COMPANION ANIMAL NUTRITION

Organic matter disappearance and production of short- and branched-chain fatty acids from selected fiber sources used in pet foods by a canine in vitro fermentation model[1]

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[1]The authors would like to thank Renew Biomass for sponsoring this work.

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Abstract

Dietary fibers can influence a dog's overall health, but high concentrations of soluble dietary fibers can cause soft stools. An in vitro model could be useful to predict the rate fibers are fermented once they reach the colon. Pet food companies are constantly searching for new ingredients to differentiate their products from competitors. Miscanthus grass (**MG**), pea fiber (**PF**), and sorghum bran (**SB**) are novel fiber sources that could be alternatives to standards like cellulose (**CE**) and beet pulp (**BP**). The objectives of the study were to determine the effects of fiber source on organic matter disappearance (**OMD**), estimated organic matter disappearance (**EOMD**), and fermentation end-product concentrations using an in vitro fermentation procedure and dog fecal inoculum. Total dietary fiber (**TDF**) residues from MG, CE, BP, PF, and SB were fermented in vitro with buffered dog feces. Fecal samples were collected and maintained in anaerobic conditions until the dilution and inoculation. Test tubes containing the fibrous substrates were incubated for 4, 8, and 12 h at 39 °C. Short-chain fatty acids (**SCFA**), branched-chain fatty acids (**BCFA**), OMD, and EOMD were determined for each fiber source and time point. Beet pulp had the highest OMD, EOMD, and SCFA production of all tested fiber sources (38.6% OMD, 26.2% EOMD, 2.72 mmol SCFA/g of substrate). Sorghum bran led to greater concentrations of BCFA (59.86 µmol/g of substrate) and intermediate OMD and EOMD compared to the other tested fibers. Cellulose and MG were poorly fermented with the lowest OMD, EOMD, SCFA, and BCFA compared to other fibers. In conclusion, MG could be used as an insoluble minimally fermentable replacement fiber for CE in dog foods.

Keywords: beet pulp, cellulose, fermentation, miscanthus grass, pea fiber, sorghum bran

Introduction

Various fiber sources are used in pet foods with different purposes, such as energy dilution, gut health, and hairball management (Castrillo et al., 2001; Loureiro et al., 2014; Floerchinger et al., 2015). For these purposes, select fibers and various inclusion levels have been used. Fiber type (soluble vs. insoluble fibers) and the concentration of the fiber in the diet can impact nutrient utilization and stool quality (Fahey et al., 1990a, 1990b, Wichert et al., 2002). Cellulose (**CE**) and beet pulp (**BP**) are

considered standard fiber sources in pet foods and have been extensively studied in dog models (Fahey et al., 1990a, 1990b; Sunvold et al., 1995a, 1995b; Wichert et al., 2002; Prola et al., 2010). However, pet food companies continue to explore alternative ingredients to sustain industry growth and consumer demand. Miscanthus grass (**MG**), sorghum bran (**SB**), and pea fiber (**PF**) could be such alternative fiber sources. However, little is known about the effects of these fiber sources on nutrient utilization, stool quality, and gut health.

Pea fiber has been previously tested in an in vitro model with canine fecal inoculum. Neutral detergent fiber levels were estimated to be about 70% (Bosch et al., 2008), thus indicating a high concentration of insoluble fibers in the ingredient. However, the authors did not evaluate its total dietary fiber (**TDF**) content. Sorghum mill feed was evaluated by Alvarenga and others (2018). The authors reported that this ingredient had 20% TDF with most of the dietary fiber being insoluble (18.3%). Similar to these, MG is a fiber source mostly composed of insoluble fibers (Donadelli et al., 2019); however, the TDF content of this ingredient was not reported. Although some information about these novel ingredients are available in the literature, it is important to further characterize the effects they could have in the colon when fermented.

Effects of fiber sources on stool quality and gut health can be evaluated using an in vitro model with canine fecal inoculum (Sunvold et al., 1995a, 1995b; de Godoy et al., 2015). Using this technique, the rate of production and the concentration of fermentation end products can be estimated. Additionally, the results can be used to guide food formulators on the purpose of each fiber source (gut health vs. energy dilution) and their inclusion levels. However, results may be inconsistent, because there is variation in fiber composition depending on the conditions that the crops were grown (Fahey et al., 1990b) and processed (Montagne et al., 2003), in addition to between-laboratory variation in the evaluation (Barry et al., 1995). Regardless, characterization of fermentability could be beneficial for pet food formulation and pet health. The hypotheses were that MG would have a limited fermentability similar to CE, and that PF and SB would have fermentabilities intermediate to CE and BP. The objectives of this study were to determine the organic matter disappearance (**OMD**), estimated organic matter disappearance (**EOMD**), and fermentation end-product production for different fiber sources using an in vitro model with dog fecal inoculum.

