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# In vitro fermentation characteristics, in vivo ileal and total tract nutrient digestibilities, and fecal microbiota responses of dogs to α-cyclodextrin[1]

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**ABSTRACT:** The objectives were to examine in vitro fermentation characteristics, in vivo nutrient digestibility, fecal microbiota, and serum lipid profiles 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, and γ-cyclodextrin. Five mixed-breed hounds were used in a Latin square design. Each experimental period comprised 14 d, including 10 d for diet adaptation and 4 d for fecal collection. Dogs were fed, twice a day, an extruded diet made with poultry byproduct meal and brewer's rice as the main ingredients. Dogs were supplemented with 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 treatment. Total tract nutrient digestibility and fecal DM concentration linearly decreased ($P < 0.05$) for treatment groups receiving ACD; no changes were observed for ileal digestibility. Serum cholesterol and triglyceride concentrations were within normal ranges for dogs and were not different among treatments. Similarly, no changes in fecal microbiota were observed. Overall, ACD supplementation appears to have no effect on nutrient absorption in the small intestine but may alter fermentation in the large bowel, which could lead to a higher proportion of propionate production as observed in the in vitro experiment.

**Key words:** canine, cyclic oligosaccharide, hindgut bacteria, in vitro, lipid metabolism, nutrient digestibility


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## INTRODUCTION

According to the American Association of Cereal Chemists (2001), dietary fibers promote beneficial physiological effects including laxation, 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 and obesity. More specifically, high low-density lipoprotein cholesterol concentrations in serum may account for a large proportion of cardiovascular disease case in western cultures (Bruckert and Rosenbaum, 2011). Similar to humans, obese dogs also suffer from hyperlipidemia, even though they are less susceptible to cardiovascular disease (Xenoulis and Steiner, 2010).

Cyclodextrins are capable of forming 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 to 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 2-fold increase in weight of cecal contents, and over a 3-fold increase in total short-chain fatty acid (**SCFA**) concentrations. At the same time, it has been reported that BCD intake enhances (1.8-fold) bile acid

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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 gastrointestinal 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).

### *In Vitro Fermentation Experiment*

***Donors and Collection Method.*** Three human fecal samples from 3 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 3 mo before or during the study. The experimental protocol was approved by the University of Illinois Institutional Review Board, and all subjects signed an informed consent before 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 (Waring Laboratory Science, Global Resources, Inc., Winsted, CT) under a stream of $CO_2$. Blended, diluted feces were filtered through 4 layers of cheesecloth and sealed in 125-mL serum bottles under $CO_2$.

**Table 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 solutions, 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 |
| $Na_2CO_3$, g/L | 4.0 |
| Cysteine HCL·$H_2O$, g/L | 0.5 |

[1]Composition: 5.4 g/L NaCl, 2.7 g/L $KH_2PO_4$, 0.18 g/L $CaCl_2 \cdot H_2O$, 0.12 g/L $MgCl_2 \cdot 6H_2O$, 0.06 g/L $MnCl_2 \cdot 4H_2O$, 0.06 g/L $CoCl_2 \cdot 6H_2O$, and 5.4 g/L $(NH_4)2SO_4$.

[2]Composition: 2.7 g/L $K_2HPO_4$.

[3]Composition: 500 mg/L EDTA (disodium salt), 200 mg/L $FeSO_4 \cdot 7H_2O$, 10 mg/L $ZnSO_4 \cdot 7H_2O$, 3 mg/L $MnCl_2 \cdot 4H_2O$, 30 mg/L $H_3PO_4$, 20 mg/L $CoCl_2 \cdot 6H_2O$, 1 mg/L $CuCl_2 \cdot 2H_2O$, 2 mg/L $NiCl_2 \cdot 6H_2O$, and 3 mg/L $Na_2MoO_4 \cdot 2H_2O$.

[4]Composition: 100 mg/L thiamin HCl, 100 mg/L D-pantothenic acid, 100 mg/L niacin, 100 mg/L pyridoxine, 5 mg/L *p*-aminobenzoic acid, and 0.25 mg/L vitamin $B_{12}$.

[5]Composition: 10 mg/L folic acid, 2 mg/L d-biotin, and 100 mg/L $NH_4HCO_3$.

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

[7]500 mg/L hemin in 10 mmol/L NaOH.

[8]250 mL/L each of *n*-valerate, isovalerate, isobutyrate, and dl-α-methylbutyrate.

