Comparison of four digestibility markers to estimate fecal output of dogs

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ABSTRACT: Twelve adult beagle dogs (10.6 ± 1.4 kg) were fed extruded dog diets in which the starch sources were whole sorghum, sorghum flour, sorghum mill-feed, or an equal combination of rice, corn, and wheat. The experiment was conducted as a replicated Latin square design digestibility study. Estimates of fecal organic matter (OM), crude protein (CP), crude fat (CF), and gross energy (GE) outputs were determined by four methods: total fecal collection (TFC), chromic oxide (Cr_2O_3), titanium dioxide (TiO_2), and acid insoluble ash (AIA). The correlation among the fecal output estimates by the four methods by partial correlation coefficients from the Error SSCP Matrix (Pearson) were considered significant at $P < 0.05$. The external markers, Cr_2O_3 and TiO_2, had a higher ($P < 0.05$) OM fecal output Pearson correlation to TFC than the intrinsic marker AIA ($R = 0.931$ for Cr_2O_3 vs. TiO_2; $R = 0.559$ for TFC vs. Cr_2O_3; $R = 0.592$ for TFC vs. TiO_2; $R = 0.291$ for AIA vs. TFC). Interestingly, TiO_2 highly correlated ($P < 0.05$) to Cr_2O_3 ($R = 0.93$ for OM), and was also correlated highly to TFC and AIA. The study suggests that TiO_2 may be a preferred marker to estimate fecal output in dogs vs. Cr_2O_3. The use of AIA represents a potential option for determining digestibility for diets in which external markers are impractical.

Key words: AIA, chromic oxide, dogs, fecal output, titanium dioxide, total fecal collection

INTRODUCTION

When a new pet food is being developed, it is common to evaluate diet digestibility in the target species to validate nutrition and safety of the product. Broadly, this provides information about acceptability, fecal output, stool consistency, nutrient utilization, and overall healthfulness. The total fecal collection method (Lindahl, 1963) is the gold standard to estimate apparent total tract digestibility (ATTD) in many animal species. This method consists of collecting all feces excreted in a given period of time. However, collecting all feces is not always possible and may be fraught with a high degree of inaccuracy (Jagger et al., 1992). Total fecal collection is time consuming, requires meticulous planning, significant effort, and full-time confinement of the animals. Dogs were confined individually for 4–5 days to allow total fecal collection is only supported by few animal care committees due to animal welfare concerns. Furthermore, animals held under full containment may step in feces, perform coprophagy, and quantitative collection may be complicated by structures and daily sanitation. This all leads to errors in true measurement of fecal output.

Furthermore, in wild species or those animals that are free-ranging, it is impractical (Atkinson et al., 1984), and application to in home testing is unreliable.

An alternative to total collection is the use of markers to estimate fecal output and ATTD. Chromic oxide is a common marker used in animal nutrition. It is the established standard for determination of metabolizable energy in pet foods according to AAFCO (2018). However, there are recognized issues with this chemical for health (Peddie et al., 1982; Sales and Janssens, 2003) and consistency in results (Moore, 1957; Jagger et al., 1992). Titanium dioxide (TiO_2) has been evaluated in a number of studies with pets (Lôbo-Junior et al., 2001; Childs-Sanford et al., 2006; Hagen-Plantinga et al., 2014) and other animal species (Peddie et al., 1982; Jagger et al., 1992; Titgemeyer et al., 2001; Smeets et al., 2015) with good results. It also has the advantage over Cr_2O_3 of being a food color additive incorporated in food up to 1% (Code of Federal Regulations, 2015), so there are fewer concerns regarding animal and worker safety. Each of these external markers must be added to the food for analysis. Conversely, acid insoluble ash (AIA) is an internal marker present in some foodstuffs, which in many cases does not need to be added externally to the food. Some researchers have evaluated AIA for use to determine fecal output in different animal species (McCarthy et al., 1974; Vogtmann et al., 1975; McCarthy et al., 1977; Atkinson et al., 1984). Markers like TiO_2 or acid insoluble ash (AIA), an internal marker, might prove beneficial to companion animal nutrition research. Therefore, the objective of the present study was to determine the relationships of these three different marker methods and TFC to estimate fecal output in dogs. The hypothesis was that all markers would be similar to TFC and would provide more animal welfare options for future digestibility studies.

MATERIALS AND METHODS

Diets and Animals

Four nutritionally complete and balanced diets (Table 1) were produced via extrusion processing as described previously by Alvarenga et al. (2018). The basal portions of the experimental diets were similar and the treatments differed primarily in the type of starch source: control (CON) a 1:1:1 mix of rice, wheat, and corn, which was exchanged for whole ground sorghum (WSD), sorghum flour (FLD), or sorghum mill-feed (MFD; Table1). External markers chromic oxide (0.25%) and titanium dioxide (0.40%) were added to the dry mix of all diets

Table 1. Experimental diets used to feed dogs: Control (CON), whole sorghum (WSD), sorghum flour (FLD) and sorghum mill-feed (MFD).