Materials and Methods

Fiber Sources Preparation and Characterization

Fiber sources (MG, Renew Biomass, Springfield, MO; CE, BP, and PF, Fairview Mills, Seneca, KS; SB, Hal Ross Flour Mill, Kansas State University; Alvarenga et al., 2018; Table 1) were selected because they have been evaluated in animal feeding studies in the Pet Food Processing Lab at Kansas State University (SB, Alvarenga et al., 2018; PF, Pontious et al., 2018; MG, CE, BP, Donadelli and Aldrich, 2019). Prior to the incubation, samples were predigested with α-amylase, protease, and amyloglucosidase (TDF assay kit, Sigma-Aldrich, catalog no. TDF100A-1KT) simulating the digestion in the small intestine of the dog. Briefly, 10 g of sample was mixed with 500 mL 0.08 M phosphate buffer (pH = 6) and 1 mL of α-amylase. The samples were placed in a water bath at 95 °C and were digested for 15 min once the sample reached 95 °C. Next, the pH was adjusted to 7.5 with 100 mL of sodium hydroxide (0.275 N) after the samples cooled to room

Table 1. Laboratory dog food and individual test fiber[1] composition (dry matter basis)

Composition, %	Food	MG	CE	BP	PF	SB
Dry matter	92.8	95.4	96.3	94.3	93.5	93.9
Crude protein	30.2	2.9*	1.7*	11.2*	3.5*	20.8*
Fat	18.2	nd	nd	nd	nd	nd
Ash	7.8	nd	nd	nd	nd	nd
Crude fiber	2.3	nd	nd	nd	nd	nd
TDF[2]	9.9	80.9	94.9	51.9	71.9	22.9
Insoluble fiber	8.9	80.9	92.9	30.6	65.1	18.5
Soluble fiber[3]	0.9	0.1	2.0	21.2	6.9	4.4

nd: not determined.
[1]MG, miscanthus grass; CE, cellulose; BP, beet pulp; PF, pea fiber; SB, sorghum bran.
[2]TDF, total dietary fiber.
[3]Calculated soluble fiber = TDF − insoluble fiber.
*Analyzed on the TDF residues used in the fermentations.

temperature and 1 mL of protease was added to the samples. Next samples were digested for 30 min once they reached 60 °C in a water bath. Then sample pH was adjusted to 4.3 with 100 mL of hydrochloric acid (0.325 N) once the samples cooled to room temperature and 1 mL of amyloglucosidase was added. Next, samples were digested for 30 min once they reached 60 °C in a water bath. After the samples were cooled to room temperature, 4 volumes of 95% ethanol were added. Samples were allowed to sit on the bench-top overnight (approximately 16 h). On the following day, the samples were filtered, then the sample was rinsed with two 100-mL volumes of 95% ethanol and two 100-mL volumes of acetone. After the filtration, samples were oven-dried overnight at 55 °C. On the following day samples were ground to pass a 1-mm screen (Retsch ZM200, Germany). Total dietary fiber (Prosky et al., 1985) and insoluble fiber (Prosky et al., 1988) were determined on the original fiber ingredients and the food provided to the dogs that served as inoculum donors. Soluble fiber was calculated for the fiber samples and food by subtracting insoluble fiber from the TDF content. Crude protein (CP; AOAC 990.03) was determined on the TDF residues of the fiber sources used for fermentations as well as on the dog food. Fat by acid hydrolysis (AOAC 954.02), ash (AOAC 942.05), and crude fiber (AOCS Ba6a-05) were determined on the dog food.

Dog Donors and Inoculum Preparation

The procedures for preparation of inoculum and incubation of the fibrous substrates were an adaptation of methods used by Sunvold et al. (1995a, 1995b). Beagle dog donors were group-housed in the Large Animal Research Center of Kansas State University. The fecal collection was approved by the institutional Animal Care and Use Committee from Kansas State University (Protocol no. 3878). Two dogs were grouped per pen with access to outside fenced exercise areas. The laboratory diet (Table 2) was provided twice daily for each dog according to their energy requirements for at least 2 wk prior to the fecal sample collection. Prior to the incubation of the fiber samples, feces were collected fresh within 15 min after defecation. Four dogs (2 neutered males and 2 spayed females) defecated within a 15-min span and their feces were collected for the preparation of the inoculum. Each fecal sample was stored in a plastic bag and the air was removed from the bag to decrease contamination with O_2. Next the bags were placed in an insulated container which was warmed to 37 °C. The fecal samples were transported to the lab and 25 ± 0.1 g from each feces was pooled to produce the inoculum.