[9]1 g/L resazurin in distilled $H_2O$.

Each substrate was fermented in vitro for 0, 3, 6, 9, and 12 h in triplicate with microbial inoculum. Triplicate tubes containing no substrate were fermented with the inoculum at each time point to enable appropriate corrections for SCFA production not arising from the substrates. A semidefined medium was used for the fermentation (Table 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 only 26 mL semidefined medium (blank tubes) or 26 mL semidefined 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 immediately processed for analyses. The pH of tube contents was measured with a standard pH meter (Denver Instrument Co., Arvada, CO).

A 2.0-mL subsample 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 × *g* at 4°C for 20 min. The supernatant was decanted and frozen at −20°C in microcentrifuge tubes. After freezing, the supernatant was thawed and centrifuged in microcentrifuge tubes at 16,000 × *g* at 20°C for 10 min. The supernatant then was transferred to gas chromatography vials and stored at −20°C until analysis.

***Chemical Analyses.*** Concentrations of SCFA were determined using GLC (Erwin et al., 1961). 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 (Agilent, Santa Clara, CA) and a glass column (180 cm by 64 mm i.d.) packed with 10% SP-1200/1% $H_3PO_4$ 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.

***Statistical Analysis.*** 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:

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

*in which* $Y$ = SCFA production; $A$ = asymptote, or maximal SCFA production expressed in micromoles per gram 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 Inst. Inc., Cary, NC). Additionally, maximal rates of SCFA production were calculated using the following equation:

$$Y + A/4 \times B,$$

in which $Y$ = maximal rate of production expressed in micromoles per hour per gram DM, $A$ = maximal SCFA production expressed in micromoles per gram 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 1 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 procedure of SAS/STAT software, version 9.2 for Windows. 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 ANOVA were fulfilled. Treatment least squares means are reported and were compared using preplanned contrasts and estimates. Standard error of the mean 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 to 8 yr) and an average initial 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 the procedure of Walker et al. (1994). The University of Illinois Institutional Animal Care and Use Committee approved all animal care procedures before initiation of the experiment. Dogs were individually housed in indoor pens (approximately 1.2 by 1.5 m) in an environmentally controlled facility (22°C and 23% relative humidity) with a 16:8 h light:dark cycle. Dogs were weighed and BCS was assessed using a 1-to-9 scale (Laflamme, 1997) throughout the experiment.

***Diets and Treatments.*** One experimental diet was formulated to meet or exceed the NRC (2006) nutrient profiles for adult dogs at maintenance. The diet consisted of poultry byproduct meal and brewer's rice as the main ingredients and Solka-floc (International Fiber Corporation, North Tonawanda, NY) as the fiber source. Chromic oxide was included as a digestion marker at 0.2% of the diet (Table 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 0, 1, 2, 3, or

**Table 2.** Ingredient (% as-fed basis) and chemical (% DM basis) composition of the experimental diet

| Item | Percent |
|---|---|
| Poultry byproduct meal, low ash | 39.0 |
| Brewer's rice | 28.9 |
| Poultry fat | 14.0 |
| Corn, yellow, ground | 10.0 |
| Solka-floc[1] | 6.5 |
| Salt | 0.7 |
| Potassium chloride | 0.5 |
| Chromic oxide | 0.2 |
| Mineral premix[2] | 0.1 |
| Vitamin premix[3] | 0.1 |
| Choline chloride | 0.1 |
| Analyzed chemical composition | |
| DM, % | 92.6 |
| | ——% DM basis—— |
| OM | 91.7 |
| CP | 30.6 |
| Acid hydrolyzed fat | 20.2 |
| Total dietary fiber | 7.3 |
| GE, kcal/kg | 5,215 |

[1]International Fiber Corporation, North Tonawanda, NY.

[2]Provided, per kilogram of diet, 120 mg iron ($FeSO_4$), 66 mg manganese (MnO), 18 mg copper ($CuSO_4$), 1.8 mg iodine ($C_2H_6N_2 \cdot 2HI$), 240 μg selenium ($Na_2SeO_3$), and 240 mg zinc (ZnO).

[3]Provided, per kilogram of diet, 10,560 IU vitamin A (vitamin A acetate), 1,056 IU vitamin D (vitamin $D_3$), 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 $B_{12}$ (vitamin $B_{12}$ supplement), 2.11 mg pyridoxine (pyridoxine hydrochloride), 79 μg biotin (d-biotin supplement), and 264 μg folic acid (folic acid supplement).