Ingredients, %	CON	WSD	FLD	MFD
Brewers rice	21.2	—	—	—
Corn	21.2	—	—	—
Wheat	21.2	—	—	—
Whole sorghum	—	64.7	—	—
Sorghum flour	—	—	63.11	—
Sorghum mill—feed	—	—	—	67.6
Chicken by-product meal	20.9	20.0	20.0	20.0
Chicken fat	5.34	5.52	6.69	3.29
Beet Pulp	4.00	4.00	4.00	4.00
Corn gluten meal	2.35	2.35	2.35	2.35
Calcium carbonate	0.75	0.35	0.26	0.67
Potassium chloride	0.49	0.52	0.65	0.19
Salt	0.46	0.45	0.47	0.43
Dicalcium phosphate	0.87	0.95	1.27	0.24
Choline chloride	0.20	0.20	0.20	0.20
Vitamin premix[1]	0.15	0.15	0.15	0.15
Trace mineral premix[2]	0.10	0.10	0.10	0.10
Natural antioxidant	0.07	0.07	0.07	0.08
Chromic oxide	0.25	0.25	0.25	0.25
Titanium dioxide	0.40	0.40	0.40	0.40

[1]Vitamin premix = calcium carbonate, vitamin E supplement, niacin supplement, calcium pantothenate, vitamin A supplement, thiamine mononitrate, pyridoxine hydrochloride, riboflavin supplement, vitamin D3 supplement, biotin, vitamin B12 supplement, and folic acid.

[2]Trace mineral premix = calcium carbonate, zinc sulfate, ferrous sulfate, copper sulfate, manganous oxide, sodium selenite, and calcium iodate.

prior to extrusion. This assures consistent uniform dosage and avoids potential losses due to fines and spillage during feeding. Diets were produced on a single screw extruder (model X25; Wenger Mfg., Sabetha, KS) and dried on a 2 pass dryer/1 pass cooler (model 4800 Wenger, Mfg., Sabetha, KS), then coated with chicken fat prior to packaging. Product was stored in ambient conditions in multi-walled poly lined bags for 4 weeks before commencement of the feeding study.

Twelve intact Beagle dogs, eight males and four females, 1–3 years old, with average body weight of 10.6 ±1.42 kg were fed the experimental diets during four periods of 9 days adaptation and 5 days total fecal collection each, in a replicated Latin square design (Kim and Stein, 2009) experiment. Total feces were collected from the Beagle dogs during each of the four collection periods twice daily (08:00 and 17:00), and animal care and handling are described by Corsato Alvarenga and Aldrich (2018). All animal testing was approved by the Kansas State University Institutional Animal Care and Use committee (IACUC; protocol number 3645) prior to the conduct of the study.

Nutrient analyses

At the conclusion of the feeding study, total feces of each dog during each period were dried in a convection oven (Cat 52755-20, Matheson Scientific, Morris Plains, NJ) at 55°C until dry to the touch (24–48 h), and then ground to pass a 0.5 mm screen in a fixed blade hammermill (Retsch, Newtown, PA, USA). A small portion of dry feces (1 g) was incinerated at 600°C for 8 h in a muffle furnace (model F30400: Thermo Scientific Thermolyne Furnace, Asheville, NC) to determine ash and organic matter (OM) content (AOAC 942.05). Fecal gross energy (GE) was determined by bomb calorimetry (model 1341, Parr Instruments Company, Moline, IL). Fecal crude protein (CP; AOAC 990.03) and crude fat (CF; AOAC 945.16) were determined at a commercial laboratory (Midwest Laboratories, Omaha, NE).

Digestibility markers measurements

Chromium was measured in feces by atomic absorption using the protocol described by Williams et al. (1962). Titanium in feces was measured using a colorimetric method according to the protocol described by Myers et al. (2004). Acid insoluble ash in feces and food was determined according to the protocol described by Van Keulen and Young

(1977), with slight modification. Briefly, 4 g of dry feces or 10 g of food were weighed in porcelain crucibles on an analytical balance (Explorer: E1RW60, OHAUS, Parsippany, NJ) and ashed at 600°C for 8 h in a muffle furnace (model F30400: Thermo Scientific Thermolyne furnace, Asheville, NC). These were then boiled in 2 N hydrochloric acid (HCl) for 5 min before being filtered under vacuum (DOA-V120-AE: Gast Manufacturing, Inc., Bonton Harbor, MI, USA) through ashless filter paper (541: Whatman, Maidstone, UK), and then ashed again at 650°C overnight (approximately 12 h). The samples were weighed after the second ashing to estimate the AIA percentage.

