individuals and those with dyslipidemia. However, assessing the impact of ACD intake in proper settings is a pre-condition to the accurate interpretation and potential use of ACD in food products. Moreover, the reasons for inconsistent responses in body weight reduction and lipid digestibility and the possible mechanisms for the hypocholesterolemic effect of ACD should be elucidated or at least partially explained.

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CHAPTER 3

SERUM LIPID PROFILES, TOTAL TRACT NUTRIENT DIGESTIBILITY, AND GASTROINTESTINAL TOLERANCE BY DOGS OF α -CYCLODEXTRIN

Abstract

The objectives were to quantify gastrointestinal tolerance, total tract nutrient digestibility, and serum lipid profiles of dogs as affected by α -cyclodextrin (ACD) supplementation and to validate the accuracy of fat analyses techniques using novel ACD-fat complexes. The ACD was hydrolyzed and free sugars and hydrolyzed monosaccharides were quantified using high performance liquid chromatography. Known amount of fats were complexed with ACD, and fat content of complexes determined using the ether extraction and acid hydrolyzed fat methods. Nine mixed-breed hounds were used in a crossover design with 3 periods of 10 d each, including 6 d for diet adaptation and 4 d for fecal collection. Dogs were fed twice daily a diet with poultry by-product meal and brewer's rice as the main ingredients, and chromic oxide (0.2%) was included as a digestion marker. Dogs were supplemented with either 0, 3, or 6 g of ACD diluted in 15 ml of water twice per day for a total of 0, 6, and 12 g ACD per day. The ACD had a very low free sugar concentration and, once hydrolyzed, released only glucose, as expected. Average daily food intake, feces production (dry matter basis), and fecal scores were not significantly different among treatments. Body weight and condition score, and serum triglycerides and cholesterol concentrations, remained unaltered throughout the duration of the experiment. Dry matter (DM), organic matter (OM), and fat digestibility coefficients were lower ($P < 0.05$) for both treatment groups compared to the control. The acid hydrolyzed fat method was valid to measure fat bound to ACD. Intake of ACD lowered fat digestibility somewhat, but not to

the extent previously reported, without affecting serum lipid concentrations or outcomes related to tolerance. Therefore, ACD supplementation appears not to decrease fat absorption and aid in weight loss programs, but might have potential in modifying serum lipid profiles.

Introduction

It has been estimated that between 66 and 70% of the U.S. adult populations is either obese or overweight (Flegal et al., 2010). This situation has led to a search for safe and practical ways to either lose or maintain body weight (BW), which includes exercise, dieting methods, and nutritional supplements. Of the nutritional supplements, α -cyclodextrin (ACD) is being commercialized as a dietary fiber with the unique ability to bind nine times its own weight in fat (Artiss et al., 2006).

α -Cyclodextrin is a non-reducing cyclic oligosaccharide comprised of six glucopyranose units linked by alpha-1,4 bonds. The shape of ACD is a conical cylinder, with hydrophilic sites on the exterior of the cylinder and the edge of the cylinder base, and hydrophobic sites on the interior and the top edge of the cylinder (Szejtli, 1998). The structure of ACD makes it both soluble in water and capable of including other apolar molecules of the appropriate size, also called guest molecules, in the axial open cavity, forming inclusion complexes (Szejtli, 2004).

It has been hypothesized that ACD is able to form a very stable complex with dietary fat that is not absorbed in the gastrointestinal tract (Artiss et al., 2006). Therefore, ACD would be a practical weight loss supplement. However, Gallaher et al. (2007) reported that animal feeding studies failed to show an increase in fecal fat, hypothesizing that conventional methods of fat extraction might be unable to extract the fat that had been bound to the ACD.

Further study of the effect of ACD supplementation and validation of current fat analysis techniques will help to determine the effectiveness of ACD in blocking fat absorption. The objectives of this study were to measure chemical composition of ACD as well as its effect on gastrointestinal tolerance, total tract nutrient digestibility, and serum lipid profiles of dogs, and to validate fat analysis techniques using novel ACD-fat complexes as substrates.

Materials and Methods

Test substrate

Commercial food-grade ACD (purity 99.4%) manufactured by Wacker Fine Chemicals, Adrian, MI (batch number 60F212) was obtained from Abbott Nutrition, Columbus, OH.

Compositional analyses and validation of fat analysis techniques

α -Cyclodextrin was analyzed for dry matter (DM) and organic matter (OM) according to AOAC (2002) methods 934.01 and 942.05, respectively. Acid hydrolyzed fat (AHF) concentrations were determined using acid hydrolysis according to AAAC (2000) method 30-14.01, followed by ether extraction (Budde, 1952). Crude protein (CP) concentrations were calculated using LECO[®] (nitrogen analyzer model FP-2000, Leco Corporation, St. Joseph, MI) nitrogen (N) values (N x 6.25) (Association of Official Analytical Chemists, 2002).

The ACD was hydrolyzed using the procedure of Hoebler et al. (1989). Hydrolyzed monosaccharides and free sugars were quantified using a Dionex DX500 HPLC system (Dionex Corporation, Sunnyvale, CA). Standards for quantification included inositol,

fucose, arabinose, rhamnose, galactose, xylose, and mannose. Free monosaccharides were injected at a volume of 25 μ L. All assays were conducted using a CarboPac PA-1 column and guard column following methods cited by Smiricky et al. (2002).

Tubes with ACD solution were prepared using 1 g of ACD and 6 ml water in each tube. Tubes were vortexed for 10 sec. Graded amounts of corn oil and lard (2, 4, 6, 8, 10, and 12 g) were placed in quadruplicate tubes containing the ACD solution and vortexed for 10 sec. After freeze drying, fat was extracted from a duplicate set of tubes containing each concentration of corn oil or lard using the acid hydrolyzed fat method described previously, whereas the other duplicate set was extracted using the ether extraction method alone, as described previously.

Tolerance and total tract nutrient digestibility

Animals

Nine mixed-breed hounds with an average age of 2.7 yr, and an average starting BW of 21.3 kg (SD=2.4), were used in this experiment. The University of Illinois Institutional Animal Care and Use Committee approved all animal care procedures prior to initiation of the experiment. Dogs were housed individually in indoor pens (approximately 1.2 x 1.5 m) in an environmentally controlled facility with a 12 h light:12 h dark cycle. Dogs were weighed and body condition score (BCS) was assessed using a 1-9 scale (Laflamme, 1997) throughout the experiment.

Diets and treatments

One experimental diet was formulated to meet or exceed the National Research Council (2006) nutrient profiles for adult dogs at maintenance. The diet consisted of poultry

by-product meal and brewer's rice as the main ingredients and Solka-floc[®] as the fiber source. Chromic oxide was included as a digestion marker at 0.2% of the diet. Complete ingredient and chemical composition data for the diets are presented in Tables

Table 3.1. The diet was prepared in extruded, dry kibble form at Kansas State University Department of Grain Science and Industry (Manhattan, KS) under the supervision of Pet Food & Ingredient Technology, Inc. (Topeka, KS).

Dogs were fed 150 g of food twice per day for a total of 300 g of food per day. Food refusals from the previous feeding were collected and weighed. Dogs had *ad libitum* access to fresh water. Dogs were supplemented immediately after feeding with either 0, 3, or 6 g of ACD diluted in 15 ml of water twice per day for a total of 0, 6 (daily amount of ACD recommended by manufacturers), and 12 g (200% recommended amount) of ACD per day.

Experimental design

The experimental design was a crossover design with three treatments and three periods. Each period consisted of two phases: 6 d for diet adaptation and 4 d for tolerance evaluation and fecal collection. Dogs were weighed, body condition score assessed, and blood collected at the beginning and at the end of each period after a 12 h fast.

Sampling procedures

A sample of approximately 500 g was taken from each bag of diet used in this experiment. Samples were composited, and a 500 g sub-sample removed, ground in a Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ) through a 2-mm screen, and stored at 4°C until analysis.

During the 4-d collection phase, all voided feces were collected from the floor of the pen and weighed. Feces were scored on a scale from 1–5, with 1 being dry, hard pellets; 2,

dry, well-formed stool; 3, soft, moist, formed stool; 4, unformed stool; and 5, watery, liquid that can be poured. Feces were stored at -20°C until composited and ground for analysis.

Tolerance to ACD supplementation was assessed based on food intake data, fecal scores, and observance of emesis, retching, and (or) non-productive emesis.

Before d-1 and after d-10 of each period, 5 ml of blood were collected via jugular venipuncture. At 1900 h on the evening before each blood sampling, any remaining food was removed, and dogs were fasted overnight (12 h), during which time they consumed only water. Because periods were consecutive, blood sample metabolite concentrations for the end of one period also were used as representative values for the start of the next period. Blood was drawn into vacutainer serum separator tubes prior to feeding the dogs. Tubes were kept at room temperature for 30 min, and centrifuged at $1,240 \times g$ at 4°C for 10 min. Serum supernatant was collected and stored at -20°C for analyses.

Chemical analyses

Frozen feces were placed in a forced air oven at 55°C until dry. Diet and dried fecal samples were ground in a Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ) through a 2-mm screen. Samples were analyzed for DM, OM, CP, and AHF following procedures described previously. Gross energy (GE) concentrations of ACD and diet were measured using oxygen bomb calorimeter (model 1261, Parr Instruments, Moline, IL). Food and fecal samples were prepared for chromium analysis according to the method of Williams et al. (1962), and chromium concentrations were measured using an atomic absorption spectrophotometer (model 3100, Perkin Elmer, Waltham, MA).

Serum total cholesterol (TC) and triglyceride (TG) concentrations were measured on a Hitachi 917 analyzer (Roche Diagnostica, Indianapolis, IN) using enzymatic kits (catalog

numbers 2016630 and 2016648, respectively). Concentrations of serum TC and TG correspond to samples of d-10 for each period. Changes in serum TC and TG concentrations were calculated by the difference in serum concentrations on the last day of the period minus serum concentrations on the first day of the period.

Calculations

Apparent total tract DM digestibilities were calculated as: $100 - (100 \times \text{marker concentration in the feed [\%]} / \text{marker concentration in the feces [\%]})$. Apparent total tract nutrient digestibilities were calculated as: $100 - 100 (\text{marker concentration in the feed [\%]} \times \text{nutrient concentration in feces [\%]} / \text{marker concentration in feces [\%]} \times \text{nutrient concentration in the feed [\%]})$.

Statistics

Data were analyzed as a crossover design using the Mixed Models procedure of SAS/STAT® software, version 9.2 for Windows® (SAS Institute Inc., Cary, NC). The statistical model included the fixed effect of dietary treatment and the random effects of period and dog. Normal distribution of residuals and homogeneity of variances were tested and assumptions for analysis of variances were fulfilled. It was assumed that there was no interaction between period and treatment, period and dog, and treatment and dog. Treatment least squares means are reported and were compared using a Bonferroni adjustment to ensure the overall protection level. Standard error of the mean (SEM) values are associated with least squares means as calculated in the Mixed Models procedure. Differences among means with a P-value of less than 0.05 were considered significant, and P-values greater than 0.05 but less than or equal to 0.10 were considered trends.

Results

Compositional analyses and validation of fat analyses techniques

Dry matter, organic matter, free sugar content, and hydrolyzed monosaccharide content are presented in Table 3.2. Percentage DM was 98.2%, with no ash content (100% OM). Mannose was the only free sugar detectable in the ACD (55.7 µg/g), whereas the only monosaccharide released after hydrolysis was glucose (1,217 mg/g).

An average of 99.9% of the fat in the tubes was recovered using the AHF method, whereas an average of 95.5% of the fat was recovered using the EE method (data not shown).

Tolerance and total tract nutrient digestibility

Some dogs experienced emesis and non-productive emesis throughout the experiment. However, this was not consistent for a specific treatment or individual, with some dogs in the control treatment experiencing these signs as well. In some cases, dogs that saw other dogs being dosed experienced non-productive emesis prior to receiving the ACD supplementation.

Body weight and BCS were not different among treatments. Intake and total tract nutrient digestibility by dogs of diets supplemented with ACD are presented in Table 3.3. Average daily food intake values were similar ($P=0.95$) among treatments throughout the study, with most of the dogs ingesting all the food they were provided. Apparent DM digestibility coefficients were relatively high, with the control treatment having a higher ($P<0.05$) value than the ACD treatments. Apparent OM digestibility coefficients followed the same trend as DM. Acid hydrolyzed fat digestibilities were high, with the control treatment having a higher value ($P<0.05$) than the ACD treatments.

Fecal characteristics of dogs supplemented with ACD are presented in Table 3.3. Fecal output expressed on a DM basis was similar among treatments. However, fecal output expressed on an as-is basis for the 12 g ACD treatment was higher than for the control treatment and not different from the 6 g ACD treatment value, which was also not different from the control treatment. Fecal output (as-is basis) per g DM consumed was higher ($P<0.05$) for both ACD treatments compared to the control. Conversely, fecal DM concentration for dogs fed the ACD treatments was lower ($P<0.05$) than for the control treatment. Despite the fact that both ACD treatments generated feces with a higher amount of water, fecal scores for dogs fed the control treatment were not different from the 6 g ACD treatment value, and only tended to be different ($P=0.07$) from those on the 12 g treatment.

Serum TC and TG concentrations and changes in serum TC and TG concentrations for dogs supplemented with ACD are presented in Table 3.4. Serum TC and TG concentrations were not different among treatments. There was no change in the mean value for either serum TC or TG concentrations in any of the treatments. However, there was a numerical decrease in serum TC concentration in three dogs when they were fed both ACD treatments. These three dogs had an initial concentration of serum TC that was higher than the reference values (2.8-8.2 mmol TC/dL serum) and had a numerical reduction of 1.89, 2.76, and 6.08 mmol TC/dL serum, respectively, after receiving the ACD treatments. After receiving the control treatment, two of the 3 dogs presented an increase in serum TC concentrations (3.0 and 2.17 mmol TC/dL serum), returning to values similar to those measured at the start of the experiment.

Discussion

The high DM and OM concentration is a result of the purification and drying processes used in the manufacture of ACD. These values coincide with the information in the inspection certificate of the manufacturer (Wacker Fine Chemicals, 2008). The presence of a low amount of mannose might be indicative of bacterial contamination of the sample or contamination of the enzyme used in the synthesis of ACD, which is of bacterial origin (Biwer et al., 2002). The release of only glucose after acid hydrolysis was expected as ACD is composed of 6 glucose molecules exclusively (Del Valle, 2004). The release of more than 1000 mg glucose/g ACD can be attributed, in part, to the fact that during hydrolysis, water is used to break polysaccharide chains into smaller chains or into simple carbohydrates. The water added to the glucose molecules during ACD hydrolysis accounts for approximately 111 mg additional ACD/g, whereas the remaining difference can be attributed to analytical error due to the multiple steps this procedure involves (Hoebler et al., 1989).

The use of ether extraction alone was insufficient to extract all the fat present in the tubes. It has been reported that ACD can form complexes with fats that are bound to the hydrophobic cavity of ACD (Szejtli, 2004). This complex formation is so strong that traditional extraction methods are not able to recover all the complexed fat (Artiss et al., 2006; Gallaher et al., 2007). The use of acid hydrolysis prior to the ether extraction process hydrolyzes the ACD, freeing the fat and, therefore, practically all the fat in the tubes was recovered when the AHF procedure was used. Consequently, the AHF method is valid for measuring fat bound to ACD.

The only signs of intolerance observed were emesis and non-productive emesis by some of the dogs. Intake of ACD has been previously reported to cause emesis. Spears et al.

(2005b) evaluated meal tolerance of ACD, β -cyclodextrin (BCD), and γ -cyclodextrin (GCD). Dogs were offered 25 g of cyclodextrin in ~240 mL distilled-deionized water using a 60-mL syringe without needle. Dogs regurgitated within 30 to 60 min of cyclodextrin consumption, with ACD being regurgitated more quickly than BCD and GCD. On the other hand, the same authors reported feeding 63.0 g of GCD in an enteral diet without regurgitation or diarrhea. Other studies involving intake of larger amounts of ACD by dogs (up to 110 g/d) and rats for 13 wk demonstrated tolerance, with transient diarrhea as the only adverse effect (Lina and Bar, 2004a, b). However, in those experiments with no tolerance problems, cyclodextrins were included in a diet matrix, so perhaps this is the reason for better tolerance of ACD by dogs. In our study, as in the cited study, ACD was provided as a solution in water using a syringe. The physical effect of putting the syringe in the mouth of the dogs or the taste of the solution might have provoked the emesis. Spears et al. (2005b) also hypothesized that the high molarity of the solution may have affected electrolyte balance or water binding, resulting in regurgitation.