Table 2. Composition of inoculation medium and anaerobic dilution solutions

Solution	Medium	Anaerobic dilution
Solution A[1], mL	330.0	37.50
Solution B[2], mL	330.0	37.50
Mineral solution[3], mL	10.00	–
Vitamin solution[4], mL	20.00	–
Folate-biotin solution[5], mL	5.00	–
Riboflavin solution[6], mL	5.00	–
Hemin solution[7], mL	2.50	–
Resazurin solution[8], mL	1.00	1.00
Water, mL	296.0	854.0
Yeast extract, g	0.50	–
Trypticase, g	0.50	–
Na$_2$CO$_3$, g	4.00	6.37
Cysteine hydrochloride, g	0.50	0.50

[1]Solution A—5.4 g sodium chloride, 5.4 g ammonium sulfate, 2.7 g potassium phosphate monobasic anhydrous, 0.18 g calcium chloride dihydrate, 0.12 g magnesium chloride hexahydrate, 0.06 g manganese chloride tetrahydrate, 0.06 g cobalt chloride hexahydrate, to 1 liter with distilled water.
[2]Solution B—2.7 g potassium phosphate dibasic anhydrous to 1 liter with distilled water.
[3]Mineral solution—500 mg of ethylenediaminetetraacetic acid, 200 mg iron (II) sulfate heptahydrate, 30 mg *m*-phosphoric acid, 20 mg cobalt chloride hexahydrate, 10 mg zinc sulfate heptahydrate, 3 mg manganese chloride tetrahydrate, 3 mg sodium molybdate dihydrate, 2 mg nickel (II) chloride hexahydrate, 1 mg copper (II) chloride dihydrate, to 1 liter with distilled water.
[4]Vitamin solution—Added to the medium by filter sterilization after other reagents were sterilized in autoclave. Weigh 100 mg thiamin hydrochloride, 100 mg pantothenic acid, 100 mg niacin, 100 mg pyridoxine hydrochloride, 10 mg ammonium carbonate, 5 mg ρ-aminobenzoic acid, 0.25 mg vitamin B-12, to 1 liter with distilled water.
[5]Folate-biotin solution—100 mg ammonium carbonate, 10 mg folic acid, 2 mg biotin, to 1 liter with distilled water.
[6]Riboflavin solution—130 mg HEPES, 1 mg riboflavin, to 1 liter with distilled water.
[7]Hemin solution—50 mg hemin, 40 mg sodium hydroxide, to 100 mL with distilled water.
[8]Resazurin solution—100 mg resazurin to 100 mL with distilled water.

Next, the pooled fecal sample was mixed with 1 liter of anaerobic dilution solution (1:10 feces:dilution solution, wt:vol; Table 2) under copper scrubbed CO$_2$. Once completely mixed, the solution was filtered through 4 layers of cheese cloth under copper scrubbed CO$_2$. The solution was kept at 39 °C until inoculation of sample tubes.

Incubation Preparation and OMD Determination

Fiber samples were weighed (310 ± 0.1 mg, in triplicate) in 50-mL centrifuge tubes for each one of the 4 time points (0, 4, 8, and 12 h). To each tube, 26 mL of media solution (Table 2) was added. Next, each tube was flushed with copper scrubbed CO$_2$ and closed with a rubber stopper equipped with a 1-way valve. Tubes were then placed in the refrigerator overnight to allow hydration of the fibers. On the following day, the samples were placed in the water bath at 39 °C for 1 h prior to the inoculation. In addition to the tubes with the fiber samples, 4 tubes for each time point were filled with media solution to be used as blanks. We decided to use 4 tubes for the blanks instead of 3 tubes based on previous experiments that showed a greater variation in the blanks.

Tubes were inoculated with 4 mL of inoculum (filtered anaerobic dilution solution) starting tubes from time 12 h, then 8 h, and lastly 4 h. After inoculation, tubes were flushed with copper scrubbed CO$_2$, closed with a rubber stopper equipped with a 1-way valve, and incubated in water bath at 39 °C for the predetermined time points. After each incubation time, two 1-mL subsamples from each tube were transferred to microcentrifuge tubes for fermentation end-product determination using the methods described by Elwakeel et al. (2013). The remaining liquid and solid residue in the centrifuge tube was transferred to a beaker, mixed with 112 mL of 95% ethanol, and allowed to rest overnight. On the following day, the samples were filtered using a dried preweighed ashless Whatman filter paper (catalog no. 1541-110) and rinsed with two 10-mL volumes of 95% ethanol and two 10-mL volumes of acetone. Next, residues and filter were dried in a convection oven overnight at 105 °C. The dry weight of the filter and residue was recorded the following day. Organic matter disappearance was calculated as follows:

$$\mathrm{OMD} = 1 - \frac{\mathrm{OM\ residue} - \mathrm{OM\ blank}}{\mathrm{Initial\ OM}}$$

wherein OM residue is the organic matter in the sample after the incubation and filtration in g, OM blank is the organic matter in the blank after incubation and filtration in g, and Initial OM is the initial organic matter in the sample prior to incubation in g. Additionally, OMD was estimated (EOMD) based on the short-chain fatty acid (**SCFA**) production. For the calculation to be feasible, 2 assumptions were used: all the acetate, propionate, and butyrate were produced from anhydrous glucose (molecular weight of 162 g/mol); 1 molecule of glucose will yield 2 acetates, 2 propionates, or 1 butyrate. Therefore, the glucose mass needed to produce the concentrations of SCFA was calculated as follows:

