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Effects of graded inclusion levels of raw garbanzo beans on apparent total tract digestibility, fecal quality, and fecal fermentative end-products and microbiota in extruded feline diets

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Abstract

Garbanzo beans (GB; *Cicer arietinum*) are a readily available pulse crop that have gained popularity as a plant-based protein source in the pet food industry. However, raw GB contain anti-nutritional factors that can reduce digestibility and cause digestive upsets in pets that are undesirable to owners. The objective of this study was to determine the effects of the inclusion of raw or cooked GB in extruded feline diets on macronutrient digestibility, gastrointestinal tolerance, and fermentative end-products in cats. Five diets were formulated to contain raw GB at 0%, 7.5%, 15%, or 30% or cooked GB at 30%. Ten adult, male cats (mean age: 1.0 ± 0.0 yr, mean BW: 4.7 ± 0.4 kg) were used in a replicated 5 × 5 Latin square design. Each period consisted of 14 d, with 10 d of diet adaptation followed by 4 d of total fecal and urine collection. At the end of each period, 4 mL of blood were collected and analyzed for a serum chemistry and complete blood count to ensure all animals remained healthy throughout the study. Cats were fed twice daily and food intake was calculated to maintain body weight. Food intake was highest ($P < 0.05$) for cats fed 0% raw GB (72.2 g/d, dry matter basis [DMB]) compared with GB inclusions of 7.5% or greater (average 70.3 g/d, DMB). Dry matter and organic matter apparent total tract digestibility (ATTD) were lowest ($P < 0.05$) for cats consuming the 30% cooked GB diet (77.3% and 81.7%, respectively). Cats fed 7.5% raw GB had greater ($P < 0.05$) crude protein ATTD (86.2%) than cats fed 15% raw GB (82.3%) or 30% cooked GB (81.6%). Total short-chain fatty acid concentrations were highest ($P < 0.05$) for 30% cooked GB at 682 μmol/g but not different ($P > 0.05$) than 15% GB (528 μmol/g) or 30% raw GB (591 μmol/g) diets. In terms of fecal microbial abundance, the predominant phyla were Firmicutes, Bacteroidota, and Actinobacteria. Cats fed the 0% GB diet had a greater relative abundance of Firmicutes (62.1%) and Fusobacteria (4.0%) than the remaining diets (average 54% and 1.6%, respectively). In conclusion, all inclusion levels of raw GB resulted in high digestibility (average > 80%) and ideal fecal scores (average 2.9), demonstrating their adequacy as a protein source in feline diets up to a 30% inclusion level.

Key words: cat, chickpea, digestibility, garbanzo bean, microbiota, plant protein

Abbreviations

AHF	acid hydrolyzed fat
ATTD	apparent total tract digestibility
BCFA	branched-chain fatty acids
CBC	complete blood count
CML	carboxymethyllysine
CP	crude protein
DM	dry matter
DMB	dry matter basis
FL	fructoselysine
FS	furosine
GB	garbanzo bean
GE	gross energy
HMF	hydroxymethylfurfural
HPLC	high-performance liquid chromatography
OM	organic matter
OTU	operational taxonomic unit
TDF	total dietary fiber

Introduction

Garbanzo beans (GB; *Cicer arietinum*) are a globally important protein source in human nutrition. They are classified as pulses which is a subset of legumes harvested for the dry grain (McCrory et al., 2010). GB are highly adaptable to a variety of growing conditions, including tropical and temperate environments (Bulbula and Urga, 2018), which have a direct impact on their physical appearance and macronutrient composition (Jukanti et al., 2012). In North America, the Kabuli type dominates due to its efficient growth in temperate climates and is characterized by a large, smooth seed coat (Gupta et al., 2019).

In companion animal nutrition, GB have gained popularity as a novel protein source in grain-free and vegetarian commercial diets. However, the inclusion of raw GB in pet food diets can have a negative impact on protein digestibility, especially at greater levels due to the presence of anti-nutritional factors (Gilani et al., 2012). It is well known that heat processing improves organoleptic characteristics and nutrient digestibility of GB, while eliminating anti-nutritional factors (Savage and Morrison, 2003; van Rooijen et al., 2014a). Cooking GB prior to inclusion in pet diets eliminates the effects of anti-nutritional factors but increases manufacturing costs. This created a need to evaluate the maximum inclusion of raw GB in pet diets without negatively affecting nutrient digestibility or fecal characteristics. Therefore, the objective of this study was to determine the effects of graded inclusion levels of raw GB in feline diets on macronutrient apparent total tract digestibility (ATTD), gastrointestinal tolerance, fermentative end-products, and intestinal microbiota populations. It was hypothesized that greater inclusions of raw GB would have decreased ATTD of macronutrients and result in poor fecal quality compared with cooked GB or 0% GB.