Intake of ACD has been reported to result in a decrease in BW gain of rats (Artiss et al., 2006; Kishino et al., 2009) and humans (Grunberger et al., 2007), which was attributed to a decreased fat absorption. In these studies, authors observed a decrease in BW gain and not a BW decrease. On the contrary, Comerford et al. (2011), in a double-blinded crossover study with 28 healthy, overweight humans, reported a decrease of 0.41 kg BW when 6 g of ACD were supplemented to their regular diets for 30 d.

Body weight and BCS of the dogs in our study remained unaltered during the duration of the experiment. The reason for this difference might be attributed to the fact that the dogs were fed an amount of food calculated to meet their energy requirements and maintain their

BW, whereas rats and humans were fed *ad libitum*. The lack of BW loss in our study also might be attributed to the shorter period of ACD supplementation.

Apparent DM, OM, and AHF digestibility coefficients were high and comparable to previously reported digestibility coefficients for diets with similar ingredient matrices (Middelbos et al., 2007; Faber et al., 2011). The reduction in DM and OM digestibility was, in part, due to the reduction in AHF digestibility. Acid hydrolyzed fat digestibility was reduced in a linear fashion, and might be due to ACD intake. Other studies have reported lower apparent fat digestibility due to ACD intake by rats (Gallaher et al., 2007) and γ -cyclodextrin (GCD) intake by dogs (Spears et al., 2005a). It has been hypothesized that ACD and fats form a complex in the stomach that remains bound through the gastrointestinal tract, preventing absorption in the small intestine and, therefore, fat digestibility is decreased in a ratio of 9 g of fat per g of ACD (Artiss et al., 2006; Grunberger et al., 2007). However, in these studies, authors failed to detect differences in fat excretion, and calculated this ratio based on the difference in body weight gain among treatments. Intake of ACD by the dogs decreased fat digestibility by approximately 1 percentage unit, which represents a reduction of absorption of ~0.5 g fat for a mean ACD intake of 9 g/dog/d. Therefore, this effect appears to be not as strong as previously reported.

Fecal DM and fecal output expressed on an as-is basis were affected by ACD intake. α -Cyclodextrin is not degraded by hydrolytic-enzymatic digestion, but is partially fermentable in the large intestine (Del Valle, 2004). Fermentation of ACD produces short-chain fatty acids (SCFA) that influence wet fecal output (Sunvold et al., 1995). Even though there were differences in fecal DM content, fecal scores were not different. This is not

unexpected, as several studies have reported a lack of correlation between fecal DM and fecal score (Grieshop et al., 2002; Gajda et al., 2005; Spears et al., 2005a).

Due to the amphiphilic nature of cyclodextrins, they have been used to bind cholesterol in a variety of substrates such as milk, butter, sperm, and experimental diets (Choi and Toyoda, 1998; Somogyi et al., 2006; Alonso et al., 2009). More specifically, the hypocholesterolemic effect of ACD in different species has been demonstrated (Artiss et al., 2006; Grunberger et al., 2007; Wagner et al., 2007; Wagner et al., 2008; Comerford et al., 2011). In this study, three dogs were hypercholesterolemic at the beginning of the experiment. This situation was not planned nor was the experiment designed with that information in mind. It was evident that the numerical trend for decreased serum TC concentrations occurred when the hypercholesterolemic dogs received ACD supplementation. However, the remaining 6 dogs had normal serum TC concentrations throughout the experiment that were not affected by ACD intake. Therefore, the reduction in serum TC failed to reach statistical significance. Differences in serum TC concentration responses also can be attributed to unique features of cholesterol metabolism in the dog. Cholesterol ester transfer protein (CETP) facilitates the transfer of cholesterol in exchange for TG from high-density lipoprotein (HDL) to low-density lipoprotein and very-low-density lipoprotein (VLDL). The dog lacks CETP activity, which results in high concentrations of HDL-cholesterol, facilitating the re-direction of cholesterol to the liver for clearance (Bailhache et al., 2004). This lack of CETP activity makes the dog very efficient in metabolizing cholesterol and not a good model for studying cholesterol metabolism in humans.

In conclusion, although ether extraction alone is not a valid method to measure fat content in ACD-fat complexes, the AHF procedure is valid for measuring fat bound to ACD. Intake of ACD appears to be well tolerated by dogs, resulting in a 1 percentage unit decrease in DM and OM total tract digestibilities, which was caused mostly by a reduction in AHF digestibility. The effect of reduction in fat digestibility by ACD intake appears to be not as strong as previously reported. Therefore, ACD supplementation appears not to be an efficient strategy for weight loss programs. Fecal characteristics of dogs consuming ACD were not drastically affected, with only a slight, but significant, increase in water content and a trend for higher fecal scores. Intake of ACD numerically decreased serum TC concentrations in hypercholesterolemic dogs, but failed to reduce serum TC concentrations in normocholesterolemic dogs, probably due to a self-limiting mechanism that prevents cholesterol concentration from dropping too low. Supplementation with ACD might have potential in reducing serum TC concentrations, but this effect should be studied in a more suitable animal model or in the human.

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Tables

Table 3.1. Ingredient (% as-fed basis) and chemical (% dry matter basis) composition of the experimental diet

Item	%
Poultry byproduct meal, low ash	39.00
Poultry fat	14.00
Corn, yellow, ground	10.00
Brewer's rice	28.85
Solka floc ^{®1}	6.50
Mineral premix ²	0.10
Vitamin premix ³	0.10
Choline chloride	0.10
Sodium chloride	0.65
Potassium chloride	0.50
Chromic oxide	0.20
<i>Analyzed chemical composition</i>	
Dry matter	92.60
Organic matter	91.70
Crude protein	30.60
Acid hydrolyzed fat	20.16
Gross energy, kcal/kg	5,215.01

¹International Fiber Corporation, North Tonawanda, NY.

Table 3.1. (Cont.)

²Provided per kilogram of diet: 120 mg iron (FeSO₄); 66 mg manganese (MnO); 18 mg copper (CuSO₄); 1.8 mg iodine (C₂H₆N₂·2HI); 240 µg selenium (Na₂SeO₃); 240 mg zinc (ZnO).

³Provided per kilogram of diet: 10,560 IU vitamin A (vitamin A acetate); 1056 IU vitamin D (vitamin D₃); 105 IU vitamin E (dl- α -tocopherol); 0.53 mg vitamin K (menadione sodium bisulfate complex); 2.64 mg thiamine (thiamine mononitrate); 23.76 mg niacin (niacin supplement); 3.43 mg riboflavin (riboflavin supplement); 13.2 mg pantothenic acid (d-calcium pantothenate); 66 µg vitamin B₁₂ (vitamin B₁₂ supplement); 2.11 mg pyridoxine (pyridoxine hydrochloride); 79 µg biotin (D-biotin supplement); 264 µg folic acid (folic acid supplement).

Table 3.2. Dry matter, organic matter, free sugar content, and hydrolyzed monosaccharide content of α -cyclodextrin

Item	Concentration
Dry matter, %	98.2
Organic matter, % ¹	100.0
<i>Free sugars</i>	
Mannose, $\mu\text{g/g DM}$	55.7
<i>Hydrolyzed monosaccharides</i> ²	
Glucose, mg/g DM	1,217.0 ³

¹ On a dry matter basis

² Corrected for free sugar concentrations.

³ Water added to the glucose molecules during ACD hydrolysis accounts for approximately 111 mg additional ACD/g. The remaining difference can be attributed to analytical error due to the multiple steps this procedure involves.

Table 3.3. Intake (g/d, as-fed basis) and digestibilities (%) for dogs fed diets supplemented with α -cyclodextrin (ACD)

Item	Treatment			SEM ¹
	Control	6 g ACD	12 g ACD	
Intake	285.6	285.2	281.6	10.70
----- Total tract digestibility, % -----				
Dry matter	77.9 ^a	76.3 ^b	76.4 ^b	0.44
Organic matter	82.7 ^a	81.2 ^b	81.0 ^b	0.41
Acid hydrolyzed fat	95.1 ^a	94.0 ^b	93.6 ^b	0.20
----- Fecal characteristics -----				
Fecal output (as-is), g/d	123.6 ^a	146.4 ^{ab}	150.5 ^b	8.01
Fecal output (DM), g/d	58.3	62.7	61.4	2.38
Fecal output (as-is) per g				
DM consumed	0.43 ^a	0.51 ^b	0.54 ^b	0.03
Fecal DM, %	47.5 ^a	43.7 ^b	41.2 ^b	1.55
Fecal score ²	2.7	2.8	3.1	0.13

^{a,b,c} Means in the same row with unlike superscript letters differ (P<0.05).

¹SEM = Pooled standard error of the mean.

² Scores based on the following scale: 1 = dry, hard pellets; 2 = dry, well-formed stool; 3 = soft, moist, formed stool; 4 = unformed stool; 5 = watery, liquid that can be poured.

Table 3.4. Serum cholesterol and triglyceride concentrations, and changes in concentration, for dogs supplemented with α -cyclodextrin (ACD)¹

Item	Treatment			SEM ²
	Control	6 g ACD	12 g ACD	
Serum cholesterol, mmol/dL	6.65	6.26	6.57	1.09
Serum triglycerides, mmol/dL	0.54	0.47	0.56	0.07
Δ Serum cholesterol, mmol/dL	0.43	-0.74	-0.46	0.54
Δ Serum triglycerides, mmol/dL	-0.01	-0.04	0.02	0.07

¹Concentrations were measured on d-10 of each period. Change was measured between d-1 and 10 of each period.

²SEM = Pooled standard error of the mean.

CHAPTER 4

***IN VITRO* FERMENTATION CHARACTERISTICS, *IN VIVO* ILEAL AND TOTAL TRACT NUTRIENT DIGESTIBILITIES, AND FECAL MICROBIOTA RESPONSES OF DOGS TO α -CYCLODEXTRIN**

Abstract

The objectives were to examine *in vitro* fermentation characteristics, *in vivo* nutrient digestibility, fecal microbiota responses, and serum lipid profiles of dogs as affected by α -cyclodextrin (ACD) supplementation. Short-chain fatty acid (SCFA) production was measured after *in vitro* fermentation for 3, 6, 9, and 12 h of ACD, β -cyclodextrin (BCD), and γ -cyclodextrin (GCD). A log function curve was fitted using non-linear regression, and maximal production, maximal rate of production, and time to attain maximal rate of production were estimated. Five mixed-breed hounds were used in a Latin square design with 5 periods of 14 d each, including 10 d for diet adaptation and 4 d for fecal collection. Dogs were fed twice a day a diet with poultry by-product meal and brewer's rice as the main ingredients, and chromic oxide (0.2%) was included as a digestion marker. Dogs were supplemented with either 0, 1, 2, 3, or 4 g of ACD diluted in 15 ml of water twice daily for a total of 0, 2, 4, 6, and 8 g ACD/d. Maximal *in vitro* production of total SCFA was lowest for ACD. However, the greatest maximal production of propionate was noted for ACD. Average daily food intake and ileal nutrient digestibility were not significantly different among treatments. Total tract nutrient digestibility and fecal dry matter concentration decreased ($P < 0.05$) linearly for treatment groups receiving ACD. Fecal output expressed on an as-is basis, on a DM basis, and on an as-is per g DM intake basis increased linearly ($P < 0.05$) with increasing ACD supplementation. Bifidobacteria, *Clostridium perfringens*,

and *E. coli* were not different among treatments. Serum cholesterol and triglyceride concentrations were within normal ranges for dogs and were not different among treatments. Intake of ACD did not alter serum lipid concentrations or ileal nutrient digestibility, but resulted in a linear decrease in total tract nutrient digestibility. This points to ACD fermentation in the large bowel, which could lead to a higher proportion of propionate production as observed in the *in vitro* experiment. Therefore, ACD supplementation appears to have no effect on nutrient absorption in the small intestine, but may alter fermentation in the large bowel.

Introduction

According to the American Association of Cereal Chemists (2001), dietary fibers promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation. The blood cholesterol attenuation effect has been proven for some soluble fibers including psyllium, β -glucan, pectin, and guar gum (Bazzano, 2008). This physiological effect is important because hyperlipidemia has been clearly linked to the risk of cardiovascular disease (CVD). More specifically, high LDL-cholesterol (LDL-C) concentrations in serum may account for a large proportion of CVD cases in western cultures (Bruckert and Rosenbaum, 2011).

Cyclodextrins, a group of cyclic oligosaccharides with cylindrical shape and hydrophilic sites on the exterior and hydrophobic sites on the interior, are capable of forming inclusion complexes with apolar substances such as cholesterol (Szejtli, 2004), thus blocking its absorption. It has been reported that intake of α -cyclodextrin (ACD), β -cyclodextrin (BCD), and γ -cyclodextrin (GCD) can result in a 15-20% decrease in serum total cholesterol (TC) concentrations (Favier et al., 1995; Kaewprasert et al., 2001).

Cyclodextrin intake by rats has been reported to result in a 60% increase in the weight of cecal tissues, over a two-fold increase in weight of cecal contents, and over a 3-fold increase in total SCFA concentrations. At the same time, it has been reported that BCD intake enhances (1.8 fold) bile acid excretion (Levrat et al., 1994). Consequently, the hypocholesterolemic effect of cyclodextrins appears to be mediated, at least partially, by changes in fermentation patterns in the large intestine.

Studying the effects of ACD supplementation on ileal nutrient digestibilities, fermentation patterns, and GI microbiota will help determine the mechanism of ACD action as regards cholesterol metabolism. The objectives of this study were to examine *in vitro* fermentation characteristics, *in vivo* nutrient digestibility, fecal microbiota responses, and serum lipid profiles of dogs as affected by ACD supplementation.

Materials and Methods

Samples

Commercial food-grade ACD (purity 99.4%) manufactured by Wacker Fine Chemicals, Adrian, MI (batch number 60F212) was obtained from Abbott Nutrition, Columbus, OH. Carbohydrates used during the *in vitro* experiment also included commercial food-grade BCD and GCD (Wacker Fine Chemicals, Adrian, MI).

***In vitro* digestion experiment**

Donors and collection method

Three human fecal samples from three individual male volunteers were pooled to serve as the source of inoculum. All donors consumed their normal diet, were greater than 18 yr old, were free of gastrointestinal disease, and had not received antibiotics at least three

months prior to or during the study. The experimental protocol was approved by the University of Illinois Institutional Review Board, and all subjects signed an informed consent prior to initiation of the experiment. On the morning of the experiment, each donor provided a fresh fecal sample (within 15 min of defecation) collected using a Commode Specimen Collection System (Sage Products, Crystal Lake, IL).

Substrates

Approximately 500 mg of ACD, BCD, or GCD were placed in tubes in triplicate for in vitro fermentation measurements.

Procedure

On the experiment day, fecal samples from donors were received and maintained at 37°C until inoculum was prepared. Anaerobic inoculum was prepared from fresh fecal samples within 15 min of defecation. Equal amounts of feces from each donor were mixed together and diluted 1:10 (wt/vol) in anaerobic dilution solution (Bryant and Burkey, 1953) by blending for 15 sec in a Waring blender under a stream of CO₂. Blended, diluted feces were filtered through four layers of cheesecloth and sealed in 125 ml serum bottles under CO₂.

Each substrate was fermented in vitro for 0, 3, 6, 9, and 12 h in triplicate with the fecal microbiota obtained from pooling fresh feces from the three donors. Triplicate tubes containing no substrate were fermented with the inoculum at each time point to enable appropriate corrections for short-chain fatty acid (SCFA) production not arising from the substrates. The composition of the semi-defined medium used for the fermentation is presented in Table 4.1. All components except for the vitamin solutions were mixed before autoclave sterilization of the medium. Filter-sterilized vitamin solutions were added just

before dispensing the medium, which was maintained under anaerobic conditions at all times after preparation.

Aliquots (26 ml) of medium were aseptically transferred to 50 ml centrifuge tubes and capped with rubber stoppers with 1-way Bunsen valves. All tubes were stored at 4°C for approximately 12 h to enable hydration of the substrates before initiating fermentation. Tubes were placed in a 37°C water bath approximately 30 min before inoculation.

Diluted feces (4 ml) were inoculated into tubes containing either 26 ml semi-defined medium only (blank tubes) or 26 ml semi-defined medium and substrate. Tubes were incubated at 37°C with periodic mixing for the respective fermentation times. At the appropriate time, tubes were removed from the 37°C incubator and processed immediately for analyses. The pH of tube contents was measured with a standard pH meter (Denver Instrument Co., Arvada, CO, USA).

A 2.0 ml sub-sample was taken from each tube for SCFA analyses. Samples to be analyzed for SCFA were mixed with 0.5 ml of 250 g/L m-phosphoric acid, precipitated at room temperature for 30 min, and then centrifuged at 20,100 x g at 4°C for 20 min. The supernatant was decanted and frozen at -20°C in microfuge tubes. After freezing, the supernatant was thawed and centrifuged in microfuge tubes at 13,000 x g at 20°C for 10 min. The supernatant then was transferred to cryovials and stored at -20 °C until analysis.