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

wherein Glucose is the mass of glucose in g, acetate is the moles of acetate produced in each tube after 4, 8, or 12 h of incubation, propionate is the moles of propionate produced in each tube after 4, 8, or 12 h of incubation, and butyrate is the moles of butyrate produced in each tube after 4, 8, or 12 h of incubation. Estimated organic matter disappearance was calculated by dividing the mass of glucose needed to produce the SCFA by the substrate OM.

Statistical Analysis

The experiment was performed as a completely randomized design, with 50-mL centrifuge tube being the experimental unit. Data were analyzed using the general linear model procedure (SAS, v. 9.4). Treatment means were separated using Fisher's Least Significant Difference. Differences were considered significant at $P < 0.05$, and trends were considered when $0.05 < P < 0.10$.

Results and Discussion

Fiber composition was variable with respect to the insoluble, soluble, and TDF (Table 1). Crude protein content of the CE substrate was greater than concentrations reported by Sunvold et al. (1995a, 1995b) and de Godoy et al. (2015). However, the TDF content was within the range from reports in the literature (Sunvold et al., 1995a, 1995b; de Godoy et al., 2015). Crude protein content of BP substrate was higher than values reported by Fahey

et al. (1990a, 1990b) and Bosch et al. (2008); conversely, the TDF value was lower than reports by Sunvold et al. (1995a, 1995b) and de Godoy et al. (2015). Pea fiber substrate CP was lower compared to report by Bosch et al. (2008). Sorghum bran TDF content was similar to that reported by Alvarenga et al. (2018), although the protein content was higher. These differences in composition of the ingredients are known to occur when comparing agricultural by-products. This variation was noted by other authors and it could be due to differences in the conditions that these crops were produced (Fahey et al., 1990b) and differences in processing conditions to generate such products (Montagne et al., 2003). In addition, Barry et al. (1995) reported that there is variation in TDF analysis among different laboratories analyzing the same fiber source using the same method. The test fibers can be categorized by their CP, insoluble and soluble fibers content. In this case, CE and MG have a high content of insoluble fiber and low content of CP and soluble fiber. Beet pulp has a higher concentration of soluble fiber compared to the other test fiber sources. Finally, PF and SB have intermediate concentrations of insoluble and soluble fibers, but SB has a much higher CP content than PF.

The results and discussion for OMD, EOMD, SCFA, and branched-chain fatty acid (**BCFA**) concentration presented here will be based on the 12-h time point unless otherwise specified (Tables 3 and 4). In general terms, EOMD values were smaller than OMD values. The higher values of OMD could be related to the grinding step after the simulated small intestinal digestion procedure, possibly, the grinding decreased the particles to an extent that they passed through the paper filter, overestimating the substrate disappearance. This most likely occurred for BP, PF, and SB, because these fiber sources' higher soluble fiber content created clumps. Conversely, CE and MG did not for clumps. Organic matter disappearance and EOMD were greater for BP compared to other tested fibers regardless of the time point (Table 3; $P < 0.05$). After 12 h of incubation, MG and CE had the lowest OMD and EOMD compared to the other fiber sources and PF and SB had intermediate values (1.3, 2.6, 6.9, and 10.6%, respectively; Table 3). The negative OMD value for MG on time 8 h (–0.8%), while unlikely, could be an error associated with the inoculum. It was not possible to separate all the fecal particles when filtering with the cheese cloth (i.e., the solution had a brown color after the filtration); therefore, some residual material from the inoculum might account for this result. Differently, the EOMD for MG at 8 h was 0.52%; while this is a very low value, this technique of estimating the substrate disappearance by the SCFA yields may be an alternative to OMD and provide a more accurate estimation.