Materials and Methods

Experimental design

All animal care protocols used in this study were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign. All methods were performed in accordance with the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Ten adult, intact, male Domestic Shorthaired cats (average age: 1.0 ± 0.0 yr, average weight: 4.7 ± 0.4 kg) were used in a replicated 5 × 5 Latin square design. Experimental periods consisted of a total of 14 d with a 10 d diet adaptation phase followed by a 4 d total fecal and urine collection phase. A 4 mL, fasted, blood sample was collected on day 14 from all cats and analyzed for serum metabolites and complete blood count (CBC) at the University of Illinois Veterinary Medicine Diagnostics Laboratory (Urbana, IL). The cats were housed in a temperature-controlled room in the Edward R. Madigan Laboratory at the University of Illinois at Urbana-Champaign. The room was kept on a 14 h light/10 h dark schedule. The cats were group housed except during mealtimes during the diet adaptation phases and for the duration of the fecal and urine collection periods. The cats were fed twice daily with access to food for 2 h (0800 to 1000 and 1500 to 1700 h). They had ad libitum access to water throughout the study. When individually housed, the cats were kept in cages (73.6 cm long × 152.4 cm wide) which provided visual contact with the majority of cats in the room. During collection phases, the cats had access to litter-free collection boxes to collect total fecal and urine samples.

Cats were randomly assigned to one of five diets formulated with either 0%, 7.5%, 15%, or 30% raw GB, or 30% cooked GB (Table 1). GB were included at the expense of poultry byproduct meal and rice to provide diets with similar chemical composition (Table 2). All diets were formulated to be complete and balanced according to AAFCO (2018) recommended values for adult cats at maintenance. Diets were processed at Wenger Manufacturing (Wenger Manufacturing, Inc., Sabetha, KS). Extrusion parameters (Table 3) were adjusted for each diet to ensure consistency among final product characteristics, such as texture, density, and kibble size. Metabolizable energy requirements were used to calculate individualized food intake for maintaining body weight. Food refusals were measured after each meal throughout the duration of the study. Body weight and body condition were recorded weekly. When necessary, food intake was adjusted accordingly at the beginning of the adaptation phase to maintain body weight.

Sample collection and preparation

A fresh fecal sample was collected from each cat within 15 min of defecation. Fecal samples were analyzed for pH, dry matter (DM), short-chain fatty acids (SCFA), branched-chain fatty acids (BCFA), ammonia, phenols/indoles, and fecal microbiota. Fecal scores were measured using a 5-point scoring system: 1 = hard, dry pellets; 2 = hard formed, remains firm and soft; 3 = soft, formed and moist stool; 4 = soft, unformed stool; or 5 = watery, liquid that can be poured. DM was analyzed in duplicate and dried for 48 h in a 105 °C, forced-air oven. Aliquots of the fresh fecal sample were collected for BCFA, SCFA, and ammonia analyses by placing 4 g of feces into a 30-mL Nalgene bottle containing 4 mL of 2 N hydrochloric acid. Phenols/indoles were collected in duplicate by weighing 2 g of feces into identical plastic tubes and covered with Parafilm. Fecal samples allocated for fermentative end-product analysis were stored at –20 °C. Fecal samples allocated for microbiota were stored in 2-mL cryovials and stored at –80 °C until analysis.

Total feces and urine were collected simultaneously for the duration of the 4-d collection phase of the study. Weight and fecal scores were initially recorded for all fecal samples and then stored at –20 °C until analyzed to determine macronutrient ATTD. Total urine was collected from the litter-free collection boxes containing 5 mL of 2 N hydrochloric acid and weighed. A subsample representing 25% of the total urine weight was collected and stored at –20 °C until analysis.