Chemical analyses

Concentrations of SCFA were determined using gas-liquid chromatography. Briefly, concentrations of acetate, propionate, and butyrate were determined in the supernatant of the tubes using a Hewlett-Packard 5890A Series II gas-liquid chromatograph and a glass column (180 cm x 64 mm i.d.) packed with 10% SP-1200/1% H₃PO₄ on 80/100 mesh

Chromosorb WAW (Supelco Inc., Bellefonte, PA). Short-chain fatty acid concentrations were corrected for the quantities of SCFA produced in the blank tubes.

Statistics

Data for SCFA production were fitted to a logistic model equation to determine the rate of production and the time to attain maximal rate of production for each substrate. This function is frequently used to model biological, and especially microbial, growth (Koops and Grossman, 1993). It is a sigmoidal curve that can describe accelerating and, after passing through an inflection point, decelerating phases of growth (Koops and Grossman, 1991). Production of SCFA was fitted to the following equation:

$$Y = A/(1 + e^{-(t-C)/B})$$

where Y = SCFA production, A = asymptote, or maximal SCFA production expressed in $\mu\text{mol/g DM}$, t = incubation time in hours, C = time in hours at which the rate of SCFA production is maximum (the inflection point), and B = a measure of the duration of SCFA production in hours.

Variables (A, B, and C) were estimated for each substrate using the Nonlinear Regression procedure of SAS/STAT® software, version 9.2 for Windows® (SAS Institute Inc., Cary, NC, Copyright 2008). Additionally, maximal rates of SCFA production were calculated using the following equation:

$$Y=A/4*B$$

where Y = maximal rate of production expressed in $\mu\text{mol/h/g DM}$, A = maximal SCFA production expressed in $\mu\text{mol/g DM}$, and B = measure of the duration of SCFA production in hours.

Because maximal rate of production values were calculated from the variables that were estimated using the logistic model equations, only one value per substrate was obtained. Therefore, these parameters could not be compared in the classical statistical manner.

Data regarding pH were analyzed as a randomized block design using the Mixed Models procedure of SAS/STAT® software, version 9.2 for Windows® (SAS Institute Inc., Cary, NC, Copyright 2008). The statistical model included the fixed effects of pull time and substrate. Normal distribution of residuals and homogeneity of variances were tested and assumptions for analysis of variances were fulfilled. Treatment least squares means are reported and were compared using preplanned contrasts and estimates. Standard error of the mean (SEM) values are associated with least squares means as calculated in the Mixed Models procedure. Contrasts with a P-value of less than 0.05 were considered significant, and P-values greater than 0.05 but less than or equal to 0.10 were considered trends.

Ileal and total tract nutrient digestibility

Animals

Five female purpose-bred hound-mix dogs with an average age of 5.4 yr (3-8 yr), and an average starting BW of 23.1 kg (SD=2.5), were used in this experiment. Dogs previously had been surgically prepared with a T-shaped cannula proximal to the ileocecal junction according to the procedure of Walker et al. (1994). The University of Illinois Institutional Animal Care and Use Committee approved all animal care procedures prior to initiation of the experiment. Dogs were housed individually in indoor pens (approximately 1.2 x 1.5 m) in an environmentally controlled facility (22°C; 23% relative humidity) with a 16 h light:8 h

dark cycle. Dogs were weighed and body condition score (BCS) was assessed using a 1-9 scale (Laflamme, 1997) throughout the experiment.

Diets and treatments

One experimental diet was formulated to meet or exceed the National Research Council (2006) nutrient profiles for adult dogs at maintenance. The diet consisted of poultry by-product meal and brewer's rice as the main ingredients and Solka-floc[®] as the fiber source. Chromic oxide was included as a digestion marker at 0.2% of the diet. Complete ingredient and chemical composition data for the diets are presented in Table 4.2. The diet was prepared in extruded, dry kibble form at Kansas State University Department of Grain Science and Industry (Manhattan, KS) under the supervision of Pet Food & Ingredient Technology, Inc. (Topeka, KS).

Dogs were fed 150 g of food twice per day for a total of 300 g of food per day. Food refusals from the previous feeding were collected and weighed. Dogs had *ad libitum* access to fresh water. Dogs were supplemented immediately after feeding with either 0, 1, 2, 3, or 4 g of ACD diluted in 15 ml of water twice per day for a total of 0, 2, 4, 6, and 8 g ACD/d.

Experimental design

The experimental design was a Latin square design with 5 treatments, 5 dogs, and 5 periods. Each period consisted of two phases: 10 d for diet adaptation and 4 d for fecal collection. Dogs were weighed, body condition score assessed, and blood collected at the beginning and at the end of each period after a 12 h fast.

Sampling procedures

A sample of approximately 500 g was taken from each bag of diet used in this experiment. Samples were composited, and a 500 g sub-sample removed, ground in a Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ) through a 2-mm screen, and stored at 4°C until analysis.

During the 4-d collection phase, all voided feces were collected from the floor of the pen and weighed. Feces were scored on a scale from 1–5, with 1 being dry, hard pellets; 2, dry, well-formed stool; 3, soft, moist, formed stool; 4, unformed stool; and 5, watery, liquid that can be poured. Feces were stored at –20°C until composited and ground for analysis. On d-14 of each period, fresh fecal samples were collected within 15 min of defecation, flash frozen in liquid nitrogen, and stored at -80°C for bacterial analyses.

Ileal effluent was collected 3 times/d over 4 d, with an interval of 4 h between the start of collections, and with individual ileal collections lasting 1 h. Ileal collection times were adjusted by one hour from the previous day's collection time for a total of 12 samples per animal per 4-d collection period. For example, sample times on collection day one were 0800, 1200, and 1600; on day two, samples were collected at 0900, 1300, and 1700, etc. Ileal effluent was collected into a sterile sampling bag by attaching the bag to the cannula extension with a rubber band. Before bag attachment, cannula barrels were scraped clean using a spatula. During collections, dogs wore Bite-Not collars (Bite-Not Products, San Francisco, CA) to prevent the dog from removing the sample bag. Dogs were encouraged to move freely during collections. After collection, a cannula plug was placed in the barrel and the cannula site was cleaned with a dilute betadine solution. Ileal samples were frozen at

-20°C in their individual bags until further analyses. After all samples were collected, ileal effluent from each dog was composited by period and re-frozen at -20°C.

Before d-1 and after d-14 of each period, 5 ml of blood were collected via jugular venipuncture. At 1900 h on the evening before each blood sampling, any remaining food was removed, and dogs were fasted overnight (12 h), during which time they consumed only water. Because periods were consecutive, blood metabolite concentrations at the end of one period were used as baseline values for the start of the next period. Blood was drawn into vacutainer serum separator tubes prior to feeding the dogs. Tubes were kept at room temperature for 30 min and centrifuged at 1,240 x g at 4°C for 10 min. Serum was collected and stored at -20°C for analyses.

Chemical analyses

Frozen feces were placed in a forced air oven at 55°C until dry. Ileal effluent was lyophilized in a Dura-Dry MP microprocessor-controlled freeze-dryer (FTS Systems, Stone Ridge, NY). Once dry, ileal effluent was ground with mortar and pestle. Diet and dried fecal samples were ground in a Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ) through a 2-mm screen.

Dry matter (DM) and organic matter (OM) concentrations were determined according to AOAC (2002) methods 934.01 and 942.05, respectively. Acid hydrolyzed fat (AHF) concentrations were determined using acid hydrolysis according to AAAC (2000) method 30-14.01, followed by ether extraction (Budde, 1952). Crude protein (CP) concentrations were calculated using LECO[®] (nitrogen analyzer model FP-2000, Leco Corporation, St Joseph, MI) nitrogen (N) values (N x 6.25) (Association of Official Analytical Chemists, 2002). Gross energy (GE) concentrations of ACD and diet were measured using oxygen

bomb calorimeter (model 1261, Parr Instruments, Moline, IL). Food and fecal samples were prepared for chromium analysis according to the method of Williams et al. (1962), and chromium concentrations were measured using an atomic absorption spectrophotometer (model 3100, Perkin Elmer, Waltham, MA).

Serum total cholesterol (TC) and triglyceride (TG) concentrations were measured on a Hitachi 917 analyzer (Roche Diagnostica, Indianapolis, IN) using enzymatic kits (catalog numbers 2016630 and 2016648, respectively). Concentrations of serum TC and TG correspond to d-14 samples for each period. Changes in serum TC and TG concentrations were calculated by the difference in serum concentrations on the last day of the period minus serum concentrations on the first day of the period.

Calculations

Apparent total tract DM digestibilities were calculated as: $100 - (100 \times \text{marker concentration in the feed [\%]} / \text{marker concentration in the feces [\%]})$. Apparent total tract nutrient digestibilities were calculated as: $100 - 100 (\text{marker concentration in the feed [\%]} \times \text{nutrient concentration in feces [\%]} / \text{marker concentration in feces [\%]} \times \text{nutrient concentration in the feed [\%]})$.

Apparent ileal DM digestibilities were calculated as: $100 - (100 \times \text{marker concentration in the feed [\%]} / \text{marker concentration in the ileal effluent [\%]})$. Apparent ileal nutrient digestibilities were calculated as: $100 - 100 (\text{marker concentration in the feed [\%]} \times \text{nutrient concentration in ileal effluent [\%]} / \text{marker concentration in ileal effluent [\%]} \times \text{nutrient concentration in the feed [\%]})$.

Statistics

Data were analyzed as a Latin square design using the Mixed Models procedure of SAS/STAT® software, version 9.2 for Windows® (SAS Institute Inc., Cary, NC, Copyright 2008). The statistical model included the fixed effect of dietary treatment and the random effects of period and dog. Normal distribution of residuals and homogeneity of variances were tested and assumptions for analysis of variances were fulfilled. It was assumed that there was no interaction between period and treatment, period and dog, and treatment and dog. Treatment least squares means are reported and were compared using pre-planned orthogonal polynomial contrasts. Linear, quadratic, cubic, and quartic effects of ACD supplementation were analyzed. Standard error of the mean (SEM) values are associated with least squares means as calculated in the Mixed Models procedure. Contrasts with a P-value of less than 0.05 were considered significant, and P-values greater than 0.05 but less than or equal to 0.10 were considered trends.

Microbiota quantification

Microbiota populations were quantified using plating and molecular techniques. For plating, collected fecal samples (15-20 g as-is weight) were homogenized by vortexing with 10 ml of peptone water (0.1% peptone). Serial dilutions were made of each sample, and 100 µl aliquots of each dilution were plated on TPY agar to culture bifidobacteria and LBS agar to culture lactobacilli. All dilutions and plating were done in an anaerobic environment. Plates were done in triplicate and incubated at 37°C for 48 h in an anaerobic chamber (Coy Lab Products, Grass Lake, MI). Anaerobic gas composition was 5% hydrogen, 5% carbon dioxide, and 90% nitrogen. All of the colonies on a given plate were counted and that number was divided by the volume of inoculation. The resulting number was multiplied by

the appropriate serial dilution factor (10, 100, 1000, etc.) and normalized per gram of fecal sample to yield colony forming units (CFU)/g.

Bacterial DNA in feces was extracted and purified from frozen samples using QIAamp DNA stool mini kits (Qiagen, Valencia, CA) using the repeated bead beating plus column (RBB+C) method described by Yu and Morrison (2004). Fecal DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). *Escherichia coli*, *Clostridium perfringens*, bifidobacteria, and lactobacilli were quantified via quantitative polymerase chain reaction (qPCR) analysis using specific primers. Amplification was performed on a set of triplicate reactions for each bacterial group within each sample according to the procedures previously described by Hernot et al. (2009).

Results

***In vitro* digestion experiment**

Short-chain fatty acid production profiles resulting from fermentation of cyclodextrins are presented in Table 4.3. Total SCFA production profiles are presented in Figures

Figure 4.1. Maximal production of total SCFA was highest ($P < 0.05$) for BCD, followed by GCD and ACD (values were statistically similar). However, the maximal rate of production was numerically highest for GCD, which also attained maximal rate of production most rapidly ($P < 0.05$) followed by BCD. The ACD exhibited the numerically lowest maximal rate of production and took the longest time to reach it ($P < 0.05$).

Acetate production (Figure 4.2) followed the same basic trend as total SCFA production, with BCD exhibiting the highest maximal acetate production but with GCD having the highest maximal rate of acetate production and the shortest time to attain it.

Again, ACD exhibited the lowest maximal acetate production, the lowest maximal rate of acetate production, and took the longest time to reach it.

Propionate production followed a very distinctive profile (Figure 4.3), with ACD resulting in the highest maximal propionate production ($P<0.05$), the highest (numerical) maximal rate of propionate production, and the longest time to attain maximal rate of production ($P<0.05$).

Butyrate production profiles (Figure 4.4) also were unique, with GCD resulting in the highest maximal butyrate production ($P<0.05$) and the numerically highest maximal rate of production, and with a time to attain maximal rate of production intermediate to those of BCD and ACD.

Changes in pH after *in vitro* fermentation of ACD, BCD, and GCD are presented in Table 4.4. The pH was 6.9 for all cyclodextrins at the 0 h pull time. After 3 h of fermentation, both BCD and GCD exhibited a greater change in pH (-0.5) than ACD (-0.3). At the 6-h pull time, GCD had the greatest pH change (-1.8), followed by BCD (-1.4), then ACD (-1.1). Changes in pH after fermentation for 9 and 12 h were not different among treatments or between these two pull times.

Ileal and total tract nutrient digestibility

Body weight and BCS of dogs were not different among treatments (data not shown). Intake and digestibilities by dogs of diets supplemented with ACD are presented in Table 4.5. Average daily food intakes were similar among treatments throughout the study, with most of the dogs ingesting all the food they were provided. However, there was a cubic effect on intake because of ACD supplementation as dogs ingesting 2 and 8 g ACD/d had higher intakes than dogs ingesting 4 and 6 g ACD/d. Ileal digestibility of DM, OM, CP, and

AHF were not different among treatments. On the contrary, total tract digestibilities of DM, OM, CP, and AHF exhibited a linear decrease as ACD intake increased.

Ileal DM values and fecal characteristics of dogs are presented in Table 4.6. Ileal DM concentration increased linearly ($P=0.01$) as ACD intake increased. Fecal output expressed on an as-is basis ($P<0.01$), on a DM basis ($P=0.03$), and on an as-is per g DM intake basis increased linearly ($P<0.01$) with increasing ACD supplementation. At the same time, fecal DM content decreased linearly ($P<0.01$) and, consequently, fecal scores increased linearly ($P=0.01$).

Quantification by plating of fecal bifidobacteria and lactobacilli from dogs fed diets supplemented with ACD are presented in Table 4.7. There was a numerical increase in both bifidobacteria ($P=0.11$) and lactobacilli ($P=0.12$). Fecal microbiota quantified by qPCR for dogs fed diets supplemented with ACD are presented in Table 4.8. Bifidobacteria, *Clostridium perfringens*, and *E. coli* were not different among treatments. Total microbe quantification using a universal primer was not different among treatments either (data not shown). Lactobacilli exhibited a quadratic ($P=0.04$) and a cubic ($p=0.02$) response to increasing ACD intake.

Serum cholesterol and triglyceride concentrations, and changes in concentration, for dogs supplemented with ACD are shown in Table 4.9. Serum cholesterol and triglyceride concentrations were within normal ranges for dogs and were not different among treatments. Changes in concentration were not different from zero ($P>0.05$) and were similar among treatments.

Discussion

Fermentation of ACD resulted in a unique SCFA pattern, with greater production of propionate than of acetate. In general, *in vitro* fermentation of different fiber sources yields acetate as the major end-product, comprising 67% of total SCFA (Cummings, 1997). This high production of propionate when ACD is fermented could partially explain the hypocholesterolemic effect observed in previous experiments as propionate can inhibit the enzyme, HMG-CoA reductase (Levrat et al., 1994), the key regulatory enzyme for endogenous cholesterol synthesis. Although ACD had the highest maximal rate of propionate production, the time to attain maximal production was greatest for ACD relative to BCD and GCD.

The less rigid structure of GCD favors its microbial fermentation (Oros et al., 1990). Changes in pH and time to attain maximal production of total SCFA indicate that GCD is more readily fermented than BCD, which, in turn, is fermented faster than ACD. In general, pH decreased as fermentation time increased. However, there was no change in pH between the 9-h and 12-h pull times. This lack of difference observed between these pull times indicates lack of further fermentation because of the depletion of substrates and the potential build-up of waste products. The lack of difference in pH change among groups after 9 and 12 h of fermentation indicates that all substrates were equally fermented at those pull times.