The OMD of BP was lower when compared to values reported by Sunvold et al. (1995a, 1995b); however, CE OMD was similar. This could be a result of the lower TDF content of the BP used in this experiment compared to the one used by Sunvold et al. (1995a; 51.86% vs. 68.4%, respectively); therefore, with less organic matter fermented and the OMD would be lower. Sorghum bran in this experiment had similar TDF content to the rice bran evaluated by Sunvold et al. (1995a), although the OMD for rice bran was higher than OMD for SB (34.8% vs. 10.6%, respectively). Although the TDF content of these fiber sources was similar, the soluble fiber content of rice bran likely is much higher than the SB. Pea fiber maximum rate of gas production was reported to be about half of the rate of BP by Bosch et al. (2008); however, the OMD of PF about 6 times lower than BP OMD (38.6% vs. 6.9%, respectively, for BP and PF). Although the fermentation rate may be faster for BP, the OMD values agree with the soluble fiber content in these fiber sources, wherein BP has about 4 times more soluble fibers than PF (Table 1). As noted previously, the MG and CE OMD and EOMD were similar, which could be due to their high insoluble and low soluble fiber composition; thereby, less material was available for the microorganisms to ferment, resulting in lower substrate fermentation compared to the other tested fibers (Table 3).

Acetate concentration was highest for BP, followed by PF and SB, and lowest for MG and CE ($P > 0.05$; Table 4). In the animal, acetate is absorbed and transported by the blood stream to various peripheral organs. In these organs, acetate can be used as fuel source (e.g., muscle) or be deposited as fat (e.g., adipose tissue; Bergman, 1990). Most of propionate is converted to glucose in the liver (Bergman, 1990). For our work, propionate concentration increased over time for all fiber sources. Additionally, BP had the highest production (0.558 mmol/g of substrate), SB had the second highest production (0.227 mmol/g of substrate), followed by PF (0.127 mmol/g of substrate), and MG and CE had the lowest values (0.023 and 0.018 mmol/g of substrate, respectively).

Butyrate is perhaps the most important fermentation end product for animal health. This SCFA promotes health benefits such as prevention of colonic cancer (McIntyre et al., 1993; Wong et al., 2005; Comalada et al., 2006) and chronic inflammation (Roediger, 1990; Vernia et al., 2003; Hamer et al., 2008), promotion of satiety (Delzenne et al., 2005; Karaki et al., 2007), improvement of defense barriers in the colon (Deplancke and Gaskins, 2001; Gaudier et al., 2004), and decreases in oxidative stress (Rosignoli et al., 2001; Toden et al., 2007). Butyrate is the preferred fuel source for colonocytes (Velazquez et al., 1997; Hamer et al., 2008). In this study, butyrate concentration was highest for

Table 3. Organic matter disappearance (OMD, %) and estimated organic matter disappearance (EOMD, %) of fermented fibers sources inoculated with dog feces for 4, 8, and 12 h

Fermentation time, h	Fiber source[1]					SEM	P-value
	MG	CE	BP	PF	SB		
OMD, %							
4	2.60[c]	3.69[bc]	29.82[a]	7.60[b]	5.83[bc]	1.58	<0.0001
8	–0.79[c]	1.79[c]	31.86[a]	6.47[b]	6.43[b]	0.98	<0.0001
12	1.27[c]	2.63[c]	38.63[a]	9.60[b]	10.62[b]	0.61	<0.0001
EOMD, %							
4	1.25[c]	0.25[c]	17.89[a]	3.95[b]	3.30[b]	0.33	<0.0001
8	0.52[c]	0.71[c]	23.43[a]	7.47[b]	5.54[b]	0.66	<0.0001
12	1.09[c]	0.31[c]	26.22[a]	6.15[b]	7.19[b]	0.50	<0.0001

[1]MG, miscanthus grass; CE, cellulose; BP, beet pulp; PF, pea fiber; SB, sorghum bran.
[a–c]Means in the same row with unlike superscripts differ.

Table 4. Short-chain fatty acids (SCFA), branched-chain fatty acids (BCFA), and total fatty acids (SCFA + BCFA) production from fermented fibers sources inoculated with dog feces for 4, 8, and 12 h, expressed in a mmol or µmol/g of substrate (dry matter basis)