Table 1. Ingredient composition of feline diets containing graded inclusion levels of garbanzo beans (GB)

	Dietary treatment				
Ingredient	0% GB	7.5% GB	15% GB	30% GB raw	30% GB cooked
Cooked Garbanzo Bean	–	–	–	–	30.00
Raw Garbanzo Bean	–	7.50	15.00	30.00	–
Poultry by-product meal	38.39	36.56	35.14	30.79	30.79
Rice	30.00	25.64	19.46	8.27	8.27
Corn gluten meal	10.76	10.50	10.50	10.50	10.50
Poultry fat	10.00	10.00	10.00	10.16	10.16
Corn	5.00	5.00	5.00	5.00	5.00
Dried beet pulp	2.50	2.50	2.50	2.50	2.50
Palatant	1.00	1.00	1.00	1.00	1.00
Potassium chloride	0.98	–	–	–	–
Ca carbonate	–	0.49	0.61	0.98	0.98
Dical. phosphate	0.58	–	–	–	–
Salt	0.30	0.30	0.30	0.30	0.30
Vitamin premix[1]	0.18	0.18	0.18	0.18	0.18
Mineral premix[2]	0.18	0.18	0.18	0.18	0.18
Choline chloride	0.12	0.12	0.12	0.12	0.12
BHT (antioxidant)	0.02	0.02	0.02	0.02	0.02

[1]Provided per kg diet: 10.8 mg copper ($CuSO_4$), 0.36 mg selenium (Na_2SeO_3), 150 mg zinc ($ZnSO_4$, ZnO), 2,562.8 IU vitamin A, 254 IU vitamin D3, and 32.1 IU vitamin E.
[2]Provided per kg diet: 17.4 mg manganese ($MnSO_4$), 284.3 mg iron ($FeSO_4$), 17.2 mg copper ($CuSO_4$), 2.2 mg cobalt ($CoSO_4$), 166.3 mg zinc ($ZnSO_4$), 7.5 mg iodine (KI), and 0.2 mg selenium (Na_2SeO_3).

Table 2. Analyzed chemical composition and energy content of feline diets containing graded inclusion levels of garbanzo beans (GB)

	Dietary treatment				
Item	0% GB	7.5% GB	15% GB	30% raw GB	30% cooked GB
Dry matter, %	93.4	92.1	92.8	91.9	94.1
			Dry matter basis		
Crude protein, %	40.6	37.4	37.3	35.8	37.5
Acid hydrolyzed fat, %	19.5	17.5	19.1	18.6	19.2
Ash, %	8.2	8.2	7.3	8.3	6.9
Total dietary fiber, %	12.7	12.4	11.4	13.9	13.7
Soluble, %	5.4	5.8	2.5	5.5	3.6
Insoluble, %	7.3	6.7	8.8	8.5	10.1
Gross energy, kcal/g	5.4	5.2	5.3	5.3	5.4

A 4 mL, fasted, blood sample was collected via jugular venipuncture from all cats at the end of each experimental period to be used as a health check. All cats were sedated prior to collection using 0.9 mL/kg of a mix of dexmedetomidine (0.062 mg/mL), ketamine (62.4 mg/mL), and butorphanol (2.5 mg/mL). Sedation was reversed by giving 0.1 mL of Atipemezole (5 mg/mL) to each cat. Serum chemistry was analyzed using 3 mL of blood collected in a serum separator vacutainer tube. Complete blood count (CBC) was analyzed using the remaining 1 mL of blood collected in an EDTA vacutainer tube (Becton, Dickinson and Company, Franklin Lakes, NJ).

Maillard reaction product analysis

Samples from each diet were analyzed for the presence of Maillard reaction products (MRP). The analyzed MRP were hydroxymethylfurfural (HMF), furosine (FS), carboxymethyllysine (CML), and fructoselysine (FL). Reactive lysine was calculated using the FS procedure according to Pahm et al. (2008).

Samples were analyzed for HMF using a modified high-performance liquid chromatography (HPLC) procedure according to Vorlová et al. (2006). A 100-mg dried sample was homogenized for 30 min with 1.3 mL of 1.2% (w/v) glacial acetic acid solution in water and 50 µL of Carraz I and 50 µL of Carraz II reagents (Carrez Clarification Kit, Sigma-Aldrich, St. Louis, MO). The homogenous sample was centrifuged (model 5416C Eppendorf Centrifuge, Brinkman Instruments, Inc., Westbury, NY) for 30 min at a rate of 10,000 × *g*. Supernatant was filtered through 0.2 µm PTFE filter. An isocratic HPLC system was used with Alliance 2695 separation module (Waters Company), an Inertsil ODS-3 column (25 cm × 0.46 cm i.d. × 5 µm df; MetaChem Technologies, Inc., Torrance, CA), and a 1050 Diode Array Detector (DAD, Agilent Technologies, Inc., Palo Alto, CA). Water (HPLC-grade) and methanol were added in a ratio of 90:10 (v/v) and used as a mobile phase at 1 mL/min flow rate for separations. The wavelength was detected at 284 nm using UV detector for HMF.