Comparative literature on this topic is scarce. Spears et al. (2007) evaluated the fermentation characteristics of select glucose-based polymers by canine and human fecal bacteria. Substrates included lyophilized canine ileal digesta containing maltodextrin, GCD, high molecular weight (MW) pullulan, and low MW pullulan as dogs had been fed these test substances in another study. Maltodextrin-containing ileal digesta exhibited a higher

butyrate and total SCFA production than did GCD- or pullulan-containing ileal digesta. However, there were no differences noted in time to attain maximal rate of production for acetate, propionate, or butyrate.

Apparent total tract digestibility quantifies the difference between amounts of nutrients consumed by the animal and those excreted in feces. However, feces contain a variable quantity of substances of non-dietary origin (e.g., spent enzymes, gastrointestinal tract secretions, sloughed mucosal cells, and microbes). Therefore, to more accurately evaluate nutrient digestibility, the ileal cannulated dog was used as an animal model for the human. Because both species are omnivorous monogastrics, nutrient digestibilities often are similar. Furthermore, the ileal cannulated dog model has been used in several studies to evaluate ileal digestibility of diets containing a variety of carbohydrate sources (Murray et al., 1998; Flickinger et al., 2000; Bednar et al., 2001; Spears et al., 2005). The diet used in this experiment was formulated to simulate a human diet. The Dietary Reference Intakes (2006) document recommends that protein supply 10-35% of the total energy intake and that fat supply 20-35%. In this experiment, protein supplied approximately 31% and fat 36% of the total dietary energy. Therefore, the experimental diet meets the nutrient specifications for an adult human.

Ileal digestibility coefficients for DM, OM, CP, and AHF were high and comparable to previously reported digestibility coefficients for diets with similar ingredient matrices (Middelbos et al., 2007; Faber et al., 2011). Intake of ACD did not alter ileal nutrient digestibility. Other studies have reported nutrient digestibility unaltered by ACD intake. Artiss et al. (2006) studied the effects of ACD on weight reduction and blood lipids in rats. Rats were divided into 4 groups and fed ad libitum for a period of 6 weeks a low fat (LF; 4%

fat) diet, LF+ACD (0.4%), high fat (HF; 40% fat), and HF+ACD (4%). Intake of ACD did not alter fecal excretion of fat between the LF diets (0.16 g) or between the HF diets (0.29 g). Spears et al. (2005) evaluated the effect of GCD on ileal and total tract apparent nutrient digestibilities by dogs. Intake of GCD did not affect ileal digestibility of DM, OM, or CP, but fat digestibility was reduced two percentage units by GCD at a 25% dietary inclusion rate (~120 g/d).

Total tract digestibility of all nutrients decreased linearly, even though there was no difference in ileal nutrient digestibility. Differences between ileum and feces represent the contribution of the large bowel to total tract nutrient digestibility. Because dietary protein and fat are scarcely absorbed by the large intestine (Nordgaard and Mortensen, 1995), differences between apparent ileal and total tract nutrient digestibilities can be explained by modifications caused by microbes in the hindgut. Microbial biomass represents approximately 50% of feces (Cummings, 1997), and microbial growth requires protein and fatty acids, which they can synthesize from carbon skeletons and non-protein nitrogen. Therefore, microbes are considered the primary source of endogenous fat and protein excreted in feces. As previously reported, ACD is not degraded by hydrolytic-enzymatic digestion, but is partially fermentable in the large intestine (Del Valle, 2004). Fermentation of ACD may increase the synthesis of endogenous fat and CP by microbes, which, in turn, will increase endogenous losses of fat and CP and reduce CP and AHF apparent total tract digestibilities (Kil et al., 2010). At the same time, this reduction in CP and AHF apparent digestibility lowers DM and OM digestibility and, thus, increases fecal output. Another consequence of ACD fermentation is the production of SCFA, which influences fecal DM (Sunvold et al., 1995). Short-chain fatty acids are rapidly and almost completely absorbed

by non-ionic diffusion mechanisms, but also by active transport mediated by a sodium-coupled transporter, thereby fostering the absorption of sodium and water (Roy et al., 2006). However, because SCFA are weak anions, they may exert osmotic pressure in the colon and increase fecal water content (Roberfroid, 1993), thus explaining the decrease in fecal DM and subsequent increase when ACD was supplemented.

Fermentability of ACD and its particular SCFA production profile might indicate possible changes in hindgut microbiota. Therefore, in order to screen for changes in microbiota, lactobacilli and bifidobacteria were quantified by plating in the control treatment and the treatment with the highest ACD intake (8 g). Bifidobacteria CFU were almost 1 log unit greater in the group receiving ACD, whereas lactobacilli were 0.8 log units greater in the same group. Quantification by qPCR did not find significant effects of ACD intake on bifidobacteria, *Escherichia coli*, or *Clostridium perfringens*. However, there was a quadratic and cubic effect of ACD intake on lactobacilli. This effect is evident by the decrease in lactobacilli for dogs receiving 6 g ACD, whereas there was an increase for those receiving 8 g. Even though this effect reached statistical significance, the biological significance of this effect is difficult to determine. However, it is important to note that what was quantified in this experiment was total CFU of bifidobacteria and lactobacilli. Therefore, it is possible that ACD might alter composition of microbial populations within those genera, but not alter total CFU values.

The lack of effect of ACD intake on serum cholesterol concentrations is consistent with previous experiments performed in our laboratory (see chapter 3). It has been reported that the lack of cholesterol ester transfer protein (CETP) activity in dogs results in high concentrations of HDL-cholesterol, which facilitates the re-direction of cholesterol to the

liver for clearance (Bailhache et al., 2004), thus making the dog a poor model for studying cholesterol metabolism in humans.

In summary, intake of ACD did not alter serum lipid concentrations or ileal nutrient digestibility values, but resulted in a linear decrease in total tract nutrient digestibility, indicating active ACD fermentation in the large bowel. It is very likely that such fermentation produces a higher proportion of propionate as observed in the *in vitro* fermentation of ACD. Therefore, ACD supplementation appears not to decrease nutrient absorption in the small intestine and its potential in modifying serum lipid profiles might be at least partially due to an altered fermentation in the large bowel. However, further studies with more suitable animal models, or humans themselves, are needed to fully elucidate the mechanisms for the hypocholesterolemic effect of ACD.

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Tables

Table 4.1. Composition of medium used for *in vitro* fermentation of cyclodextrins.

Component	Concentration in medium
Solution A, ml/L ¹	330.0
Solution B, ml/L ²	330.0
Trace mineral solution, ml/L ³	10.0
Water-soluble vitamin mix, ml/L ⁴	20.0
Folate/biotin solution, ml/L ⁵	5.0
Riboflavin solution, ml/L ⁶	5.0
Hemin solution, ml/L ⁷	2.5
Short-chain fatty acid mix, ml/L ⁸	0.4
Resazurin, ml/L ⁹	1.0
Distilled water, ml/L	296.0
Yeast, g/L	0.5
Trypticase, g/L	0.5
Na ₂ CO ₃ , g/L	4.0
Cysteine HCl · H ₂ O, g/L	0.5

¹Composition, g/L: NaCl, 5.4; KH₂PO₄, 2.7; CaCl₂ · H₂O, 0.18; MgCl₂ · 6H₂O, 0.12; MnCl₂ · 4H₂O, 0.06; CoCl₂ · 6H₂O, 0.06; and (NH₄)₂SO₄, 5.4.

²Composition: K₂HPO₄, 2.7 g/L.

Table 4.1. (Cont.)

³Composition, mg/L: EDTA (disodium salt), 500; FeSO₄ · 7H₂O, 200; ZnSO₄ · 7H₂O, 10; MnCl₂ · 4H₂O, 3; H₃PO₄, 30; CoCl₂ · 6H₂O, 20; CuCl₂ · 2H₂O, 1; NiCl₂ · 6H₂O, 2; Na₂MoO₄ · 2H₂O, 3.

⁴Composition, mg/L: thiamin · HCl, 100; d-pantothenic acid, 100; niacin, 100; pyridoxine, 100; p-aminobenzoic acid, 5; vitamin B₁₂, 0.25.

⁵Composition, mg/L: folic acid, 10; d-biotin, 2; NH₄HCO₃, 100.

⁶Composition: riboflavin, 10 mg/L in 5 mmol/L of HEPES.

⁷Hemin, 500 mg/L in 10 mmol/L NaOH.

⁸250 ml/L each of n-valerate, isovalerate, isobutyrate, and DL- α -methylbutyrate.

⁹Resazurin, 1 g/L in distilled H₂O.

Table 4.2. Ingredient (% as-fed basis) and chemical (% dry matter basis) composition of the experimental diet.

Item	%
Poultry byproduct meal, low ash	39.00
Poultry fat	14.00
Corn, yellow, ground	10.00
Brewer's rice	28.85
Solka floc ^{®1}	6.50
Mineral premix ²	0.10
Vitamin premix ³	0.10
Choline chloride	0.10
Salt	0.65
Potassium chloride	0.50
Chromic oxide	0.20
<i>Analyzed chemical composition</i>	
Dry matter	92.60
Organic matter	91.70
Crude protein	30.60
Acid hydrolyzed fat	20.16
Total dietary fiber	13.70
Gross energy, kcal/kg	5,215.01

¹International Fiber Corporation, North Tonawanda, NY.

Table 4.2. (Cont.)

²Provided per kilogram of diet: 120 mg iron (FeSO₄); 66 mg manganese (MnO); 18 mg copper (CuSO₄); 1.8 mg iodine (C₂H₆N₂·2HI); 240 µg selenium (Na₂SeO₃); 240 mg zinc (ZnO).

³Provided per kilogram of diet: 10,560 IU vitamin A (vitamin A acetate); 1056 IU vitamin D (vitamin D₃); 105 IU vitamin E (dl- α -tocopherol); 0.53 mg vitamin K (menadione sodium bisulfate complex); 2.64 mg thiamine (thiamine mononitrate); 23.76 mg niacin (niacin supplement); 3.43 mg riboflavin (riboflavin supplement); 13.2 mg pantothenic acid (d-calcium pantothenate); 66 µg vitamin B₁₂ (vitamin B₁₂ supplement); 2.11 mg pyridoxine (pyridoxine hydrochloride); 79 µg biotin (D-biotin supplement); 264 µg folic acid (folic acid supplement).

Table 4.3. Short-chain fatty acid production profiles during *in vitro* fermentation of cyclodextrins.

Item	Cyclodextrin			SEM ¹
	α	β	γ	
	-----Total SCFA-----			
Maximal production, $\mu\text{mol/g DM}$	6,655.8 ^b	7,326.4 ^a	6,927.2 ^b	91.1
Duration of production, h	1.25 ^a	1.24 ^a	0.98 ^b	0.07
Maximal rate of production, $\mu\text{mol/h/g DM}$	1,331.2	1,477.1	1,767.1	N/E ²
Time to attain maximal rate of production, h	6.4 ^a	4.9 ^b	4.2 ^c	0.1
	-----Acetate-----			
Maximal production, $\mu\text{mol/g DM}$	2,763.9 ^c	3,786.1 ^a	3,463.5 ^b	33.0
Duration of production, h	1.39 ^a	1.15 ^b	0.91 ^c	0.05
Maximal rate of production, $\mu\text{mol/h/g DM}$	497.1	823.1	951.5	N/E ²
Time to attain maximal rate of production, h	5.7 ^a	4.5 ^b	3.9 ^c	0.1
	-----Propionate-----			
Maximal production, $\mu\text{mol/g DM}$	3,409.6 ^a	2,895.6 ^b	2,360.5 ^c	51.9
Duration of production, h	0.96 ^{ab}	1.26 ^a	0.75 ^b	0.09
Maximal rate of production, $\mu\text{mol/h/g DM}$	887.9	574.5	786.8	N/E ²
Time to attain maximal rate of production, h	6.7 ^a	5.4 ^b	4.1 ^c	0.1
	-----Butyrate-----			
Maximal production, $\mu\text{mol/g DM}$	423.6 ^c	644.4 ^b	1,133.1 ^a	15.2
Duration of production, h	1.58	1.37	1.41	0.12
Maximal rate of production, $\mu\text{mol/h/g DM}$	67	117.6	200.9	N/E ²
Time to attain maximal rate of production, h	6.3 ^a	5 ^c	5.7 ^b	0.1

Table 4.3. (Cont.)

^{a,b,c} Estimates in the same row with unlike superscript letters differ ($P < 0.05$).

¹SEM = pooled standard error of the mean.

²N/E = Not estimable.

Table 4.4. Changes in pH after *in vitro* fermentation of α -cyclodextrin, β -cyclodextrin, and γ -cyclodextrin¹.

Pull time, h	Cyclodextrin			SEM ²
	α	β	γ	
3	-0.3 ^a	-0.5 ^b	-0.5 ^b	0.03
6	-1.1 ^a	-1.4 ^b	-1.8 ^c	0.03
9	-2.0	-1.9	-2.0	0.03
12	-2.1	-2.0	-2.0	0.03

^{a,b,c} Means in the same row with unlike superscript letters differ (P<0.05).

¹Change was measured between pH at 0 h and pH at each pull time.

²SEM = pooled standard error of the mean.

Table 4.5. Intake (g/d, as-fed basis) and ileal and total tract nutrient digestibilities (%) by dogs fed diets supplemented with α -cyclodextrin (ACD).

Item	Treatment					SEM ¹	Contrasts			
	Control	2 g ACD	4 g ACD	6 g ACD	8 g ACD		Linear	Quadratic	Cubic	Quartic
Intake	266.7	290.2	274.1	271.8	297.5	14.5	0.48	0.97	0.05	0.27
----- Ileal digestibility, % -----										
Dry matter	69.2	69.8	67.1	66.6	68.2	2.13	0.24	0.43	0.22	0.62
Organic matter	75.0	75.4	72.6	71.7	73.5	2.09	0.16	0.39	0.22	0.72
Crude protein	67.5	71.0	69.2	69.6	71.6	3.39	0.20	0.90	0.19	0.57
Acid hydrolyzed fat	93.5	94.6	93.0	93.1	93.8	0.88	0.64	0.77	0.12	0.31
----- Total tract digestibility, % -----										
Dry matter	77.5	77.5	77.7	76.5	76.3	0.49	0.02	0.27	0.51	0.22
Organic matter	82.5	82.4	82.3	81.4	81.1	0.44	<0.01	0.39	0.68	0.46
Crude protein	84.0	83.0	83.2	82.0	81.3	0.53	<0.01	0.44	0.49	0.09
Acid hydrolyzed fat	94.3	94.5	94.1	93.5	93.5	0.19	<0.01	0.33	0.04	0.81

¹SEM = pooled standard error of the mean.

Table 4.6. Ileal dry matter values (%) and fecal characteristics of dogs fed diets supplemented with α -cyclodextrin (ACD).

Item	Treatment					SEM ¹	Contrasts			
	Control	2 g ACD	4 g ACD	6 g ACD	8 g ACD		Linear	Quadratic	Cubic	Quartic
Ileal dry matter, %	13.4	13.4	14.7	15.8	15.3	1.10	0.01	0.60	0.16	0.93
----- Fecal characteristics -----										
Fecal output (as-is), g/d	122.7	139.4	135.0	142.4	159.6	8.47	<0.01	0.50	0.07	0.41
Fecal output (DM), g/d	55.5	60.5	56.4	59.0	65.2	3.17	0.03	0.33	0.12	0.36
Fecal output (as-is) per g DM										
consumed	0.50	0.52	0.53	0.57	0.58	0.02	<0.01	0.95	0.76	0.43
Fecal DM, %	45.5	43.4	41.9	41.6	41.0	0.94	0.17	<0.01	0.11	0.28
Fecal score ²	2.6	2.7	2.8	2.8	2.8	0.09	0.01	0.49	0.70	0.98

¹SEM = pooled standard error of the mean.

² Scores based on the following scale: 1 = dry, hard pellets; 2 = dry, well-formed stool; 3 = soft, moist, formed stool; 4 = unformed stool; 5 = watery, liquid that can be poured.

Table 4.7. Fecal colony forming units (log CFU/g feces) of bifidobacteria and lactobacilli quantified by plating techniques for dogs fed diets supplemented with 8 g α -cyclodextrin (ACD)/d.

Item	Treatment		SEM ¹	P-value
	Control	8 g ACD		
Bifidobacteria	7.74	8.23	0.13	0.11
Lactobacilli	8.39	9.19	0.36	0.12

¹SEM = pooled standard error of the mean.

Table 4.8. Fecal microbiota (log CFU/g DM feces) quantified by quantitative polymerase chain reaction for dogs fed diets supplemented with α -cyclodextrin (ACD).

Item	Treatment					SEM ¹	Contrasts			
	Control	2 g ACD	4 g ACD	6 g ACD	8 g ACD		Linear	Quadratic	Cubic	Quartic
Bifidobacteria	8.8	8.7	8.9	8.7	8.8	0.24	0.92	0.87	0.83	0.18
Lactobacilli	10.2	10.2	10.2	9.6	10.8	0.21	0.34	0.04	0.02	0.10
<i>Escherichia coli</i>	11.5	11.6	11.6	11.4	11.4	0.19	0.19	0.31	0.24	0.85
<i>Clostridium perfringens</i>	10.6	10.3	10.5	10.5	10.3	0.30	0.66	0.95	0.29	0.83

¹SEM = pooled standard error of the mean.