Fermentation time, h	Fiber sources[1]					SEM	P-value
	MG	CE	BP	PF	SB		
Acetate, mmol/g of substrate							
4	0.12[d]	0.03[e]	1.48[a]	0.35[b]	0.22[c]	0.028	<0.0001
8	0.04[d]	0.05[d]	1.85[a]	0.53[b]	0.35[c]	0.033	<0.0001
12	0.08[c]	0.02[c]	2.05[a]	0.51[b]	0.44[b]	0.049	<0.0001
Propionate, mmol/g of substrate							
4	0.008[c]	0.010[c]	0.294[a]	0.067[b]	0.072[b]	0.0048	<0.0001
8	0.011[d]	0.027[d]	0.452[a]	0.118[c]	0.156[b]	0.0081	<0.0001
12	0.023[d]	0.018[d]	0.558[a]	0.127[c]	0.227[b]	0.0123	<0.0001
Butyrate, µmol/g of substrate							
4	4.26[d]	−3.44[d]	65.26[a]	16.83[c]	27.67[b]	2.48	<0.0001
8	3.78	3.13	90.87	97.23	44.23	30.61	0.1422
12	7.80[d]	−2.81[e]	105.25[a]	28.24[c]	51.44[b]	2.58	<0.0001
SCFA[2], mmol/g of substrate							
4	0.14[c]	0.03[c]	1.83[a]	0.43[b]	0.32[b]	0.034	<0.0001
8	0.06[d]	0.08[d]	2.40[a]	0.75[b]	0.55[c]	0.051	<0.0001
12	0.11[c]	0.04[c]	2.72[a]	0.67[b]	0.72[b]	0.063	<0.0001
Isobutyrate, µmol/g of substrate							
4	0.72[c]	−0.56[c]	5.86[b]	1.37[c]	10.02[a]	0.68	<0.0001
8	1.77[c]	2.08[c]	11.69[b]	3.03[c]	19.74[a]	0.80	<0.0001
12	4.35	18.90	12.25	1.12	21.03	7.61	0.3290
Isovalerate, µmol/g of substrate							
4	3.94[bc]	0.40[c]	9.07[b]	6.84[b]	29.90[a]	1.70	<0.0001
8	4.50[c]	5.77[c]	15.05[b]	5.11[c]	37.25[a]	1.61	<0.0001
12	6.84[b]	1.37[c]	8.91[b]	−1.52[c]	35.71[a]	1.31	<0.0001
Valerate, µmol/g of substrate							
4	0.32[ab]	0.00[b]	0.64[a]	0.00[b]	0.00[b]	0.20	0.1695
8	0.00[c]	0.00[c]	0.00[c]	1.28[b]	2.57[a]	0.20	<0.0001
12	0.56[b]	0.24[b]	0.24[b]	−1.52[c]	3.13[a]	0.32	<0.0001
BCFA[3], µmol/g of substrate							
4	4.99[cd]	−0.16[d]	15.57[b]	8.21[c]	39.93[a]	2.17	<0.0001
8	6.27[c]	7.86[c]	25.74[b]	9.42[c]	59.56[a]	2.04	<0.0001
12	11.75[b]	20.50[b]	21.41[b]	−1.92[b]	59.86[a]	7.91	0.0030
TOTAL[4], mM/g of substrate							
4	0.14[b]	0.03[c]	1.85[a]	0.44[b]	0.36[b]	0.036	<0.0001
8	0.06[c]	0.09[c]	2.42[a]	0.76[b]	0.61[b]	0.052	<0.0001
12	0.13[c]	0.06[c]	2.74[a]	0.66[b]	0.44[b]	0.064	<0.0001

[1]MG, miscanthus grass; CE, cellulose; BP, beet pulp; PF, pea fiber; SB, sorghum bran.
[2]SCFA, short-chain fatty acids; sum of acetate, propionate, and butyrate.
[3]BCFA, branched-chain and minor fatty acids; sum of isobutyrate, isovalerate, 2-methylbutyrate, and valerate.
[4]TOTAL, total short-chain, branched-chain, and minor fatty acids; sum of SCFA and BCFA.
[a]–[e]Means in the same row with unlike superscripts differ at ($p < 0.05$).

BP and lowest for CE (105.25 vs. −2.81 µmol/g of substrate, respectively). Similar results were also reported by Sunvold et al. (1995a, 1995b) and de Godoy et al. (2015). Sorghum bran led to the second highest butyrate production, followed by PF, and then MG ($P < 0.05$; Table 4). The butyrate production at 12 h of incubation was lower for the SB compared to rice bran (Sunvold et al., 1995a; 51.44 µmol/g of substrate vs. 0.26 mmol/g of organic matter), thus rice bran might be considered a better fiber source for pet foods aiming at gut health claims. Butyrate production was greater for MG than CE ($P < 0.05$). This result could be related to how these ingredients are produced. Cellulose is a purified ingredient made from wood chips, in which most of the soluble fibers and lignin have been removed from the raw materials (Dahl, 1884). Differently, MG is produced from the ground dried canes of *Miscanthus giganteus* without any purification steps. Therefore, the higher concentrations of other constituents than CE from the plant cell wall are not removed from the final

ingredient which allowed them to be fermented and result in a higher concentration of butyrate and isovalerate (Table 4). Short-chain fatty acid production was greatest for BP, intermediate for SB and PF, with MG and CE producing the lowest production of SCFA. Similar results were reported by Barry et al. (1995), wherein the production of SCFA of sugar beet fiber was higher than that of CE (25.7 vs. 1.7 mmol/L, respectively). In addition, these authors reported a high variation when comparing results from different laboratories. For example, sugar beet fiber SCFA content varied from 6.0 to 53.7 mmol/L.