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 5 × 5 Latin square design. Each period consisted of 2 phases: 10 d for diet adaptation and 4 d for fecal collection. Dogs were weighed, BCS was assessed, and blood was 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 subsample was removed, ground in a Wiley mill (model 4; Thomas Scientific, Swedesboro, NJ) through a 2-mm screen, and stored at 4°C until further analyses.

During the 4-d collection phase, all voided feces were collected from the floor of the pen and weighed. Feces were scored on a 5-point scale, with 1 being dry, hard pellets; 2 being dry, well-formed stool; 3 being soft, moist, formed stool; 4 being unformed stool; and 5 being 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, aliquoted only from the interior part of the stools, were collected within 15 min of defecation, flash frozen in liquid nitrogen, and stored at −80°C for bacterial analyses. A short time between defecation and sampling (i.e., 15 min) is important for microbial analysis to minimize DNA damage and obtain high-quality DNA for sequencing.

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 1 h from the previous day's collection time for a total of 12 samples per animal per 4-d collection period. 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 refrozen 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 before feeding the dogs. Tubes were kept at room temperature for 30 min and centrifuged at 1,240 × *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 a constant moisture level in the samples was achieved. 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 a mortar and pestle. Diet and dried fecal samples were ground in a Wiley mill (model 4; Thomas Scientific) through a 2-mm screen.

Dry matter and OM concentrations were determined according to AOAC International (Horwitz, 2002) methods 934.01 and 942.05, respectively. Acid hydrolyzed fat (AHF) concentrations were determined using acid hydrolysis according to American Association of Cereal Chemists (2000) method 30-14.01 followed by ether extraction (Budde, 1952). Crude protein concentrations were determined using LECO nitrogen values

(N × 6.25; method 992.15; Horwitz, 2002; nitrogen analyzer model FP-2000; Leco Corporation, St. Joseph, MI). Total dietary fiber concentration of the diet was measured according to Prosky et al. (1985). Gross energy concentrations of ACD and diet were measured using an oxygen bomb calorimeter (model 1261; Parr Instruments, Moline, IL). Food, ileal, 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; PerkinElmer, Waltham, MA).

Serum TC and triglyceride (TG) concentrations were measured on a Hitachi 917 analyzer (Roche Diagnostics, Indianapolis, IN) using enzymatic kits (catalog numbers 2016630 and 2016648, respectively; Roche Diagnostics). 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 d 1 of the period.

***Calculations.*** Apparent total tract DM digestibilities were calculated as 100 − (100 × marker concentration in the feed (%)/marker concentration in the feces (%)). Apparent total tract nutrient digestibilities were calculated as 100 − 100(marker concentration in the feed (%) × nutrient concentration in feces (%)/(marker concentration in feces (%) × nutrient concentration in the feed (%)).

Apparent ileal DM digestibilities were calculated as 100 − (100 × marker concentration in the feed (%)/marker concentration in the ileal effluent (%)). Apparent ileal nutrient digestibilities were calculated as 100 − 100(marker concentration in the feed (%) × nutrient concentration in ileal effluent (%)/marker concentration in ileal effluent (%) × nutrient concentration in the feed (%)).

***Microbiota Quantification.*** Microbiota populations were quantified using molecular techniques. 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 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 PCR (**qPCR**) analysis using specific primers (Middelbos et al., 2007). 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).

***Statistical Analyses.*** Data were analyzed as a Latin square design using the Mixed procedure of SAS/STAT software, version 9.2 for Windows. 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 ANOVA 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 preplanned orthogonal polynomial contrasts. Linear and quadratic effects of ACD supplementation were analyzed. Standard error of the mean 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.

## RESULTS

### *In Vitro Fermentation Experiment*

Short-chain fatty acid production profiles resulting from fermentation of cyclodextrins are presented in Table 3. Maximal production of total SCFA was greatest ($P < 0.05$) for BCD followed by GCD and ACD (values did not statistically differ). However, the maximal rate of production was numerically greatest 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 followed the same basic trend as total SCFA production, with BCD exhibiting the greatest maximal acetate production but with GCD having the numerically greatest maximal rate of acetate production and the shortest time to attain it ($P < 0.05$). Again, ACD exhibited the lowest maximal acetate production and the lowest maximal rate of acetate production and took the longest time to reach it. Propionate production followed a very distinctive profile, with ACD resulting in the greatest maximal propionate production ($P < 0.05$), the greatest (numerical) maximal rate of propionate production, and the longest time to attain maximal rate of production ($P < 0.05$). Butyrate production profiles also were unique, with GCD resulting in the greatest maximal butyrate production ($P < 0.05$) and the numerically greatest 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. 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 ($P < 0.05$) change in pH (−0.5) than ACD (−0.3). At the 6-h pull time, GCD had the greatest ($P < 0.05$) pH change (−1.8) followed by BCD (−1.4) and then ACD (−1.1).