Table 4.9. Serum cholesterol and triglyceride concentrations, and changes in concentration, for dogs supplemented with α -cyclodextrin (ACD)¹.

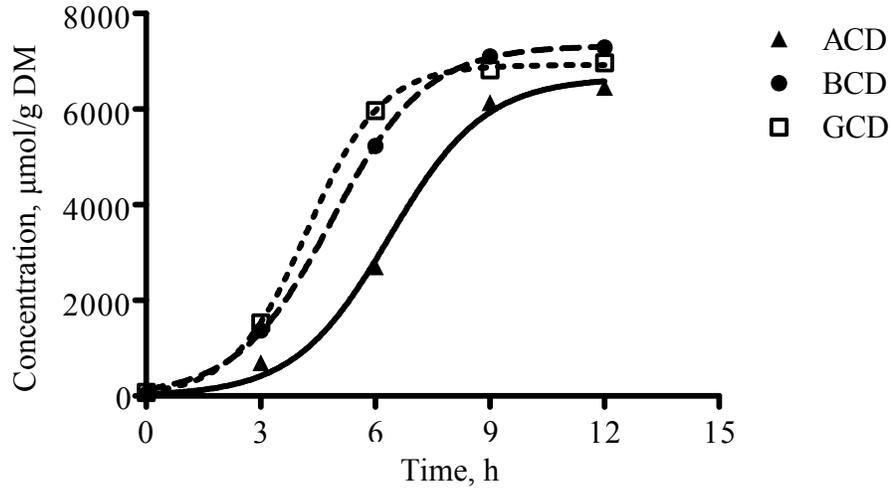
Item	Treatment					SEM ²	Contrasts			
	Control	2 g ACD	4 g ACD	6 g ACD	8 g ACD		Linear	Quadratic	Cubic	Quartic
Serum cholesterol, mmol/dL	5.5	5.5	5.5	5.4	5.4	0.39	0.74	0.94	0.93	0.71
Serum triglycerides, mmol/dL	0.6	0.6	0.5	0.6	0.6	0.05	0.48	0.98	0.38	0.08
Δ Serum cholesterol, mmol/dL	-0.1	0.5	0.1	0.1	0.3	0.29	0.86	0.74	0.22	0.58
Δ Serum triglycerides, mmol/dL	0.0	-0.1	0.2	0.0	0.0	0.08	0.87	0.31	0.31	0.08

¹Concentrations were measured on d-14 of each period. Change was measured between d-1 and 10 of each period.

²SEM = pooled standard error of the mean.

Figures

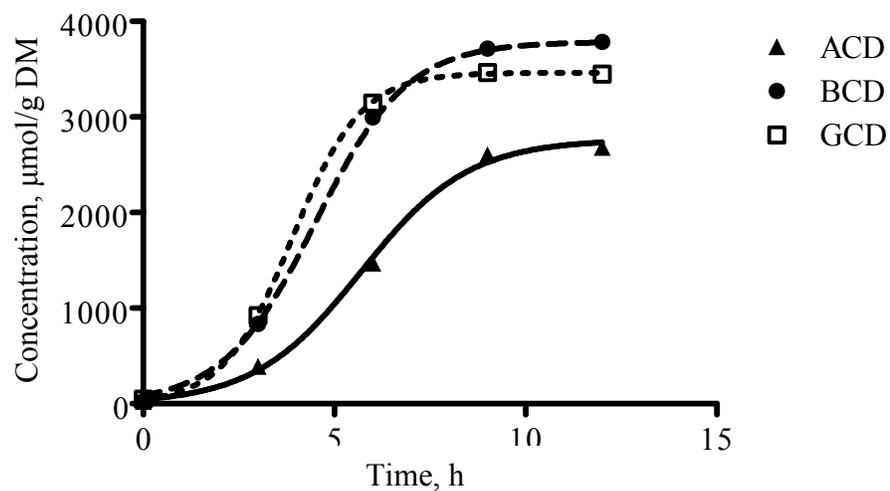
Figure 4.1. Total short-chain fatty acid production profiles during 12 h of *in vitro* fermentation of α -cyclodextrin (ACD), β -cyclodextrin (BCD), and γ -cyclodextrin (GCD)^{1,2}



¹n=3.

²Symbols represent means of the observed values. Lines represent curve fitting procedures using parameters determined by non-linear regression.

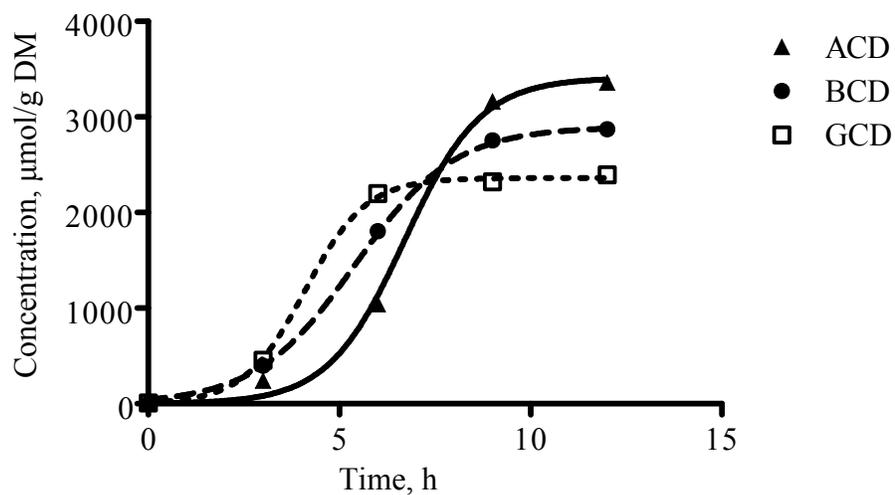
Figure 4.2. Acetate production profiles during 12 h of *in vitro* fermentation of α -cyclodextrin (ACD), β -cyclodextrin (BCD), and γ -cyclodextrin (GCD)^{1,2}



¹n=3.

²Symbols represent means of the observed values. Lines represent curve fitting procedures using parameters determined by non-linear regression.

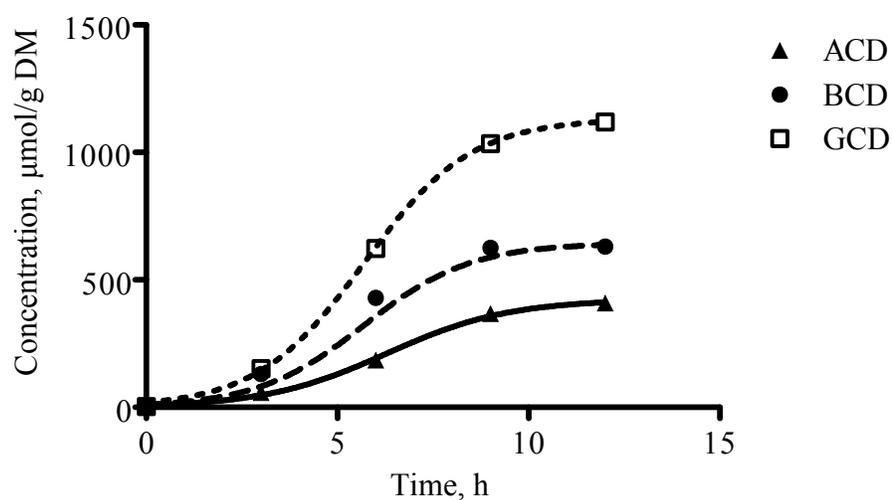
Figure 4.3. Propionate production profiles during 12 h of *in vitro* fermentation of α -cyclodextrin (ACD), β -cyclodextrin (BCD), and γ -cyclodextrin (GCD)^{1,2}



¹n=3.

²Symbols represent means of the observed values. Lines represent curve fitting procedures using parameters determined by non-linear regression.

Figure 4.4. Butyrate production profiles during 12 h of *in vitro* fermentation of α -cyclodextrin (ACD), β -cyclodextrin (BCD), and γ -cyclodextrin (GCD)^{1,2}



¹n=3.

²Symbols represent means of the observed values. Lines represent curve fitting procedures using parameters determined by non-linear regression.

CHAPTER 5

EFFECTS OF α -CYCLODEXTRIN SUPPLEMENTATION OF HAMSTERS ON APPARENT NUTRIENT DIGESTIBILITY, BILE ACID EXCRETION, CECAL MICROBIOTA AND SHORT-CHAIN FATTY ACID CONCENTRATIONS, SERUM LIPID PROFILES, AND LIVER GENE EXPRESSION

Abstract

The objectives were to examine apparent nutrient digestibility, bile acid excretion, cecal microbiota and short-chain fatty acid (SCFA) concentrations, serum lipid profiles, and liver gene expression in hamsters as affected by α -cyclodextrin (ACD) supplementation. Forty Golden hamsters (*Mesocricetus auratus*) were used in a completely randomized experimental design with a 2 x 2 factorial arrangement of treatments (two ACD concentrations [0, 2%] and two supplemental cholesterol concentrations [0, 0.5%]). The control consisted of a semipurified diet with casein, cornstarch, lard, and soybean oil as the major ingredients. To produce the treatment diets, a portion of the cornstarch was replaced with 2% ACD (A), 0.5% cholesterol (C), or 2% ACD and 0.5% cholesterol (AC). Body weight gain, apparent nutrient digestibility, and fecal characteristics were not different among treatments. Fecal bile acid excretion was increased ($P<0.01$) by cholesterol intake, but not by ACD intake. Cecum and cecal content weights were increased ($P<0.01$) by ACD intake, whereas cholesterol intake tended ($P=0.08$) to decrease cecal content weight expressed on a dry matter (DM) basis. The 2% ACD alone resulted in higher propionate concentrations in cecal contents. Total CFU of lactobacilli ($P=0.04$) and total microbes ($P=0.03$) in cecal contents were greater for hamsters ingesting ACD than for those not ingesting ACD. Intake of diets containing ACD tended ($P=0.10$) to increase expression of

7 α -hydroxylase (CYP7A1), whereas intake of diets containing cholesterol resulted in a reduction ($P=0.01$) in expression of HMG-CoA reductase. α -Cyclodextrin intake resulted in reduced cholesterol concentrations in the serum of normocholesterolemic hamsters, but failed to reduce cholesterol concentrations of hamsters fed diets supplemented with 0.5% cholesterol. The hypocholesterolemic effect of ACD appears to result from a combination of factors including up-regulation of cholesterol degradation through CYP7A1, changes in hindgut fermentation profiles, and, perhaps, changes in hindgut microbiota.

Introduction

Hyperlipidemia has been clearly linked to the risk of cardiovascular disease (CVD; Law et al., 1994). Cholesterol-lowering medications are widely used for this reason; however, nutritional interventions provide unique opportunities to decrease CVD risk throughout life. Cyclodextrins are cyclic oligosaccharides comprised of glucopyranose units in a cylinder shape. The family of cyclodextrins includes α -cyclodextrin (ACD) with 6 glucose units, β -cyclodextrin (BCD) with 7, and γ -cyclodextrin (GCD) with 8 (Szejtli, 1998). Cyclodextrins have been reported to decrease blood cholesterol concentrations in many species, including humans (Kaewprasert et al., 2001; Garcia-Mediavilla et al., 2003; Wagner et al., 2008; Comerford et al., 2011). More specifically, intake of ACD decreased serum cholesterol concentrations in hypercholesterolemic dogs. However, cholesterol concentrations in serum were unaffected in dogs with normal cholesterol concentrations (see chapter 3).

How ACD lowers plasma cholesterol concentrations is not completely defined although several hypotheses have been proposed. These hypotheses include binding or sequestration of bile acids leading to an increase in fecal bile acid excretion, formation of

fat-ACD complexes leading to lipid malabsorption (Wagner et al., 2008), and suppression of hepatic sterol synthesis by ACD fermentation products. *In vitro* fermentation of ACD resulted in greater propionate production compared with acetate and butyrate (see chapter 4). Interestingly, propionate has been reported to reduce cholesterol biosynthesis (Levrat et al., 1994). It also has been hypothesized that changes in microbial populations due to ACD fermentation could lead to an increase in excretion of bile acids. However, the data currently available are inconsistent, suggesting that multiple mechanisms may contribute to the hypocholesterolemic effects of ACD.

The lipoprotein profile of the hamster resembles more closely that of humans than do the profiles of rats (Zhang et al., 2009). In addition, the hamster has plasma cholesteryl ester transfer protein (CETP) activity similar to that of humans, whereas other animal models like the dog, rabbit, rat, and mouse have virtually no plasma CETP activity (Tsutsumi et al., 2001; Bailhache et al., 2004). Therefore, hamsters are the preferred model to study efficacy of cholesterol-lowering functional foods.

Feeding ACD to hypercholesterolemic and normocholesterolemic hamsters may elucidate possible mechanisms of action of ACD on cholesterol metabolism. The objectives of this study were to evaluate apparent nutrient digestibility, fecal excretion of bile acids, short-chain fatty acids (SCFA) and microbiota in cecal contents, serum lipid profiles, and liver gene expression of hamsters as affected by ACD and cholesterol supplementation.

Materials and Methods

Animals

Forty Golden hamsters (*Mesocricetus auratus*; Harlan Sprague-Dawley, Inc.; Indianapolis, IN) with an average age of 6 wk and an average starting body weight (BW) of

98.1 g (SD=4.2) were used in this experiment. Hamsters were housed individually in wire-bottom cages in an environmentally controlled facility (22°C; 23% relative humidity) with a 12 h light:12 h dark cycle. The University of Illinois Institutional Animal Care and Use Committee approved all animal care procedures prior to initiation of the experiment. Hamsters were weighed and health condition assessed throughout the experiment.

Diets and treatments

Commercial food-grade α -cyclodextrin (ACD) (purity 99.4%) manufactured by Wacker Fine Chemicals, Adrian, MI (batch number 60F212) was obtained from Abbott Nutrition, Columbus, OH. Cholesterol (purity 95%) manufactured by Dishman Netherlands B.V. (Veenendaal, Netherlands) also was used in this experiment.

Four experimental diets were formulated to meet or exceed the National Research Council (1995) nutrient profiles for growing hamsters. The control consisted of a semipurified diet with casein, cornstarch, lard, and soybean oil as the major ingredients. To produce the treatment diets, a portion of the cornstarch was substituted with 2% ACD (diet A), 0.5% cholesterol (diet C), or 2% ACD and 0.5% cholesterol (diet AC). Complete ingredient and chemical composition data for the diets are presented in Table 5.1. Diets were prepared by Research Diets, Inc. (New Brunswick, NJ).

Hamsters were fed *ad libitum*. Food refusals from the previous feeding were collected and weighed daily. Hamsters also had *ad libitum* access to fresh water.

Experimental design

The experimental design was a completely randomized design with a 2 x 2 factorial arrangement of treatments, with two ACD concentrations (0, 2%) and two cholesterol concentrations (0, 0.5%). Hamsters were allowed 7 d for adaptation to the facilities. During

this time they were fed the control diet, and then on d-8, hamsters were randomized into one of four experimental treatments that they were fed for 28 d. Hamsters were weighed and health status checked at arrival and then weekly until the end of the experiment.

Sampling procedures

A sample of approximately 500 g was taken from each diet used in this experiment. Samples were ground in a Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ) through a 2-mm screen and stored at 4°C until analysis.

On d 23 to 28, feces were collected from the pan below the cage and stored at -20°C until composited and ground for analysis. Hamsters were divided into three groups. Starting at 0500 on d 28, any remaining food was removed every hour from a group of hamsters. Animals were fasted for 6 h, during which time they could consume only water. Hamsters were weighed and anesthetized by intraperitoneal injection of a ketamine (200 mg/kg) and xylazine (10 mg/kg) mixture. Once animals had lost consciousness, blood was collected via cardiac puncture until death by exsanguination. Blood was drawn into vacutainer serum separator tubes and kept on ice until processing and centrifuged at 1,240 x g at 4°C for 10 min. Serum supernatant was collected and stored at -20°C for analyses.

After hamsters were euthanized, the cecum was clamped, removed, and weighed. Cecal contents then were squeezed through an incision and distributed into the following containers for storage and analysis: ~0.1 g in pre-weighed aluminum pans for DM; ~ 0.2 g in microfuge tubes with three volumes (~ 0.6 ml) of HCl (0.67 N) and stored at -20 °C for SCFA; the remainder of the cecal contents was placed in labeled cryovials, flash frozen in liquid nitrogen, and stored at -80 °C for bacterial analyses. The emptied cecum was washed

with distilled water and weighed. Samples of liver were taken, flash frozen in liquid nitrogen, and stored at -80°C for subsequent analyses.