In addition to the SCFA, minor and BCFA were determined (Table 4). For the sake of this discussion, valerate will be grouped with the BCFA because its concentration was much lower than the other measured fermentation end products; however, it is important to state that valerate is a straight chain fatty acid, not branched. Unlike the SCFA, branched volatile fatty acids are exclusively produced from amino acids rather than

Table 5. Short-chain fatty acids (SCFA) and branched-chain fatty acid (BCFA) expressed as a percentage of total fatty acids (dry matter basis)

Fermentation time, h	Fiber source[1]					SEM	P-value
	MG	CE	BP	PF	SB		
% of total							
Acetate							
4	86.5[a]	61.1[bc]	79.7[ab]	79.1[ab]	61.5c	4.16	0.0107
8	67.4[ab]	49.9[c]	76.5[a]	71.2[ab]	57.0[bc]	5.45	0.0357
12	66.7[ab]	45.7[b]	75.0[a]	77.0[a]	56.3[ab]	9.08	0.1561
Propionate							
4	3.3[b]	15.3[ab]	15.2[ab]	14.40[b]	19.9[a]	3.55	0.1930
8	18.4[b]	38.6[a]	16.7[b]	15.9[b]	25.8[ab]	4.84	0.0447
12	17.9[b]	31.2[a]	20.4[b]	19.1[b]	29.3[a]	2.71	0.0153
Butyrate							
4	2.4	8.8	3.5	3.8	7.6	4.40	0.8031
8	4.2	2.0	3.7	11.6	7.3	3.79	0.4503
12	6.1a	−4.9[b]	3.9[a]	4.2[a]	6.6[a]	1.04	<0.0001
SCFA[2]							
4	95.5[abc]	91.7[bc]	99.2[a]	98.1[ab]	89.0[c]	2.13	0.0325
8	89.9[b]	90.4[b]	98.9[a]	98.7[a]	90.1[b]	0.60	<0.0001
12	90.7	72.0	99.2	100.3	92.3	9.57	0.2998
Isobutyrate							
4	0.6	2.7	0.3	0.3	2.8	0.98	0.2204
8	2.7[ab]	2.2[b]	0.5[c]	0.4[c]	3.3[a]	0.26	<0.0001
12	3.5	25.3	0.4	0.2	2.7	9.60	0.3602
Isovalerate							
4	3.8[bc]	5.6[ab]	0.5[c]	1.5[c]	8.2[a]	1.27	0.0097
8	7.4[a]	7.4[a]	0.6[b]	0.7[b]	6.1[a]	0.63	<0.0001
12	5.4[a]	2.3[b]	0.3[c]	−0.2[c]	4.6[a]	0.31	<0.0001
Valerate							
4	0.15	0	0.03	0	0	0.066	0.4865
8	0[c]	0[c]	0[c]	0.17[b]	0.43[a]	0.035	<0.0001
12	0.43[a]	0.42[a]	0.01[b]	−0.23[b]	0.40[a]	0.109	0.0041
BCFA[3]							
4	4.50[abc]	8.3[ab]	0.84[c]	1.85[bc]	11.0[a]	2.13	0.0325
8	10.06[a]	9.59[a]	1.10[b]	1.27[b]	9.85[a]	0.60	<0.0001
12	9.3	28.0	0.8	−0.3	7.7	9.57	0.2998

[1]MG, miscanthus grass; CE, cellulose; BP, beet pulp; PF, pea fiber; SB, sorghum bran.
[2]SCFA, short-chain fatty acids; sum of acetate, propionate, and butyrate.
[3]BCFA, branched-chain and minor fatty acids; sum of isobutyrate, isovalerate, 2-methylbutyrate, and valerate.
[a-c]Means in the same row with unlike superscripts differ.