**Table 3.** Short-chain fatty acid (SCFA) production profiles during in vitro fermentation of cyclodextrins

| Item | Cyclodextrin α | Cyclodextrin β | Cyclodextrin γ | Pooled SEM |
|---|---|---|---|---|
| Total SCFA | | | | |
| Maximal production, μmol/g DM | 6,656[b] | 7,326[a] | 6,927[b] | 91.1 |
| Duration of production, h | 1.3[a] | 1.2[a] | 1.0[b] | 0.1 |
| Maximal rate of production, μmol ·h$^{-1}$·g DM$^{-1}$ | 1,331 | 1,477 | 1,767 | N/E[1] |
| Time to attain maximal rate of production, h | 6.4[a] | 4.9[b] | 4.2[c] | 0.1 |
| Acetate | | | | |
| Maximal production, μmol/g DM | 2,764[c] | 3,786[a] | 3,464[b] | 33.0 |
| Duration of production, h | 1.4[a] | 1.2[b] | 0.9[c] | 0.1 |
| Maximal rate of production, μmol ·h$^{-1}$·g DM$^{-1}$ | 497.1 | 823.1 | 951.5 | N/E |
| Time to attain maximal rate of production, h | 5.7[a] | 4.5[b] | 3.9[c] | 0.1 |
| Propionate | | | | |
| Maximal production, μmol/g DM | 3,410[a] | 2,896[b] | 2,361[c] | 51.9 |
| Duration of production, h | 1.0[ab] | 1.3[a] | 0.8[b] | 0.1 |
| Maximal rate of production, μmol ·h$^{-1}$·g DM$^{-1}$ | 887.9 | 574.5 | 786.8 | N/E |
| Time to attain maximal rate of production, h | 6.7[a] | 5.4[b] | 4.1[c] | 0.1 |
| Butyrate | | | | |
| Maximal production, μmol/g DM | 423.6[c] | 644.4[b] | 1,133[a] | 15.2 |
| Duration of production, h | 1.6 | 1.4 | 1.4 | 0.1 |
| Maximal rate of production, μmol ·h$^{-1}$·g DM$^{-1}$ | 67 | 117.6 | 200.9 | N/E |
| Time to attain maximal rate of production, h | 6.3[a] | 5.0[c] | 5.7[b] | 0.1 |

[a–c]Estimates within a row with different superscripts differ ($P < 0.05$); $n = 3$.

[1]N/E = not estimable.

Changes in pH after fermentation for 9 and 12 h were not different among treatments or between these 2 pull times.

### *Apparent 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 5. Average daily food intakes were similar among treatments throughout the study, with most of the dogs ingesting all the food they were provided. Ileal digestibility of DM, OM, CP, and AHF were not different among treatments. On the contrary, total tract digestibilities of DM ($P = 0.016$), OM ($P = 0.004$), CP ($P < 0.0001$), and AHF ($P = 0.001$) exhibited a linear decrease as ACD intake increased.

Ileal DM concentration linearly increased ($P = 0.008$) as ACD intake increased. Fecal output expressed on an as-is basis ($P < 0.0001$), on a DM basis ($P = 0.033$), and on an as-is per gram DM intake basis linearly increased ($P < 0.0001$) with increasing ACD supplementation (Table 6). At the same time, fecal DM content linearly decreased ($P = 0.001$) and, consequently, fecal scores linearly increased ($P = 0.009$).

**Table 4.** Changes in pH after in vitro fermentation of α-cyclodextrin, β-cyclodextrin, and γ-cyclodextrin[1]

| Pull time, h | Cyclodextrin α | Cyclodextrin β | Cyclodextrin γ | Pooled SEM |
|---|---|---|---|---|
| 3 | −0.3[a] | −0.5[b] | −0.5[b] | 0.0 |
| 6 | −1.1[a] | −1.4[b] | −1.8[c] | 0.0 |
| 9 | −2.0 | −1.9 | −2.0 | 0.0 |
| 12 | −2.1 | −2.0 | −2.0 | 0.0 |

[a–c]Means within a row with different superscripts differ ($P < 0.05$); $n = 3$.