Chemical analyses

Frozen feces were lyophilized in a Dura-Dry MP microprocessor-controlled freeze-dryer (FTS Systems, Stone Ridge, NY). Once dry, feces were ground in a Wiley mini-mill (3383-L10 Series, Thomas Scientific, Swedesboro, NJ) through a 1-mm screen.

Dry matter (DM) and organic matter (OM) concentrations were determined according to AOAC (2002) methods 934.01 and 942.05, respectively. Acid hydrolyzed fat (AHF) concentrations were determined using acid hydrolysis according to AAAC (2000) method 30-14.01, followed by ether extraction (Budde, 1952). Crude protein (CP) concentrations were calculated using LECO[®] (nitrogen analyzer model FP-2000, Leco Corporation, St Joseph, MI) nitrogen (N) values ($N \times 6.25$) (AOAC, 2002). Gross energy (GE) concentrations of diets were measured using oxygen bomb calorimeter (model 1261, Parr Instruments, Moline, IL).

Samples to be analyzed for SCFA were thawed and mixed with 0.5 ml of 250 g/L m-phosphoric acid, precipitated at room temperature for 30 min, and then centrifuged at 20,100 x g at 4°C for 20 min. The supernatant was decanted and frozen at -20°C in microfuge tubes. After freezing, the supernatant was thawed and centrifuged in microfuge tubes at 13,000 x g at 20°C for 10 min. The supernatant then was transferred to cryovials and stored at -20 °C until analysis. Concentrations of SCFA and branched-chain fatty acids (BCFA) were determined using gas-liquid chromatography. Briefly, concentrations of acetate, propionate, butyrate, isobutyrate, isovalerate, and valerate were determined in the supernatant of the tubes using a Hewlett-Packard 5890A Series II gas-liquid chromatograph

and a glass column (180 cm x 64 mm i.d.) packed with 10% SP-1200/1% H₃PO₄ on 80/100 mesh Chromosorb WAW (Supelco Inc., Bellefonte, PA). Short-chain fatty acid concentrations were corrected for the quantities of SCFA produced in the blank tubes.

Serum total cholesterol (TC) and triglyceride (TG) concentrations were measured on a Hitachi 917 analyzer (Roche Diagnostica, Indianapolis, IN) using enzymatic kits (catalog numbers 2016630 and 2016648, respectively).

Bile acids in feces were extracted and purified according to the procedure described by Locket and Gallaher (1989). Briefly, 2 ml of ethanol were added to 100 mg of sample in screw-cap tubes and incubated for 30 min at room temperature. Tubes were refluxed at 100°C for 15 min, centrifuged at 1,500 x g at 4°C for 10 min, and supernatant removed and retained. The pellet was resuspended in 2 ml 80% ethanol, refluxed, centrifuged as before, and supernatant pooled with the previous one. The pellet was resuspended in 2 ml chloroform/methanol (1:1, v/v), followed by reflux, centrifugation, and removal of supernatant. Pooled supernatants were dried under argon gas and stored at -20°C until purification. For partial purification, pellets were resuspended in 0.6 ml methanol and 2.4 ml distilled water (DW). Reverse-phase C18 (500 mg) bonded-phase cartridges (Sep-pak, Waters Corporation, Milford, MA) were solvated with 4 ml methanol, followed by 4 ml DW. Then, the sample solution was loaded into the cartridge and successively washed with DW and hexane, and eluted with methanol. Eluents were dried under argon gas and stored at -20°C until quantification.

Bile acids were quantified using enzymatic methods as described by MacDonald (1976). The enzyme, 3 α -hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* (Catalog No. H1506, Sigma-Aldrich, St. Louis, MO), was used to oxidize the 3 α -hydroxyl

group of bile acids in the presence of NAD. The yield of NADH was estimated by the increase in absorbance at 340 nm on a Beckman DU-640 spectrophotometer (Beckman Coulter Inc., Brea, CA), and bile salt concentrations calculated based on a standard curve using cholic acid.

Microbiota quantification

Bacterial DNA from cecal contents was purified using QIAamp DNA stool mini kits (Qiagen, Valencia, CA) using the repeated bead beating plus column (RBB+C) method described by Yu and Morrison (2004). Fecal DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). *Escherichia coli*, bifidobacteria, and lactobacilli were quantified via quantitative polymerase chain reaction (qPCR) analysis using specific primers, whereas for the total population of bacteria, a universal primer set (341F/534R; Applied Biosystems, Foster City, CA) was used. Amplification was performed on a set of triplicate reactions for each bacterial group within each sample according to the procedures previously described by Hernot et al. (2009).

Gene expression

Total RNA was extracted from liver samples using the Qiazol Lysis Reagent protocol (Qiagen, Valencia, CA). Extracted RNA was quantified using a spectrophotometer (NanoDrop ND-1000, Nano-Drop Technologies, Wilmington, DE). Ribonucleic acid samples were subjected to a 7x gDNA Wipeout Buffer and then converted to complementary DNA (cDNA) using a Quantitect[®] Reverse Transcription kit (Qiagen, Valencia, CA). Wipeout of gDNA and reverse transcription was performed in a thermocycler (model PTC-200, Biorad, Hercules, CA) with the program set for 2 min at

42°C, 2 min at 4°C, 15 min at 42°C, 3 min at 95 °C, and then cooled to 4 °C. The cDNA synthesized was stored at -20°C.

Quantitative real time PCR was performed using the Prime Time qPCR Assay (Integrated DNA Technologies, Coralville, IA). The cDNA was amplified by PCR where a target cDNA (HMG-CoA reductase, 7 α -dehydroxylase [CYP7A1], and low-density lipoprotein receptor [LDL-R]) and reference cDNA (glyceraldehyde 3-phosphate dehydrogenase[GAPDH]) were amplified using Taqman (Invitrogen, Carlsbad, CA), an oligonucleotide probe with a 5' fluorescent reporter dye (6-FAM) and a 3' non-fluorescent quencher dye (NFQ). Primers and probes used for real-time PCR were: GAPDH, F: GAACATCATCCCTGCATCCA, R: CCAGTGAGCTTCCCGTTCA, probe: CTTGCCCACAGCCTTGGCAGC; HMG-CoA reductase, F: CGAAGGGTTTGCAGTGATAAAGGA, R: GCCATAGTCACATGAAGCTTCTGTA, probe: ACGTGCGAATCTGCT; CPY7A, F: GGTAGTGTGCTGTTGTATATGGGTTA, R: ACAGCCCAGGTATGGAATCAAC, probe: CACCTGCTTTCCTTCTCC; LDL-R, F: GCCGGGACTGGTCAGATG, R: ACAGCCACCATTGTTGTCCA, probe: GCACTCATTGGTCCT GCAGTCCTT (Zhang et al., 2009). Fluorescence was determined on an ABI PRISM 7900HT-sequence detection system (Applied Biosystems, Forest City, CA). To normalize gene expression, a parallel amplification of endogenous GAPDH was performed for each gene. Reactions with no reverse transcription and no template were included as negative controls. Data were analyzed using the comparative threshold cycle (C_t) method (Livak and Schmittgen, 2001) and results were expressed as fold-change relative to the control treatment.

Calculations

Apparent total tract nutrient digestibility was calculated as: $100 \times [(\text{food intake} \times \text{nutrient concentration in the food}) - (\text{voided feces} \times \text{nutrient concentration in feces})] / (\text{food intake} \times \text{nutrient concentration in the food})$.

Statistics

Data were analyzed as a completely randomized experimental design using a 2 x 2 factorial arrangement of treatments using the Mixed Models procedure of SAS/STAT[®] software, version 9.2 for Windows[®] (SAS Institute Inc., Cary, NC, Copyright 2008). The statistical model included the main effect of ACD and cholesterol, and the interaction. Normal distribution of residuals and homogeneity of variances were tested and assumptions for analysis of variances were fulfilled. Treatment least squares means are reported and were compared using a Bonferroni adjustment to ensure the overall protection level. Standard error of the mean (SEM) values are associated with least squares means as calculated in the Mixed Models procedure. Differences among means with a P-value of less than 0.05 were considered significant, and P-values greater than 0.05 but less than or equal to 0.10 were considered trends.

Results

Chemical composition was similar among dietary treatments (Table 5.1). Food intake and apparent nutrient digestibility data are presented in Table 5.2. There was no ACD x CHOL interaction effect for daily food intake. Intake of diets containing ACD was greater ($P < 0.01$) than that of diets with no ACD, whereas intake of diets containing cholesterol tended to be greater ($P = 0.07$) than that of diets with no cholesterol. The control treatment

exhibited the lowest intake ($P<0.05$), whereas the AC treatment resulted in the highest intake. Although intakes were different among treatments, there was no difference in BW gain at the end of the experiment (data not shown). Apparent nutrient digestibility values were high and similar among treatments, resulting in no main or interaction effects.

Fecal DM, fecal output expressed on an as-is and DM basis, and fecal output expressed on an as-is basis per g DM consumed were not different among treatments, as there were no main or interaction effects (Table 5.3).

Data regarding fecal excretion of bile acids are presented in Table 5.3. Concentration of bile acids per g fecal DM was higher ($P<0.01$) for hamsters fed cholesterol treatments, whereas intake of ACD resulted in lower ($P=0.03$) concentrations of fecal bile acids per g DM. However, when bile acids were expressed on an as-is basis, there was no main effect of ACD ($P=0.27$) on fecal bile acid concentrations. Cholesterol intake increased ($P<0.01$) daily fecal excretion of bile acids. Treatment C exhibited the highest ($P<0.05$) daily fecal excretion of bile acids, followed by treatments A and control. The AC treatment, resulted in an intermediate daily fecal bile acid excretion, which was not statistically different from all other treatments.

Cecal characteristic data are presented in Table 5.4. The control exhibited the lowest ($P<0.05$) cecal weights, both with and without cecal contents, whereas treatment A resulted in the highest cecal weights, followed by treatments C and AC. Intake of ACD increased cecal weights ($P<0.01$); however, ACD did not increase full cecal weight when cholesterol was fed simultaneously. Cecal content weight expressed on an as-is basis exhibited the same trend as cecal weights. Treatment A resulted in the highest ($P<0.05$) weight, followed by treatments C and AC, with the control having the lowest weight. Cecal content weight

expressed on a DM basis was similar for treatments C, AC, and control, whereas treatment A resulted in the highest weight. Intake of diets containing ACD resulted in lower ($P=0.04$) cecal DM concentrations compared with treatments with no ACD.

Concentration, total contents, and cecal SCFA molar proportion data are presented in Table 5.5. Concentrations of acetate, butyrate, and total SCFA expressed on a DM basis were not different among treatments. Treatment A resulted in the highest ($P<0.05$) propionate concentration (DM basis) followed by treatments C, control, and AC (values were statistically similar). In general, concentration of SCFA expressed on an as-is basis followed the same trend as concentrations expressed on a DM basis, except for total SCFA that tended to be lower ($P=0.07$) with cholesterol intake. Total contents of SCFA in cecum followed a different pattern. Treatment A resulted in the highest amounts of acetate, propionate, butyrate, and total SCFA in cecum, whereas there were no statistical differences among the remaining treatments. Even though there was a significant ($P=0.03$) ACD x cholesterol interaction effect, proportions of acetate and butyrate in cecal contents were not different among treatments ($P>0.05$). However, treatment A resulted in the highest propionate molar proportion, whereas treatments AC and control resulted in the lowest molar proportion of propionate. Treatment C resulted in an intermediate molar proportion of propionate, which was not different ($P>0.05$) from values for the other treatments.

Cecal microbiota data are presented in Table 5.6. Bifidobacteria and lactobacilli CFU per g cecal DM were not different among treatments. The control treatment resulted in the highest *E. coli* counts per g DM, followed by treatment AC. Treatments A and C resulted in the lowest values. Total microbes quantified using universal primers resulted in a statistically significant ACD x cholesterol interaction effect ($P=0.02$), but there were no

differences among treatment groups ($P>0.05$). Microbial population densities per g of cecal contents (as-is basis) followed the same trend as that expressed on a DM basis. Total CFU of lactobacilli ($P=0.04$) and total microbes ($P=0.03$) in cecal contents were greater for hamsters fed ACD than for those not fed ACD. Cholesterol intake resulted in a reduction in *E. coli* populations in cecal contents.

Concentrations of cholesterol and TG in serum are presented in Table 5.7. Intake of cholesterol resulted in higher concentrations of cholesterol in serum. This effect was not ameliorated by simultaneous ACD intake. Treatments C and AC (statistically similar) resulted in the highest serum cholesterol concentrations, followed by control. Treatment A resulted in a reduction in cholesterol concentrations in serum (lowest value among treatments). Concentrations of TG in serum were not different among treatments. However, cholesterol intake resulted in higher TG concentrations in serum when compared with hamsters fed no cholesterol.

Liver gene expression data are presented in Table 5.8. Expression of the LDL receptor was not different among treatments. Intake of ACD resulted in a 2-fold increase in CYP7A1 expression ($P<0.10$). Intake of diets containing cholesterol resulted in a reduction in expression of HMG-CoA reductase, with treatment AC resulting in the lowest expression.

Discussion

Experimental diets were isocaloric and isonitrogenous. However, concentrations of AHF in diets containing cholesterol were approximately 0.5 percentage units higher than those of diets without it. The AHF method extracts, after acid hydrolysis, lipid materials with mixed ethers. Cholesterol is included within these lipid materials and, thus, is quantified as part of the AHF fraction.

To evaluate the effect of ACD intake on cholesterol metabolism, the Golden hamster was used as an animal model for the human. The lipoprotein profile of the hamster resembles more closely that of humans than do the profiles of rats (Zhang et al., 2009). In addition, the hamster has plasma CETP activity similar to that of humans, whereas other animal models like the dog, rabbit, rat, and mouse have virtually no plasma CETP activity (Tsutsumi et al., 2001; Bailhache et al., 2004).

In general, food intake values were similar to those reported in previous studies of hamsters ingesting semipurified diets (Riottot et al., 1993; Terpstra et al., 1998). The increased intake of diets A, C, and AC compared to the control might have been caused by the novelty effect of these diets, which were not fed during the 7-d adaptation period. However, these differences in food intake did not alter BW gain. Also, higher food intakes did not affect apparent nutrient digestibility values.

Even though most ACD is not digested in the small intestine and up to 98% reaches the large bowel (Andersen et al., 1963), intake of ACD did not alter fecal output, indicating active ACD fermentation by microbiota in the hindgut. This is consistent with the increased weight of cecal tissues, weight of cecal contents, and increased cecal SCFA contents exhibited by hamsters on treatment A. Microbiota changes also are in agreement with these outcomes as intake of ACD increased total CFU of lactobacilli and total bacteria (quantified by using universal primers) in cecal contents.

Cecal contents of acetate, propionate, and butyrate were higher for treatment A. However, when expressed as a molar proportion, propionate was the only SCFA that differed significantly among treatments. This indicates that although ACD fermentation resulted in greater amounts of all SCFA, propionate production was preferentially increased.

This is similar to our *in vitro* results (see chapter 4). However, fermentation of ACD seems to be reduced by addition of cholesterol to the diet. It has been hypothesized that ACD can bind fat and cholesterol and form a very stable complex (Grunberger et al., 2007).

Apparently this complexation renders ACD unavailable for fermentation by the hindgut microbiota.

The only organ that can degrade cholesterol is the liver, and the greatest proportion of cholesterol is degraded through synthesis of bile acids secreted into the small intestine, reabsorbed in the hindgut, and recycled by the liver. Therefore, the increase in fecal excretion of bile acids by hamsters ingesting diets with supplemental cholesterol is probably due to a reduced efficiency of reabsorption of bile acids in the hindgut in order to maintain homeostasis of the bile acid pool.

It has been reported that ACD intake decreases serum cholesterol concentrations in humans (Comerford et al., 2011), rats (Kaewprasert et al., 2001; Garcia-Mediavilla et al., 2003), and mice (Wagner et al., 2008). In study 1 (chapter 3), a numerical decrease in serum cholesterol concentration of hypercholesterolemic dogs was noted. However, cholesterol concentrations in serum of normocholesterolemic dogs were not reduced by ACD intake (see chapters 3 and 4). Intake of treatment A resulted in a reduction in serum cholesterol concentration when compared to control. However, ACD failed to decrease serum cholesterol concentrations in hypercholesterolemic hamsters that had received diets with 0.5% supplemental cholesterol. The hypocholesterolemic effect of ACD seems to be insufficient to reduce serum cholesterol concentrations in dietary cholesterol-induced hypercholesterolemia. It has been reported that the hypocholesterolemic effect of some ingredients is mediated by up-regulation of CYP7A1 expression (Bartley et al., 2010; Kim

et al., 2010; Reena et al., 2011) and, consequently, by an increase in the catabolism of cholesterol. It appears that, even though ACD intake resulted in a 2-fold increase ($P < 0.10$) in expression of CYP7A1, the increase in cholesterol degradation could not overcome the excess cholesterol provided by the diet.