Fecal Output Calculations

There were in total 48 fecal samples (12 dogs × 4 diets) used to calculate fecal output according to TFC or marker method. Total fecal collection fecal output (using organic matter as an example) was determined by the following equation:

$$\text{OM fecal output (TFC)} = \%\text{OM in feces} \times \text{total feces on DM basis (g)} \quad (1)$$

Fecal output determined by external markers Cr_2O_3 and TiO_2, and internal marker AIA (using organic matter as an example) were calculated using the following equation:

$$\text{OM fecal output (markers)} = \frac{\%\text{OM in feces} \times \text{marker ingested in food (g)}}{\%\text{marker in feces}} \quad (2)$$

Statistical Analysis

Pearson correlation coefficients between methods used to estimate fecal output (TFC, AIA, Cr_2O_3, and TiO_2) were determined with multivariate analysis of variance (MANOVA) on three response variables: diet, period, and dog, using the generalized linear model (GLM) procedure with the aid of a statistical software (SAS, version 9.4). Correlation coefficients were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The experimental diets comprised of 63.6% cereals from equal proportions of rice, corn, and wheat (CON), or a similar amount of sorghum as whole (WSD), flour (FLD), or mill-feed (MFD;

Table 2. Nutrient analysis of experimental diets control (CON), whole sorghum (WSD), sorghum flour (FLD) and sorghum mill-feed (MFD) fed to dogs.

Nutrient	CON	WSD	FLD	MFD
Dry matter, %	93.9	93.6	94.8	93.8
Organic matter, %	92.7	93.5	93.4	93.1
Crude protein, %	21.5	21.4	21.2	23.8
Crude fat, %	12.10	10.7	10.25	9.48
Gross Energy, kcal/g	4,311	4,329	4,490	4,415
TDF, %	4.73	7.57	4.33	16.39
Ash, %	7.24	6.52	6.59	6.86

Table 3. Partial Correlation Coefficients from the Error SSCP Matrix[1] (Pearson) evaluating methods[2] to determine organic matter, crude protein, crude fat and gross energy fecal outputs by dogs in which dietary treatment data were pooled.

	Organic matter		Crude protein		Crude fat		Gross energy	
	R^2	P-value	R^2	P-value	R^2	P-value	R^2	P-value
TFC vs. Cr_2O_3	0.559	<0.0001	0.569	<0.0001	0.635	<0.0001	0.563	<0.0001
TFC vs. Ti_2O	0.592	<0.0001	0.613	<0.0001	0.685	<0.0001	0.600	<0.0001
TFC vs. AIA	0.291	0.0446	0.326	0.0238	0.410	0.0038	0.328	0.0038
Cr_2O_3 vs. Ti_2O	0.931	<0.0001	0.914	<0.0001	0.919	<0.0001	0.929	<0.0001
Cr_2O_3 vs. AIA	0.749	<0.0001	0.731	<0.0001	0.774	<0.0001	0.776	<0.0001
Ti_2O vs. AIA	0.842	<0.0001	0.821	<0.0001	0.861	<0.0001	0.864	<0.0001

[1]Coefficient of correlation significant at $P < 0.05$.

[2]TFC = total fecal collection; Cr_2O_3 = chromic oxide; TiO_2 = titanium dioxide; AIA = acid insoluble ash.

Table 1). Dietary treatments met the goal of being similar in crude protein (range 21.2–23.8%), crude fat (range 9.48–12.10%; Table 2), vitamins and minerals.

Estimation of animal dietary utilization in measures such as digestibility typically rely on calculating the difference between intake and output. Intake is typically controlled and easily measured. However, fecal output, while ostensibly a single measure, can be challenging for a myriad of reasons. Measurement of fecal output can be done by collection of all feces excreted during a determined period, which is the gold standard method, or by collecting fecal aliquots and measuring the proportion of a marker. It is generally considered that a substance can be used as a marker for digestibility if it is unabsorbed and undigested by the animal, if it mixes homogenously with the digesta through the gut, and if it is pharmacologically inactive within the digestive tract (Schneider and Flatt, 1975; Maynard et al., 1979).