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

Fecal microbiota quantified by qPCR for dogs fed diets supplemented with ACD are presented in Table 7. Bifidobacteria, *C. perfringens*, and *E. coli* were not different among treatments. *Lactobacilli* exhibited a quadratic ($P = 0.04$) response to increasing ACD intake.

Serum cholesterol and TG concentrations and changes in concentration for dogs supplemented with ACD are shown in Table 8. Serum cholesterol and TG concentrations were within normal ranges for dogs and were not different among treatments. Changes in concentration were not different from 0 ($P > 0.05$) and were similar among treatments.

## DISCUSSION

Alpha-cyclodextrin is a fiber source suitable for human and companion animal nutrition. For this reason, this substrate and other cyclodextrin analogs were tested using human fecal inoculum to assess the fermentative profiles of these substrates in vitro and in vivo using the canine model to understand the impact these substrates on ileal and total tract nutrient digestibility. Even though the domestic dog belongs to the order Carnivora, it has an omnivore feeding behavior and shares similar gastrointestinal anatomy and physiology, dietary patterns, metabolic processes, and gastrointestinal diseases (Swanson and Schook, 2006; Swanson et al., 2011). Therefore, a similar fermentative pattern between these species is expected.

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, making up 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 3-hydroxy-3-methyl-glutaryl-CoA reductase (Levrat et al., 1994), the key regulatory enzyme

**Table 5.** Intake (g/d, as-fed basis) and apparent ileal and total tract nutrient digestibilities (%) by dogs fed diets supplemented with α-cyclodextrin (ACD)

| | α-Cyclodextrin | | | | | *P*-value[1] | |
|---|---|---|---|---|---|---|---|
| Item | 0 g ACD | 2 g ACD | 4 g ACD | 6 g ACD | 8 g ACD | Linear | Quadratic |
| Intake | 266.7 | 290.2 | 274.1 | 271.8 | 297.5 | 0.48 | 0.97 |
| Ileal digestibility, % | | | | | | | |
| DM | 69.2 | 69.8 | 67.1 | 66.6 | 68.2 | 0.244 | 0.427 |
| OM | 75.0 | 75.4 | 72.6 | 71.7 | 73.5 | 0.164 | 0.390 |
| CP | 67.5 | 71.0 | 69.2 | 69.6 | 71.6 | 0.200 | 0.899 |
| Acid hydrolyzed fat | 93.5 | 94.6 | 93.0 | 93.1 | 93.8 | 0.643 | 0.773 |
| Total tract digestibility, % | | | | | | | |
| DM | 77.5 | 77.5 | 77.7 | 76.5 | 76.3 | 0.016 | 0.271 |
| OM | 82.5 | 82.4 | 82.3 | 81.4 | 81.1 | 0.004 | 0.393 |
| CP | 84.0 | 83.0 | 83.2 | 82.0 | 81.3 | <0.001 | 0.436 |
| Acid hydrolyzed fat | 94.3 | 94.5 | 94.1 | 93.5 | 93.5 | 0.001 | 0.320 |

[1]Polynomial contrast; $n = 5$.

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- 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 buildup of waste products. Furthermore, 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 selected glucose-based polymers by canine and human fecal bacteria. Substrates included lyophilized canine ileal digesta containing maltodextrin, GCD, high-molecular-weight pullulan, and low-molecular-weight 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 nondietary 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. This animal 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).

Ileal digestibility coefficients for DM, OM, CP, and AHF were high and comparable with 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

**Table 6.** Ileal DM values (%) and fecal characteristics of dogs fed diets supplemented with α-cyclodextrin (ACD)

| | α-Cyclodextrin | | | | | *P*-value[1] | |
|---|---|---|---|---|---|---|---|
| Item | 0 g ACD | 2 g ACD | 4 g ACD | 6 g ACD | 8 g ACD | Linear | Quadratic |
| Ileal DM, % | 13.4 | 13.4 | 14.7 | 15.8 | 15.3 | 0.008 | 0.597 |
| Fecal characteristics | | | | | | | |
| Fecal output (as-is), g/d | 122.7 | 139.4 | 135.0 | 142.4 | 159.6 | 0.000 | 0.495 |
| Fecal output (DM), g/d | 55.5 | 60.5 | 56.4 | 59.0 | 65.2 | 0.033 | 0.326 |
| Fecal output (as-is) per g DM consumed | 0.49 | 0.52 | 0.53 | 0.57 | 0.58 | <0.001 | 0.950 |
| Fecal DM, % | 45.5 | 43.4 | 41.9 | 41.6 | 41.0 | 0.001 | 0.143 |
| Fecal score[2] | 2.56 | 2.65 | 2.76 | 2.83 | 2.84 | 0.009 | 0.494 |

[1]Polynomial contrast; $n = 5$.