Kaewprasert et al. (2001) evaluated the effect of dietary cyclodextrins on liver and serum lipids and cecal SCFA concentrations in male Wistar rats. Animals were divided into 4 groups and fed a basal diet and diets containing 5% ACD, BCD, or GCD. In this study, similar to findings of our study, there was no difference in BW gain among groups and, compared to the control group, rats fed ACD exhibited no differences in serum TG concentrations, a 15% decrease in serum TC concentration, an approximately 60% increase in the weight of cecal tissues, and over a two-fold increase in weight of cecal contents. Intake of ACD also resulted in over a 3-fold increase in total cecal SCFA, acetate, and propionate, and a 2-fold increase in cecal butyrate concentrations.

Similar results have been reported for β -cyclodextrin (BCD). Mediavilla et al. (2003) examined the effects of BCD on cholesterol and bile metabolism using rats divided into 4 treatment groups: control, 2% cholesterol (A), A+2.5% BCD (B), and A+5.0% BCD (C). It was reported that BCD enhanced CYP7A1 mRNA expression (B: 14%, C: 29%), and reduced plasma TG concentration (C: -38%). However, serum TC concentrations were not different among treatments receiving cholesterol (A, B, C).

Intake of diets containing ACD resulted in a 2-fold increase ($P < 0.10$) in expression of CYP7A1. This increase failed to reach significance because of the large standard error of the mean values. The major form of regulation of CYP7A1 expression appears to be feedback inhibition of gene transcription by bile acids in the enterohepatic circulation. Thus, reducing

the enterohepatic pool of bile acids by administering bile acid sequestrants results in de-repression of CYP7A1 expression (Horton et al., 1995). Therefore, it can be inferred that the up-regulation of CYP7A1 expression in this study is a response to increased bile acid excretion. However, in this study, excretion of bile acids was not affected by ACD. It is possible that bile acid extraction might have been incomplete if bile acids formed complexes with ACD, thus resulting in an underestimation of fecal excretion of bile acids for both ACD treatments. As an analogy, use of ether extraction alone was insufficient to extract all the lipids from ACD-fat complexes. Acid hydrolysis prior to ether extraction was necessary to hydrolyze the ACD and to free the lipids for accurate quantification (see chapter 3).

Cholesterol supplementation decreased expression of HMG-CoA reductase that catalyzes the rate-limiting step in cholesterol synthesis. Zhang et al. (2009) reported that both hepatic HMG-CoA reductase and its mRNA decreased in hamsters and rats in a dose-dependent manner as a result of increasing cholesterol concentrations in diets. In response to hypercholesterolemia, the expression of HMG-CoA reductase would decrease in order to maintain a constant concentration of cholesterol in plasma (Andersen et al., 1982).

In summary, ACD intake resulted in reduced cholesterol concentrations in serum of normocholesterolemic hamsters, but failed to reduce cholesterol concentrations in dietary-induced hypercholesterolemic hamsters. The hypocholesterolemic effect of ACD appears to be a combination of factors including up-regulation of cholesterol degradation through CYP7A1, changes in hindgut fermentation profiles, and, perhaps, changes in hindgut microbiota. Further research is necessary to define the mechanisms for such up-regulation and microbiota effects on bile acid degradation and excretion.

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Tables

Table 5.1. Ingredient (% as-fed basis) and chemical (% dry matter basis) composition of experimental diets containing α -cyclodextrin (ACD) and cholesterol (CHOL).

Item	Diet ¹			
	Control	A	C	AC
Cornstarch	41.3	39.3	40.8	38.8
Maltodextrin	13.2	13.2	13.2	13.2
Sucrose	5.0	5.0	5.0	5.0
Casein	22.0	22.0	22.0	22.0
Soybean oil	5.0	5.0	5.0	5.0
Lard	5.0	5.0	5.0	5.0
Solka floc ²	3.0	3.0	3.0	3.0
Mineral mix ³	3.5	3.5	3.5	3.5
Vitamin mix ⁴	1.5	1.5	1.5	1.5
L-Cystine	0.3	0.3	0.3	0.3
Choline chloride-50%	0.2	0.2	0.2	0.2
α -Cyclodextrin	-	2.0	-	2.0
Cholesterol ⁵	-	-	0.5	0.5
<i>Analyzed chemical composition</i>				
Dry matter	93.4	93.2	93.6	93.3
	----- % , dry matter basis -----			
Organic matter	97.0	97.0	97.0	96.9
Crude protein	23.1	23.0	22.4	22.5

Table 5.1. (Cont.)

Item	Diet ¹			
	Control	A	C	AC
	----- %, dry matter basis -----			
Acid hydrolyzed fat	10.7	10.6	11.1	11.2
Gross energy, kcal/kg	4,938.3	4,916.4	4,976.6	4,948.6

¹Control = 0% ACD, 0% CHOL; A = 2% ACD, 0% CHOL; C = 0% ACD, 0.5% CHOL; AC = 2% ACD, 0.5% CHOL.

²International Fiber Corporation, North Tonawanda, NY.

³Mineral mix AIN-93G-MX (Reeves et al., 1993).

⁴Vitamin mix AIN-93G-VX (Reeves et al., 1993).

⁵95% purity, Dishman Netherlands B.V., Veenendaal, Netherlands.

Table 5.2. Food intake and apparent nutrient digestibility by hamsters fed diets containing α -cyclodextrin (ACD) and cholesterol (CHOL).

Item	Main effects					ACD x CHOL interaction effect					P-value		
	ACD		CHOL		SEM ²	Treatment ¹				SEM	ACD	CHOL	ACD x CHOL
	0%	2%	0%	0.5%		Control	A	C	AC				
Intake, g/d (as-fed basis)	6.0 ^a	6.3 ^b	6.0 [#]	6.3 [*]	0.09	5.8 ^y	6.2 ^{xy}	6.1 ^{xy}	6.4 ^x	0.13	<0.01	0.07	0.88
<i>Digestibility, %</i>													
Dry matter	91.2	91.8	91.2	91.7	0.33	91.1	91.3	91.2	92.2	0.46	0.19	0.30	0.44
Organic matter	91.3	92.0	91.4	91.9	0.33	91.3	91.6	91.4	92.4	0.46	0.16	0.30	0.50
Crude protein	84.4	85.1	84.3	85.2	0.67	84.6	84	84.2	86.2	0.95	0.45	0.34	0.20

^{a,b} ACD (main effect) means in the same row with unlike superscript letters differ (P<0.05).

^{x,y,z} Treatment (interaction) means in the same row with unlike superscript letters differ (P<0.05).

^{#,*} Means in the same row with unlike superscript symbols tend to differ (P<0.10).

¹Control = 0% ACD, 0% CHOL; A = 2% ACD, 0% CHOL; C = 0% ACD, 0.5% CHOL; AC = 2% ACD, 0.5% CHOL.

²SEM = pooled standard error of the mean.

Table 5.3. Fecal characteristics and fecal excretion of bile acids by hamsters fed diets containing α -cyclodextrin (ACD) and cholesterol (CHOL).

Item	Main effects					ACD x CHOL interaction effect							
	ACD		CHOL		SEM ²	Treatment ¹				SEM	P-value		
	0%	2%	0%	0.5%		Control	A	C	AC		ACD	CHOL	ACD x CHOL
Fecal output (as-is), g/d	0.53	0.52	0.53	0.52	0.02	0.52	0.54	0.53	0.50	0.03	0.88	0.69	0.45
Fecal output (DM), g/d	0.42	0.43	0.43	0.42	0.02	0.41	0.44	0.42	0.42	0.03	0.70	0.83	0.56
Fecal output (as-is) per g DM consumed	0.10	0.09	0.9	0.9	<0.01	0.10	0.09	0.09	0.08	0.01	0.19	0.27	0.43
Fecal DM, %	80.11	82.4	80.9	81.6	1.26	80.05	81.65	80.17	83.08	1.77	0.21	0.67	0.71
<i>Bile acids in feces</i>													
Bile acids, $\mu\text{mol/g DM}$	0.95 ^a	0.88 ^b	0.78 ^d	1.05 ^c	0.02	0.81 ^y	0.74 ^y	1.08 ^x	1.02 ^x	0.03	0.03	<0.01	0.96
Bile acids, $\mu\text{mol/g}$ as-is	0.76	0.73	0.63 ^d	0.86 ^c	0.02	0.65 ^y	0.61 ^y	0.87 ^x	0.84 ^x	0.03	0.27	<0.01	0.82
Bile acids, $\mu\text{mol/d}$	0.40	0.37	0.33 ^d	0.44 ^c	0.02	0.34 ^y	0.33 ^y	0.46 ^x	0.42 ^{xy}	0.02	0.30	<0.01	0.48

Table 5.3. (Cont.)

^{a,b} ACD (main effect) means in the same row with unlike superscript letters differ (P<0.05).

^{c,d} CHOL (main effect) means in the same row with unlike superscript letters differ (P<0.05).

^{x,y,z} Treatment (interaction) means in the same row with unlike superscript letters differ (P<0.05).

^{#,*} Means in the same row with unlike superscript symbols tend to differ (P<0.10).

¹Control = 0% ACD, 0% CHOL; A = 2% ACD, 0% CHOL; C = 0% ACD, 0.5% CHOL; AC = 2% ACD, 0.5% CHOL.

²SEM = pooled standard error of the mean.

Table 5.4. Cecal characteristics of hamsters fed diets containing α -cyclodextrin (ACD) and cholesterol (CHOL).

Item	Main effects					ACD x CHOL interaction effect					P-value		
	ACD		CHOL		SEM ²	Treatment ¹				SEM	ACD	CHOL	ACD x CHOL
	0%	2%	0%	0.5%		Control	A	C	AC				
Cecum, full, g	2.75 ^a	3.81 ^b	3.39	3.17	0.19	2.31 ^z	4.47 ^x	3.21 ^y	3.15 ^y	0.26	<0.01	0.43	<0.01
Cecum, empty, g	0.96 ^a	1.33 ^b	1.11	1.17	0.07	0.79 ^z	1.44 ^x	1.13 ^y	1.21 ^{xy}	0.10	<0.01	0.61	<0.01
Cecal contents, g													
as-is basis	1.80 ^a	2.50 ^a	2.27	2.01	0.14	1.53 ^z	3.03 ^x	2.08 ^y	1.94 ^y	0.19	<0.01	0.18	<0.01
Cecal contents, g													
DM basis	0.39 ^a	0.51 ^b	0.49 [#]	0.42 [*]	0.03	0.35 ^y	0.63 ^x	0.44 ^y	0.40 ^y	0.04	<0.01	0.08	<0.01
Cecal contents													
DM, %	22.0 ^a	20.7 ^b	21.7	21.0	0.41	22.7 [*]	20.6 [#]	21.2 ^{#*}	20.8 ^{#*}	0.58	0.04	0.27	0.15

^{a,b} ACD (main effect) means in the same row with unlike superscript letters differ (P<0.05).

^{x,y,z} Treatment (interaction) means in the same row with unlike superscript letters differ (P<0.05).

^{#,*} Means in the same row with unlike superscript symbols tend to differ (P<0.10).

¹Control = 0% ACD, 0% CHOL; A = 2% ACD, 0% CHOL; C = 0% ACD, 0.5% CHOL; AC = 2% ACD, 0.5% CHOL.

²SEM = pooled standard error of the mean.

Table 5.5. Concentrations ($\mu\text{mol/g}$) expressed on a dry matter basis and on an as-is basis, total contents (μmol), and molar proportions (%) of short-chain fatty acid (SCFA) in cecal contents of hamsters fed diets containing α -cyclodextrin (ACD) and cholesterol (CHOL).

Item	Main effects				ACD x CHOL interaction effect								
	ACD		CHOL		SEM ²	Treatment ¹				SEM	P-value		
	0%	2%	0%	0.5%		Control	A	C	AC		ACD	CHOL	ACD x CHOL
<i>Dry matter basis</i>													
Acetate	84.8	89.5	89.7	84.6	3.64	87.0	92.3	82.5	86.7	5.14	0.36	0.34	0.91
Propionate	26.1	29.2	30.1 ^c	25.3 ^d	1.50	25.7 ^y	34.4 ^x	26.6 ^y	24.1 ^y	2.12	0.15	0.03	0.01
Butyrate	19.8	22.0	20.4	21.4	1.37	18.6	22.1	21.0	21.9	1.94	0.27	0.58	0.53
Total	130.7	140.7	140.0	131.4	5.36	131.3	148.8	130.1	132.7	7.58	0.19	0.26	0.34
<i>As-is basis</i>													
Acetate	18.6	18.4	19.3 [#]	17.6 [*]	0.67	19.7	18.9	17.4	17.8	0.95	0.82	0.09	0.54
Propionate	5.7	6.0	6.4 ^c	5.3 ^d	0.30	5.8 ^{xy}	7.0 ^x	5.6 ^{xy}	5.0 ^y	0.42	0.49	0.01	0.03
Butyrate	4.3	4.5	4.4	4.5	0.29	4.2	4.6	4.5	4.5	0.41	0.62	0.81	0.76
Total	28.6	28.9	30.1 [#]	27.4 [*]	1.01	29.7	30.5	27.5	27.3	1.43	0.85	0.07	0.73

Table 5.5. (Cont.)

Item	Main effects					ACD x CHOL interaction effect							
	ACD		CHOL		SEM	Treatment				SEM	P-value		
	0%	2%	0%	0.5%		Control	A	C	AC		ACD	CHOL	ACD x CHOL
<i>Total contents</i>													
Acetate	33.6 ^b	47.0 ^a	44.7 [#]	35.9 [*]	3.32	30.6 ^y	58.9 ^x	36.6 ^y	35.1 ^y	4.70	<0.01	0.07	<0.01
Propionate	10.6 ^b	16.3 ^a	15.8	11.1	1.44	8.8 ^y	22.8 ^x	12.5 ^y	9.8 ^y	2.03	<0.01	0.03	<0.01
Butyrate	8.6 [#]	12.0 [*]	10.8	9.7	1.23	6.6 ^y	15.0 ^x	10.5 ^{xy}	8.9 ^{xy}	1.74	0.06	0.53	<0.01
Total	52.8 ^b	75.3 ^a	71.3 [#]	56.7 [*]	5.72	45.9 ^y	96.8 ^x	60.0 ^y	53.8 ^y	8.08	<0.01	0.07	<0.01
<i>Molar ratios</i> ³													
Acetate	64.6	63.5	64.0	64.1	1.20	66.4	61.6	62.7	65.5	1.69	0.56	0.97	0.03
Propionate	19.9	20.6	21.1 [#]	19.4 [*]	0.68	19.2 ^y	23.0 ^x	20.6 ^{xy}	18.2 ^y	0.96	0.48	0.08	<0.01
Butyrate	15.5	15.9	14.9	16.5	0.73	14.3	15.4	16.7	16.3	1.03	0.75	0.11	0.47

^{a,b} ACD (main effect) means in the same row with unlike superscript letters differ (P<0.05).

^{c, d} CHOL (main effect) means in the same row with unlike superscript letters differ (P<0.05).

Table 5.5. (Cont.)

^{x, y, z} Treatment (interaction) means in the same row with unlike superscript letters differ (P<0.05).

^{#, *} Means in the same row with unlike superscript symbols tend to differ (P<0.10).

¹Control = 0% ACD, 0% CHOL; A = 2% ACD, 0% CHOL; C = 0% ACD, 0.5% CHOL; AC = 2% ACD, 0.5% CHOL.

²SEM = pooled standard error of the mean.

³Molar proportions were calculated as: 100 x cecal contents of individual SCFA/cecal contents of total SCFA.

Table 5.6. Microbiota (log CFU) in cecal contents of hamsters fed diets containing α -cyclodextrin (ACD) and cholesterol (CHOL).

Item	Main effects				SEM ²	ACD x CHOL interaction effect				SEM	P-value		
	ACD		CHOL			Treatment ¹					ACD	CHOL	ACD x CHOL
	0%	2%	0%	0.5%		Control	A	C	AC				
<i>Dry matter basis</i> ³													
Bifidobacteria	10.2	10.2	10.1	10.3	0.06	10.2	10.1	10.2	10.3	0.09	0.95	0.14	0.14
Lactobacilli	11.6	11.7	11.7	11.7	0.08	11.7	11.7	11.5	11.8	0.11	0.25	0.81	0.24
<i>Escherichia coli</i>	9.0	8.7	9.0	8.7	0.14	9.5 ^x	8.5 ^y	8.5 ^y	9.0 ^{xy}	0.20	0.22	0.13	<0.01
Total population, AU ⁴	14.1	14.1	14.1	14.1	0.05	14.2	14.0	14.0	14.2	0.07	0.49	0.67	0.02
<i>As-is basis</i> ⁵													
Bifidobacteria	9.5	9.5	9.5	9.6	0.06	9.6	9.4	9.5	9.7	0.09	0.73	0.16	0.09
Lactobacilli	11.0	11.1	11.0	11.0	0.08	11.1	11.0	10.9	11.1	0.11	0.35	0.72	0.18
<i>Escherichia coli</i>	8.3	8.0	8.3	8.0	0.14	8.8 ^x	7.9 ^y	7.8 ^y	8.2 ^{xy}	0.20	0.18	0.11	<0.01
Total population, AU	13.4	13.4	13.4	13.4	0.04	13.5 [*]	13.4 [#]	13.3 [#]	13.5 [*]	0.06	0.73	0.51	<0.01

Table 5.6. (Cont.)