Samples were analyzed for FS and CML using gas chromatography-mass (Agilent 6890N GC/5973N MSD) spectrometry according to Charissou et al. (2007). A modified defatting step was used by adding 50 mg of the dried sample and 5-mL pentane to a 15-mL glass tube with a polytetrafluoroethylene-lined cap. The samples were vortexed for 5 min and centrifuged for the

Table 3. Average Wenger X-115 single screw extruder processing conditions for feline diets containing graded inclusion levels of garbanzo beans (GB)

	Dietary treatment				
Measurement	0% GB	7.5% GB	15% GB	30% raw GB	30% cooked GB
Raw material					
Dry recipe density, kg/m^3	549.0	571.0	484.0	461.0	527.0
Dry recipe rate, kg/h	490.0	496.0	490.0	495.0	496.0
Feeder speed, rpm	53.9	47.7	54.5	51.5	51.8
Preconditioner					
Mixing intensity, %	30.0	30.0	30.0	30.0	30.0
Large side speed, rpm	263.0	263.0	263.0	263.0	263.0
Small side speed, rpm	377.0	377.0	377.0	377.0	377.0
Cylinder steam, kg/h	40.1	19.9	30.0	20.1	40.2
Cylinder water, kg/h	80.0	111.0	119.8	109.6	130.5
Cylinder discharge temp, °C	76.0	57.0	65.0	55.0	75.0
Extruder					
Speed, rpm	385.0	375.0	375.0	390.0	435.0
Motor load, %	58.6	62.9	56.1	61.1	54.4
Motor power, kW	27.1	30.5	24.8	30.1	28.1
Knife speed, rpm	2,602.0	3,000.0	3,000.0	2,101.0	2,700.0
Zone 1 temp, °C	90.0	90.0	90.0	90.0	95.0
Zone 2 temp, °C	95.0	95.0	95.0	95.0	95.0
Zone 3 temp, °C	100.0	100.0	100.0	100.0	100.0
Zone 4 temp, °C	105.0	105.0	105.0	105.0	105.0
Zone 5 temp, °C	110.0	110.0	110.0	110.0	110.0
Conehead pressure, KPA	3,191.0	3,243.0	2,739.0	4,163.0	2,637.0
Specific mechanical energy	55.4	61.6	50.6	60.9	56.5
Dryer					
Zone 1 temp, °C	107.0	104.0	104.0	111.0	104.0
Zone 2 temp, °C	48.0	54.0	55.0	55.0	55.0
Zone 3 temp, °C	80.0	75.0	75.0	81.0	74.0
Retention time—pass 1, min	23.0	15.0	15.0	15.0	15.0
Retention time—pass 2, min	10.0	10.0	10.0	10.0	10.0
Exhaust 1 temp, °C	66.0	60.0	62.0	70.0	61.0
Final product					
Extruder discharge density	371.0	399.0	393.0	412.0	437.0

separation of the particulate. Excess pentane was removed. A 50-µL internal standard solution (1.15 mg/mL of cycloleucine in water) was added. Hydrolyzation and derivatization of the defatted sample was done according to the protocol outlined by Charissou et al. (2007). van Rooijen et al. (2014b) described the calculation of FL concentration from FS.

Chemical analyses

Fecal samples were composited by experimental period for each cat and dried in a 57 °C forced-air oven. All five diets and the fecal samples were ground through a 2-mm screen using a Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ). All diets and fecal samples were analyzed in duplicate for DM, ash, organic matter (OM), acid hydrolyzed fat (AHF), crude protein (CP), gross energy (GE), and total dietary fiber (TDF). DM, ash, and OM were determined according to AOAC (2006; methods 934.01 and 942.05). CP was calculated from Leco (TruMac N, Leco Corporation, St. Joseph, MI) with nitrogen values determined according to AOAC (2006; method 992.15). AHF, used to measure total lipid content, was analyzed according to AACC (1983) and Budde (1952). GE was analyzed by bomb calorimetry (Model 6200, Parr Instruments Co., Moline, IL). TDF was analyzed according to Prosky et al. (1992).