[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 7.** Fecal microbiota (log cfu/g DM feces) quantitative polymerase chain reaction for dogs fed diets supplemented with α-cyclodextrin (ACD)

| | α-Cyclodextrin | | | | | *P*-value[1] | |
|---|---|---|---|---|---|---|---|
| Item | 0 g ACD | 2 g ACD | 4 g ACD | 6 g ACD | 8 g ACD | Linear | Quadratic |
| Bifidobacteria | 8.8 | 8.7 | 8.9 | 8.7 | 8.8 | 0.920 | 0.869 |
| Lactobacilli | 10.19 | 10.19 | 10.22 | 9.63 | 10.80 | 0.343 | 0.043 |
| Escherichia coli | 11.48 | 11.64 | 11.56 | 11.38 | 11.38 | 0.190 | 0.311 |
| Clostridium perfringens | 10.59 | 10.31 | 10.47 | 10.54 | 10.34 | 0.664 | 0.945 |

[1]Polynomial contrast; $n = 5$.

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 wk, a low-fat (4% fat) diet, a low-fat + ACD (0.4%) diet, a high-fat (40% fat) diet, and a high-fat + ACD (4%) diet. Intake of ACD did not alter fecal excretion of fat between the low-fat diets (0.16 g) or between the high-fat diets (0.29 g). Spears et al. (2005) evaluated the effect of GCD on ileal digestibility of DM, OM, or CP, but fat digestibility was reduced 2% by GCD at a 25% dietary inclusion rate (approximately 120 g/d).

Total tract digestibility of all nutrients linearly decreased, even though there was no difference in ileal nutrient digestibility. Differences between the 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 NPN. 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 nonionic 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, to screen for changes in microbiota, quantification by qPCR was used in this study. Dietary supplementation of ACD did not significant affect fecal bifidobacteria, *E. coli*, or *C. perfringens*. However, there was a quadratic 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 the bacterial genera and species analyzed. Therefore, it

**Table 8.** Serum cholesterol and triglyceride concentrations and changes in concentration for dogs supplemented with α-cyclodextrin (ACD)[1]

| | α-Cyclodextrin | | | | | *P*-value[2] | |
|---|---|---|---|---|---|---|---|
| Item | 0 g ACD | 2 g ACD | 4 g ACD | 6 g ACD | 8 g ACD | Linear | Quadratic |
| Serum cholesterol, mmol/dL | 5.5 | 5.5 | 5.5 | 5.4 | 5.4 | 0.742 | 0.938 |
| Serum triglycerides, mmol/dL | 0.6 | 0.6 | 0.5 | 0.6 | 0.6 | 0.484 | 0.989 |
| Δ Serum cholesterol, mmol/dL | −0.1 | 0.5 | 0.1 | 0.1 | 0.3 | 0.859 | 0.743 |
| Δ Serum triglycerides, mmol/dL | 0.0 | −0.1 | 0.2 | 0.0 | 0.0 | 0.877 | 0.311 |

[1]Concentations were measured on d 14 of each period. Change was measured between d 1 and 10 of each period.

[2]Polynomial contrast; $n = 5$.

is possible that ACD might alter composition of microbial populations within those genera but not alter total cfu values. Future studies should be done using high-throughput sequencing technology to better understand the impact of cyclodextrins on the gastrointestinal microbiome of dogs.

The lack of effect of ACD intake on serum cholesterol concentrations is consistent with previous experiments performed in our laboratory (Guevara et al., 2015). It has been reported that the lack of cholesterol ester transfer protein activity in dogs results in high concentrations of high-density lipoprotein cholesterol, which facilitates the redirection of cholesterol to the liver for clearance (Bailhache et al., 2004).

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 are warranted to elucidate the mechanism for the hypocholesterolemic effect of ACD; the use of obese and/or hyperlipidemic dogs might be a more responsive animal model for this purpose.

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