Item	Main effects					ACD x CHOL interaction effect					P-value		
	ACD		CHOL		SEM	Treatment				SEM	ACD	CHOL	ACD x CHOL
	0%	2%	0%	0.5%		Control	A	C	AC				
<i>Total contents</i> ⁶													
Bifidobacteria	9.7	9.8	9.7	9.8	0.06	9.7	9.8	9.8	9.9	0.09	0.22	0.24	0.95
Lactobacilli	11.2 ^b	11.4 ^a	11.3	11.3	0.09	11.2	11.5	11.1	11.4	0.13	0.04	0.56	0.98
<i>Escherichia coli</i>	8.5	8.4	8.6 ^c	8.2 ^d	0.13	8.9 ^x	8.4 ^{xy}	8.0 ^y	8.4 ^{xy}	0.18	0.70	0.03	0.02
Total population,													
AU	13.6 ^a	13.8 ^b	13.7	13.7	0.05	13.7 ^{#*}	13.8 [#]	13.6 [*]	13.7 ^{#*}	0.07	0.03	0.26	0.56

^{a,b} ACD (main effect) means in the same row with unlike superscript letters differ (P<0.05).

^{c,d} CHOL (main effect) means in the same row with unlike superscript letters differ (P<0.05).

^{x,y,z} Treatment (interaction) means in the same row with unlike superscript letters differ (P<0.05).

^{#,*} Means in the same row with unlike superscript symbols tend to differ (P<0.10).

¹Control = 0% ACD, 0% CHOL; A = 2% ACD, 0% CHOL; C = 0% ACD, 0.5% CHOL; AC = 2% ACD, 0.5% CHOL.

Table 5.6. (Cont.)

²SEM = pooled standard error of the mean.

³Expressed as log CFU/g cecal DM.

⁴AU = arbitrary units.

⁵Expressed as log CFU/g cecal contents.

⁶Expressed as log CFU in total cecal contents.

Table 5.7. Serum cholesterol and triglyceride concentrations (mmol/dL) in hamsters fed diets containing α -cyclodextrin (ACD) and cholesterol (CHOL).

Item	Main effects					ACD x CHOL interaction effect					P-value		
	ACD		CHOL		SEM ²	Treatment ¹				SEM	ACD	CHOL	ACD x CHOL
	0%	2%	0%	0.5%		Control	A	C	AC				
Serum cholesterol	4.36	4.12	2.55 ^b	5.90 ^a	0.13	2.94 ^y	2.17 ^z	5.79 ^x	6.07 ^x	0.19	0.20	<0.01	<0.01
Serum triglycerides	1.74	1.85	1.27 ^b	2.33 ^a	0.14	1.36	1.18	2.13	2.52	0.20	0.58	<0.01	0.16

^{a, b} CHOL (main effect) means in the same row with unlike superscript letters differ (P<0.05).

^{x, y, z} Treatment (interaction) means in the same row with unlike superscript letters differ (P<0.05).

^{#, *} Means in the same row with unlike superscript symbols tend to differ (P<0.10).

¹Control = 0% ACD, 0% CHOL; A = 2% ACD, 0% CHOL; C = 0% ACD, 0.5% CHOL; AC = 2% ACD, 0.5% CHOL.

²SEM = pooled standard error of the mean.

Table 5.8. Liver gene expression of hydroxymethylglutaryl (HMG)-CoA reductase, cholesterol 7 α -hydroxylase (CYP7A1), and low-density lipoprotein receptor (LDL-R) for hamsters fed diets containing α -cyclodextrin (ACD) and cholesterol (CHOL)¹.

Item	Main effects					ACD x CHOL interaction effect					P-value		
	ACD		CHOL		SEM ³	Treatment ²				SEM	ACD	CHOL	ACD x CHOL
	0%	2%	0%	0.5%		Control	A	C	AC				
HMG-CoA reductase	0.83	1.10	1.35 ^a	0.58 ^b	0.20	1.00 ^{xy}	1.70 ^x	0.66 ^{xy}	0.50 ^y	0.28	0.34	0.01	0.14
CYP7A1	1.22 [#]	2.27 [*]	1.54	1.94	0.44	1.00	2.08	1.44	2.46	0.62	<0.10	0.51	0.96
LDL-R	0.91	1.13	1.20	0.84	0.23	1.00	1.41	0.83	0.85	0.32	0.52	0.27	0.55

^{a, b} CHOL (main effect) means in the same row with unlike superscript letters differ (P<0.05).

^{x, y, z} Treatment (interaction) means in the same row with unlike superscript letters differ (P<0.05).

^{#, *} Means in the same row with unlike superscript symbols tend to differ (P<0.10).

¹Values expressed as fold-change relative to the control treatment.

²Control = 0% ACD, 0% CHOL; A = 2% ACD, 0% CHOL; C = 0% ACD, 0.5% CHOL; AC = 2% ACD, 0.5% CHOL.

³SEM = pooled standard error of the mean.

CHAPTER 6

SUMMARY

Obesity is a disorder often associated with type 2 diabetes, dyslipidemia, and cardiovascular disease (CVD). From the nutritional point of view, reduction of lipid absorption and normalization of blood lipids have been among the main goals in treating obesity. In order to reach these goals, drugs and nutritional interventions have been developed. The connection between carbohydrates and health has fueled the interest of researchers and the food industry to develop novel carbohydrates using microbial enzymes, chemicals, and (or) heat treatments. Cyclodextrins are examples of such carbohydrates.

Cyclodextrins are derived from starch via enzymatic processes. The cyclodextrin glucosyl transferase enzymes (CGT-ases) are amylolytic enzymes produced naturally by a large number of microorganisms. Cyclodextrins are non-reducing cyclic oligosaccharides comprised of α -1,4-glycosidic-linked glucopyranose units. The family of cyclodextrins is very large, including the three major cyclodextrins, α -cyclodextrin (ACD) with 6 glucose units, β -cyclodextrin (BCD) with 7, and γ -cyclodextrin (GCD) with 8. α -Cyclodextrin, also is known as Schardinger's α -dextrin, cyclohexomaltose, cyclohexaglukan, cyclohexaamylose, and C6A. However, the most standardized nomenclature is α -cyclodextrin or ACD (Szejtli, 1996).

Because cyclodextrins are linked by α -1,4 bonds, α -amylases are capable of hydrolyzing them. However, α -amylases act slowly on cyclodextrins (Szejtli, 1996). While ACD is hydrolyzed by α -amylases of fungal and bacterial origin (Saha and Zeikus, 1992), human salivary and pancreatic amylases cannot hydrolyze ACD to a significant extent (Kondo et al., 1990). For this reason, most ACD is not digested and absorbed in the small

intestine, with up to 98% reaching the large bowel (Andersen et al., 1963) where it is metabolized by the resident microbiota.

α -Cyclodextrin has a cylinder shape with hydrophilic sites on the exterior and hydrophobic sites on the interior of the cylinder (Szejtli, 1998). This structure of ACD makes it both soluble in water and capable of including other apolar molecules of the appropriate size, also called guest molecules, in the axial open cavity forming inclusion complexes (Biwer et al., 2002; Szejtli, 2004). It has been reported that ACD binds fats, forming stable emulsions. This emulsion, or complex, is hypothesized to render fats indigestible and decrease weight gain and blood cholesterol concentrations (Gallaher et al., 2007). These authors also hypothesized that ACD binds TG with such affinity that traditional methods of measuring fat excretion were unable to extract fat from the ACD-fat complex. They also hypothesized that this might be the explanation as to why animal feeding trials failed to detect an increase in fat excretion when ACD was fed.

The main objective of this research was to characterize the nutritional effects of ACD intake using *in vitro*, canine, and hamster models. To accomplish this objective, Study 1 was designed to evaluate the composition of ACD, validate the current fat analysis techniques as related to ACD-fat complexes, and quantify gastrointestinal tolerance, total tract nutrient digestibility, and blood lipid profiles of dogs as affected by ACD supplementation. Once fat analysis techniques were validated, and doses of ACD that were well tolerated by dogs were defined, Study 2 was designed to evaluate *in vivo* total tract and ileal nutrient digestibilities, fecal microbiota concentrations, and blood lipid profiles of dogs as affected by ACD supplementation. Another objective was to evaluate *in vitro* fermentation characteristics of cyclodextrins. Based on observations from the two previous studies and reports of the effect

of ACD on blood cholesterol concentrations, Study 3 was designed to evaluate apparent nutrient digestibility, cecal SCFA concentrations, cecal microbiota concentrations, blood lipid profiles, bile acid excretion, and liver gene expression of hamsters as affected by ACD and cholesterol intakes.

Hydrolysis of ACD showed that it is effectively composed of glucose only. The use of ether extraction alone was insufficient to extract all the fat in the ACD-fat complexes in agreement with previous reports (Gallaher et al., 2007). However, use of acid hydrolysis prior to ether extraction hydrolyzes ACD, freeing the fat and, consequently, all the fat in the complexes was recovered. Therefore, the acid hydrolyzed fat (AHF) method proved to be valid for measuring fat bound to ACD.

α -Cyclodextrin was shown to be generally well tolerated by dogs and hamsters and did not alter body weight (BW), body condition score, or fecal score. The only signs of intolerance observed were emesis and non-productive emesis by some of the dogs. In those instances, because ACD was provided as a solution in water using a syringe, the physical effect of putting the syringe in the mouth of the dogs or the taste of the solution might have provoked the emesis. Intake of 6 or 12 g ACD daily numerically reduced serum cholesterol concentrations in hypercholesterolemic dogs, but failed to affect serum TC concentrations in normocholesterolemic dogs.

Total tract digestibility of all nutrients decreased linearly with increasing amounts of ACD intake. However, this reduction in nutrient digestibility was much lower than what had been reported previously (Artiss et al., 2006; Grunberger et al., 2007). It also was shown that even with changes in total tract nutrient digestibility, there was no difference in ileal nutrient

digestibility. The differences between apparent ileal and total tract nutrient digestibilities can be explained by modifications caused by the hindgut microbiota.

In vitro fermentation of ACD resulted in a unique SCFA pattern, with greater production of propionate than of acetate. Differences between apparent ileal and total tract nutrient digestibilities by dogs caused by ACD intake, and the unique SCFA production profile, might indicate possible changes in hindgut microbiota. When quantified by plating, bifidobacteria CFU were almost 1 log unit greater in the group receiving ACD, whereas lactobacilli were 0.8 log units greater in the same group. Quantification by molecular techniques did not demonstrate significant effects of ACD intake on bifidobacteria, *E. coli*, or *Clostridium perfringens*. However, there was a quadratic and cubic effect of ACD intake on lactobacilli. The biological significance of this effect is difficult to determine.

To evaluate the effect of ACD intake on cholesterol metabolism, the Golden hamster was used as an animal model for the human. The lipoprotein profile of the hamster resembles more closely that of humans than do the profiles of rats (Zhang et al., 2009). In addition, the hamster has plasma cholesteryl ester transfer protein (CETP) activity similar to that of humans whereas other animal models like the dog, rabbit, rat, and mouse, have virtually no plasma CETP activity (Tsutsumi et al., 2001; Bailhache et al., 2004).

Intake by hamsters of 2% ACD in semipurified diets did not alter apparent nutrient digestibility. However, ACD intake increased weight of cecal tissues, weight of cecal contents, and increased concentrations and total contents of cecal SCFA. When expressed in molar proportions, propionate was the only SCFA that was different among treatments. This indicates that, although ACD fermentation yielded larger amounts of all SCFA, propionate production was preferentially increased. Intake of ACD resulted in a reduction in cholesterol

concentration in serum. However, ACD failed to decrease serum cholesterol concentrations in hypercholesterolemic hamsters that had received diets with 0.5% supplemental cholesterol. The hypocholesterolemic effect of ACD seems to be insufficient to reduce serum cholesterol concentrations in dietary cholesterol-induced hypercholesterolemia. Intake of diets containing ACD resulted in a 2-fold increase ($P < 0.10$) in expression of 7 α -hydroxylase (CYP7A1), the enzyme that catalyzes the rate-limiting step in cholesterol degradation to bile acids, and whose activity is regulated by the bile acids reabsorbed from the portal blood as a negative feedback mechanism (Leuschner, 2010). It can be inferred that up-regulation of CYP7A1 expression is a response to increased bile acid excretion. However, in this study, excretion of bile acids was not affected by ACD. It is possible that bile acid extraction might have been incomplete if bile acids formed complexes with ACD, thus underestimating fecal excretion of bile acids for both ACD treatments.

Previous reports of reduced fat absorption due to ACD intake (Artiss et al., 2006; Grunberger et al., 2007) are contrary to our results. Their studies inferred decreased fat absorption based on differences in BW gain; however, fat excretion was either not quantified or was not different among treatments. The same authors reported lower BW gain in subjects ingesting ACD and ingesting food *ad libitum*. Our findings differ as regards effect of ACD intake on BW. This could be explained by the fact that dogs in studies 1 and 2 were fed an amount of food calculated to meet their energy requirements and maintain their BW. However, our hamsters were fed *ad libitum*, and, perhaps due to interspecies differences, there were no differences in BW gain. Our data were in general agreement with most of literature data regarding the hypocholesterolemic effect of ACD. However, dogs,

rats, and, perhaps, dietary cholesterol-induced hypercholesterolemic hamsters, are not the ideal models to accurately quantify this effect.

To our knowledge, these data are the first to report *in vitro* fermentation profiles and hindgut microbial population and ileal digestibility responses resulting from ACD intake at amounts comparable to practical and feasible intakes for humans. Research regarding nutritional effects of ACD is vital for determining inclusion levels in foods and functionality in the body. A clear outcome of this research relates to the validation of the AHF method to measure fat that has been complexed with ACD, and to the quantification of the effect of ACD intake on ileal nutrient digestibility and hindgut microbiota.

In conclusion, ACD intake varies in its ability to affect cholesterol metabolism, nutrient digestibility, fecal characteristics, and hindgut microbiota and fermentation patterns. This variation in response is due to differences in the model used to evaluate it, inclusion level in the diet, and dietary matrix where it is supplied. The hypocholesterolemic effect of ACD appears to be a combination of multiple factors including up-regulation of cholesterol degradation through CYP7A1, changes in hindgut fermentation profiles, and, perhaps, changes in hindgut microbiota. However, further research is necessary to define the mechanisms for such up-regulation and microbiota effects on bile acid degradation and excretion.

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AUTHOR'S BIOGRAPHY

Marcial Antonio Guevara was born to Juan and Maria on June 12th, 1971, in Barquisimeto, Venezuela. In 1990, after attending military aviation school for two years, he attended the University Centroccidental Lisandro Alvarado (UCLA; Barquisimeto, Venezuela), and worked as a teaching assistant for the Animal Nutrition Department from 1994 to 1997. He received the Veterinary Medical degree with honors in October of 1997. Since August of 1997, Marcial worked for different pet food companies as an external consultant in veterinary technical support, training personnel in companion animal nutrition and technical sales, and giving talks in different places in Venezuela, Colombia, Costa Rica, and Panama. In December 1997, he married Maria J. and together they have three boys: Jesus, Jose, and Oscar. In March of 1998, concurrently with his job as a consultant, Marcial began to teach animal nutrition at UCLA. In January of 2006, Marcial began graduate studies in the Department of Animal Sciences at the University of Illinois, Urbana-Champaign, working under the supervision of Dr. George C. Fahey, Jr. Marcial's M.S thesis, which he defended in September of 2007, focused on the evaluation of select corn fibers as regards chemical composition, *in vitro* fermentation characteristics, and *in vivo* digestibility responses by dogs. After completing the Master of Science degree, he immediately began working towards the Doctor of Philosophy degree in Animal Sciences under the advisement of Dr. George C. Fahey, Jr. His research focused on the influence of α -cyclodextrin intake on nutrient digestibilities, cholesterol metabolism, and gastrointestinal microbiota and fermentation patterns. Upon completion of the doctoral degree, he will begin work as a Postdoctoral Research Associate in the Department of Animal Sciences at the University of Illinois.