Fecal concentrations of SCFA and BCFA were measured using gas chromatography (Thermo TRACE 1310 Gas Chromatography coupled with ISQ LT single quadrupole Mass Spectrometer) according to Erwin et al. (1961). Fecal ammonia concentrations were determined using gas chromatography according to Chaney and Marbach (1962). Fecal phenol and indole concentrations were analyzed through gas chromatography according to Flickinger et al. (2003).

Anti-nutritional factors and oligosaccharides

Samples were collected for all diets throughout the extrusion process. Samples were taken of the base mix, after the preconditioner, after the extruder, and the coated final diet at multiple time-points. The samples were analyzed for anti-nutritional factors and oligosaccharide content by the Experiment Station Chemical Laboratories (Columbia, MO). Samples were analyzed for trypsin inhibitors according to AACC (2006; method 22-40) and urease activity according to AACC (2006; method 22-90). Free sugar profiles of substrates were determined according to methods of Churms (1982) and Kakehi and Honda (1989).

DNA extraction, amplification, sequencing, and bioinformatics

Total DNA was extracted from fresh fecal samples using a Mo-Bio PowerSoil kit (MO BIO Laboratories, Inc., Carlsbad, CA). Quantification of DNA concentration was completed using a Qubit

2.0 Fluorometer (Life technologies, Grand Island, NY). A Fluidigm Access Array (Fluidigm Corporation, South San Francisco, CA), in combination with Roche High Fidelity Fast Start Kit (Roche, Indianapolis, IN), was used for amplification of the 16S rRNA gene. The primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), targeting a 291 bp-fragment of V4 region, were used for amplification (primers synthesized by IDT Corp., Coralville, IA; Caporaso et al., 2012). Fluidigm specific primer, forward (CS1) and reverse (CS2) tags, were added in accordance with the Fluidigm protocol. The quality of amplicons' regions and sizes were confirmed by Fragment Analyzer (Advanced Analytics, Ames, IA). A DNA pool was generated through the combination of equimolar amounts of the amplicons from each sample. The pooled samples were selected by size on a 2% agarose E-gel (Life Technologies, Grand Island, NY) and extracted using a Qiagen gel purification kit (Qiagen, Valencia, CA). The pooled, size-selected, and cleaned products were analyzed on an Agilent Bioanalyzer to confirm appropriate profile and mean sizes. The W. M. Keck Center for Biotechnology at the University of Illinois performed Illumina sequencing on a MiSeq using v3 reagents (Illumina Inc., San Diego, CA). A FASTX-Toolkit (version 0.0.14) removed the Fluidigm tags. Analysis of sequences was completed using QIIME 2 (Caporaso et al., 2012) and DADA2 (version 1.14; Callahan et al., 2016). The high-quality (quality value ≥ 20) sequence data, derived from the sequencing process, were de-multiplexed. An opened-reference OTU clustered the sequences into operational taxonomic units (OTU), choosing against the SILVA 138 references OTU database with a similarity threshold of 97% (Quast et al., 2013). The OTUs observed fewer than two times (singletons), as well as OTUs with less than 0.01% of the total observation, were discarded. An average of 44,186 reads were obtained, with a total of 4,418,616 reads. The number of reads ranged from 30,854 to 56,936 per sample. Rarefaction curves based on observed species, Chao1, and the plateaus observed in the phylogenetic distance whole tree measures suggest sufficient sequencing depth. Analysis of diversity and species richness, the dataset was rarified to 44,186 reads. Weighted and unweighted unique fraction metric (UniFrac) distances were performed by principal coordinates analysis (PCoA). This measured the phylogenetic distance between sets of taxa in a phylogenetic tree as a fraction of the branch length of the tree, based on the 97% OTU composition and abundance matrix (Lozupone and Knight, 2005).