carbohydrate sources (Bergman, 1990; Topping and Clifton, 2001; Blachier et al., 2007). This may provide some explanation for their much smaller concentrations compared to SCFA (Table 4). In general, their concentration has been reported to be about 5% to 10% of SCFA (Middelbos et al., 2007; Nery et al., 2012); however, the proportion can change if protein is used as substrate in an in vitro model (Urrego et al., 2017). Isobutyrate and isovalerate are produced from valine and leucine, respectively (Blachier et al., 2007). It should be noted that the isovalerate peak from gas chromatography analysis also includes 2-methylbutyrate, which would be produced from fermentation of isoleucine (Jackson and Jewell, 2018). Because both isovalerate and 2-methylbutyrate elute at the same peak, they will be treated as isovalerate in this manuscript. Possibly isobutyrate is the BCFA with the most importance, because of its similarities with butyrate (Dagher et al., 1996; Charney et al., 1999). Isobutyrate production was similar among fiber sources after 12 h of incubation. Differently, isovalerate production was higher for SB, followed by BP and MG, and CE and PF had the lowest contents (P < 0.05; Table 4). Valerate concentration was low among all treatments, but SB had the highest concentration (3.13 μmol/g of substrate) which was about 6 times higher than MG (the second highest numerically; 0.56 μmol/g of substrate). Valerate concentration was similar among MG, CE, and BP after 12 h of fermentation (Table 4). Valerate concentration for PF was negative and the lowest compared to the other tested substrates (Table 4). Pea fiber had similar results for the BCFA and valerate concentrations (P > 0.05; Table 4). Negative values for valerate productions are a result of blank tubes having greater concentrations than tubes with substrate. These results indicate that these fiber sources were resistant to microbial degradation and no net production of SCFA and BCFA was recorded until 8 h of fermentation. Therefore, these substrates needed more time to be utilized and even after 12 h of incubation small concentrations of fermentation end products were produced. Branched-chain fatty acids concentrations were higher for SB than all other tested fiber sources (P < 0.05; Table 4). Finally, total volatile fatty acid production was higher for BP than PF and SB, with the lowest concentrations for MG and CE (2.74, 0.66, 0.44, 0.13, and 0.06 mmol/g of substrate, respectively; Table 4).

The production of SCFA and BCFA is directly related to the content of soluble fibers and amino acid concentrations,

respectively, but not necessarily the crude protein content. For example, BP had the highest content of soluble fibers compared to the other test fibers (Table 1), and yielded the highest production of SCFA after 4, 8, and 12 h of incubation (Table 4). Similarly, when considering the protein content of the substrates, SB had a higher content of CP (Table 1) and produced the highest concentration of BCFA. Beet pulp had a higher CP concentration than PF, but the BCFA concentration after 12 h of incubation was similar to PF. In addition, MG with about 3.5 times less CP than BP produced similar concentration of BCFA to BP. As noted previously, the fermentation of valine and leucine will produce isobutyrate and isovalerate, respectively (Blachier et al., 2007). These results might be explained by considering net production of BCFA as a function of both production and consumption of BCFA by the microbial population. Although BP may have led to more total production of BCFA than MG and PF (based on greater protein content), the presumably greater microbial cell growth for BP than for MG or PF likely led to greater consumption of BCFA for production of branched-chain amino acids to support greater microbial protein synthesis.

In addition to the volatile fatty acid concentrations, their relative proportions in relation to the total were evaluated (Table 5). Similar to the concentration data, the discussion will be focused on the 12-h incubation values. In proportion to the total volatile fatty acids production, MG fermentation yielded a higher proportion of acetate, butyrate, and isovalerate compared to CE (Table 5). In addition, BP fermentation resulted in a low proportion of propionate (20.4%) compared to CE and SB (31.2% and 29.3%, respectively). However, the proportion of SCFA was similar among the tested fiber sources after 12 h of incubation (Table 5). Pea fiber fermentation resulted in lower proportions of isovalerate and valerate compared to MG, SB, and CE (Table 5). Finally, SB had high proportions of propionate and butyrate (29.3% and 6.6%, respectively; Table 5).

Although the proportions of the volatile fatty acids may change depending on the fiber sources (Barry et al., 1995; Sunvold et al., 1995a, 1995b, de Godoy et al., 2015), there is probably limited competition for the absorption among the different fermentation end products because the majority is transported by passive diffusion through the cell membrane (Bergman, 1990; Topping and Clifton, 2001). The important aspect of the fermentation is the rate of production of these products. As fermentation intensifies, there is an accumulation of flatus in the large intestine (Yamka et al., 2006) which could cause discomfort. Additionally, the increase in concentration of fermentation end products could shift the osmotic balance in the colon and favor water and sodium transport toward the lumen. Thus, feeding a diet rich in soluble and rapidly fermentable fiber could lead to flatulence and diarrhea. Therefore, the fiber source composition included in the diet should be considered to prevent these side effects.

Conclusion

Fermentation end-products content increased as the soluble fiber content of the substrate increased. Similarly, as more protein was present in the substrate, more isobutyrate and isovalerate were produced. Beet pulp generated the highest concentrations of the individual and overall total SCFA, thus from the tested fiber sources it would be the best alternative for diets targeting gut health. Pea fiber and SB were intermediate in the production of SCFA; however, SB had the highest production of valerate and isovalerate. Production of all SCFA and BCFA was lower when MG and CE were used as substrates; therefore, they could be included in weight loss diets to aid in weight control of overweight and obese animals.

Conflict of Interest

C.G.A. has previously provided consulting services for the sponsor of the project (Renew Biomass).

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