To be most effective, markers like Cr_2O_3 and TiO_2 need to be provided in a constant quantity. Addition to the diet fulfills this need. In the present study the TFC method had significant correlations with Cr_2O_3 and TiO_2 (range $R^2 = 0.559$ to 0.685 for OM, CP, CF, and GE fecal outputs; Table 3). In growing pigs, ATTD of energy and DM were slightly lower when determined by TiO_2 compared with TFC method, while estimations by Cr_2O_3 were similar to the extremes (Kavanagh et al., 2001). In a study with dogs, ATTD of DM, CP, CF, and GE determined by Cr_2O_3 were also similar to TFC (Lôbo-Junior et al., 2001). Likewise, Hill et al. (2009) reported that apparent nutrient digestibility coefficients determined by TFC and TiO_2 methods were not different in dogs. Although the correlation between TFC and external markers was intermediate in the present study, other studies suggest that both Cr_2O_3 and TiO_2 are efficient fecal markers to estimate fecal output and ATTD of nutrients in dogs.

An interesting option to avoid the extra effort associated with adding a marker is to exploit something already present in the food (Atkinson et al., 1984). Acid insoluble ash (AIA) is an intrinsic mineral material found in food that is not digested or absorbed by the animal gut. This characteristic allows it to be used as a marker and it has been reported to be successful in determining apparent total tract digestibility in many animal species (McCarthy et al., 1974; Vogtmann et al., 1975; McCarthy et al., 1977; Atkinson et al., 1984). In horses, De Marco et al. (2012) found that DM, OM, GE, and CP digestibility determined by TFC and AIA methods were very similar numerically. Studies

in dogs have also reported that AIA and TFC techniques estimate fecal output and nutrient digestibility with a high degree of accuracy (Lôbo-Junior et al., 2001; Zanatta et al., 2013). In contrast, the present study found that TFC and AIA had the poorest correlation for fecal output of all nutrients tested (R^2 = 0.291, 0.326, 0.410, and 0.328 for OM, CP, GE, and CP, respectively; Table 1). Since TFC had low to intermediate correlations with internal and external markers, our concern was that TFC was not reflective of true fecal output and may have led to the inconsistent interpretation.

External markers TiO_2 and Cr_2O_3 correlated highly for fecal outputs of all nutrients (OM, CP, CF, and GE R^2 = 0.931, 0.914, 0.919, and 0.929, respectively; Table 3). Kavanagh (2001) also found that TiO_2 and Cr_2O_3 estimated similar nutrient digestibility in growing pigs. The high correlation between external markers in the present study further confirms that TFC may have been inconsistent, which was possibly due to difficulties in collecting all feces, animals stepping on their stool, and coprophagy habits.

Despite having poor correlation with TFC, AIA in our study had an intermediate to high correlation with Cr_2O_3 (R^2 = 0.750, 0.731, 0.774, and 0.776 for OM, CP, CF, and GE, respectively; Table 3) and even higher with TiO_2 (R^2 = 0.842, 0.821, 0.861, and 0.864 for OM, CP, CF, and GE, respectively, Table 3). Equivalently, Wang et al. (2017) found that in pigs all three markers TiO_2, Cr_2O_3, and AIA gave similar results for energy and nitrogen digestibility, while TFC led to different digestibility estimates. Other studies with pigs reported similar ATTD determined by the AIA and Cr_2O_3 markers (Favero et al., 2014; Brestenský et al., 2017). This is encouraging because AIA may have beneficial applications for animals that cannot be fed prepared diets with added external markers. Thus, there is need to further explore the use of AIA as an intrinsic fecal marker for dogs. Caution must be used when determining AIA in feedstuffs with low ash levels (e.g., Alfalfa; Van Keulen and Young, 1977), as small discrepancies in analysis and sample preparation can unduly increase the variation and make interpretation difficult. Fortunately, most pet food formulas contain more than sufficient ash to be effective.

According to our study, TiO_2 was the marker that best correlated with all other three methods. Jagger et al. (1992) also found that TiO_2 was a better marker to estimate apparent total tract digestibility in pigs, when compared to Cr_2O_3. Titanium dioxide is a generally recognized as safe (GRAS) food coloring agent that is permitted in foods, including pet foods. It is commonly used to help lighten and (or) whiten foods for consumer visual appeal. Further, the method of sample preparation and analysis are simpler and require less sophisticated analytical equipment. Hence, TiO_2 would be a more readily useable method for future research to determine diet utilization and caloric content of pet diets, and it should be a good alternative to the official marker for dogs and cats (Cr_2O_3; AAFCO, 2018).

CONCLUSION

Titanium dioxide had the highest correlation to all markers in this study. It also has the benefit that it can be safely used as a food additive (Code of Federal Regulations, 2015) and the spectrophotometry method of analysis is relatively straightforward. AIA correlated intermediate to high with both Cr_2O_3 and TiO_2, but had a low correlation with TFC. There is need to further explore the use of AIA as an intrinsic marker for estimating fecal output when addition of a marker into the diet is not possible. Future evidence of an acceptable alternative marker could provide with data to discontinue the use of Cr_2O_3 as a fecal marker and select options such as TiO_2 or AIA.

The authors have no conflicts of interest.

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