Statistical analyses

Data were analyzed in SAS (SAS Institute, Inc., version 9.4, Cary, NC) using the MIXED models procedure. Fecal score data were analyzed using the GLIMMIX procedure. A fixed effect of diet and a random effect of cat was used in the model. The differences among treatment groups were reported using a Fisher-protected least significance test with a Tukey adjustment to control for the Type 1 experiment-wise error. Additionally, treatment comparisons were made using orthogonal contrasts. Statistical significance was set using a probability of $P < 0.05$. Standard errors of the means (SEM) were reported as determined from the MIXED models procedure in SAS. Statistical analysis could not be performed on the MRP, anti-nutritional factor, or oligosaccharide data because the procedures were only performed using technical replicates.

Results

Diet proximate analyses, food intake, and fecal characteristics

Ingredient composition (Table 1) of all five diets were targeted to be similar with the exception of the inclusion level of raw or cooked GB. Macronutrient composition of the dietary treatments is reported on a dry matter basis (DMB; Table 2). The CP content was the greatest for 0% GB at 40.6% with the other dietary treatments ranging from 35.8% (30% raw GB) to 37.5% (15% GB). Acid-hydrolyzed fat concentrations for all five diets were similar, ranging from 17.5% (7.5% GB) to 19.5% (0% GB). The similarities in CP and AHF among all five diets are reflected in the GE content (average 5.3 kcal/g).

Food intake (g/d, DMB) was greatest ($P < 0.05$) for 0% GB, with an intake of 72.2 g/d (Table 4). Cats fed the 7.5% GB and 30% raw GB treatments had the lowest ($P < 0.05$) food intakes at 69.7 and 69.4 g/d, respectively. However, these differences in food intake among dietary treatments can be attributed to the individualized preference of the cats with a difference of only 2.8 g/d between the highest and lowest intakes. All diets were considered to be well-accepted and the observed differences in daily food intake were not likely to affect the outcomes of this study.

Fecal output (g/d, DMB) was greatest ($P < 0.05$) for 30% cooked GB at 15.3 g/d but not significantly different from cats fed 15% or 30% raw GB, which had fecal outputs of 13.0 and 13.1 g/d, respectively (Table 4). Cats fed 0% GB and 7.5% raw GB had fewer ($P < 0.05$) fecal outputs at 12.4 and 11.5 g/d, respectively. Fecal scores (Table 4) were highest ($P < 0.05$) for the 15% raw GB treatment at 3.1, but was not statistically different from cats fed 7.5% raw GB or 30% cooked GB, both of which had fecal scores of 2.9. Despite the statistical differences in fecal scores, all fecal scores were within the ideal range of 2 to 3 with an average value of 2.9. Urine output (Table 4) was less ($P < 0.05$) for cats fed 30% raw GB than cats fed 0% GB, with no differences ($P > 0.05$) among the remaining treatments.

Table 4. Food intake, fecal scores, and fecal output of cats fed diets containing graded inclusion levels of garbanzo beans (GB)

	Dietary treatment					
Item	0% GB	7.5% GB	15% GB	30% raw GB	30% cooked GB	SEM[1]
Food intake, g/d, as-is	77.3[a]	75.6[b]	76.4[ab]	75.5[b]	75.6[b]	0.372
Food intake, g/d, DMB[2]	72.2[a]	69.7[b]	70.9[b]	69.4[c]	71.1[b]	0.346
Fecal score	2.8[b]	2.9[a]	3.1[a]	2.8[b]	2.9[a]	0.097
Fecal output, g/d, as-is	35.6[b]	33.5[b]	43.1[ab]	43.6[ab]	52.6[a]	2.634
Fecal output, g/d, DMB[2]	12.4[b]	11.5[b]	13.0[ab]	13.1[ab]	15.3[a]	0.734
Urine output, mL/d	79.9[a]	66.2[ab]	65.0[ab]	57.5[b]	64.2[ab]	4.864

[1]SEM, standard error of the mean.
[2]DMB, dry matter basis.
[a,b,c]Means within a row with different superscript letters are different ($P < 0.05$).

Anti-nutritional factors and oligosaccharides

Anti-nutritional factor content at the various stages of diet processing (Table 5) showed no trypsin inhibitor activity in 0% GB or 7.5% raw GB in the base mix, after the preconditioner, or after the extruder. Although statistical analysis could not be performed, the greatest trypsin inhibitor activity was measured in the 30% raw GB base mix (1,970.1 TIU/g, DMB) which decreased with the progression of extrusion. Interestingly, detectable trypsin inhibitor activity was measured in the final diets containing 0% GB (682.9 TIU/g) and 15% raw GB (267.3 TIU/g). Urease activity was negligible in all diets at all processing stages.

Oligosaccharide content of the dietary treatments at various stages of processing (Table 5) showed numerical increases with increasing inclusion levels of GB. In the final diet, raffinose content was 0.03% (DMB) in 0% GB and 0.18% (DMB) in 30% cooked GB. Because oligosaccharides are heat-stable, oligosaccharide content was fairly equivalent for the individual diets at all processing stages. The verbascose content was negligible for all diets at all processing stages.

Maillard reaction products

All diets were analyzed for the presence of HMF, FS, FL, and CML (Figure 1). Although statistical analysis could not be conducted on these data, diets with 0% GB and 7.5% raw GB had comparable total MRP content (132.8 vs. 125.7 μg/g, respectively). The dietary treatments were considered to have minimal heat damage due to the high percentage of reactive lysine remaining after extrusion (Figure 2). The reactive lysine content ranged from 99.6% (DMB) in the 30% cooked GB diet to 99.8% in the 15% GB diet.

Apparent total tract macronutrient and energy digestibility

Cats fed 30% cooked GB had less ($P < 0.05$) DM and OM digestibility compared with the other diets which did not differ ($P > 0.05$) from each other (Table 6). The ATTD of DM of cats fed 30% cooked GB treatment was 77.3% with the other four dietary treatments having an average DM digestibility of 82.4%. The 7.5% raw GB had the highest ($P < 0.05$) CP ATTD at 86.2% but was not different ($P > 0.05$) from 0% GB (83.9%) or 30% raw GB (86.4%). The 30% cooked GB treatment had lowest ($P < 0.05$) CP ATTD at 81.6% and lowest ($P < 0.05$) AHF ATTD at 91.5%. Metabolizable energy was lowest ($P < 0.05$) for 7.5% raw GB (4.21 kcal/g), but only different ($P < 0.05$) from 30% cooked GB (4.43 kcal/g).

Fecal fermentative end-products and serum chemistry

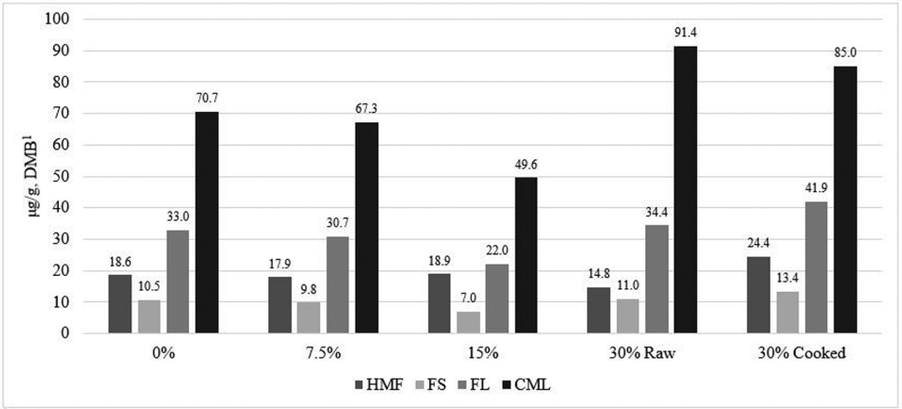
Total fecal concentrations of SCFA (Table 7) were highest ($P < 0.05$) for the 30% cooked GB treatment at 434.6 μmol/g (DMB), but not different ($P > 0.05$) from the 15% raw or 30% raw GB treatments (345.0 and 373.6 μmol/g, respectively). Fecal acetate and propionate concentrations were highest ($P > 0.05$) for 30% cooked GB at 434.6 and 206.8 μmol/g, respectively. No differences ($P > 0.05$) were observed among dietary treatments in butyrate concentrations.

Total phenol and indole concentrations (Table 7) were highest ($P < 0.05$) at 3.2 μmol/g for 0% GB. There were no differences ($P > 0.05$) among other dietary treatments for phenols and indoles. Total fecal BCFA concentrations (Table 7) were not significantly different ($P > 0.05$) among dietary treatments with concentrations ranging from 22.1 μmol/g (30% cooked GB) to 31.0 μmol/g (0% GB).

Table 5. Anti-nutritional factors and oligosaccharide content of feline diets containing garbanzo beans (GB) at various processing stages

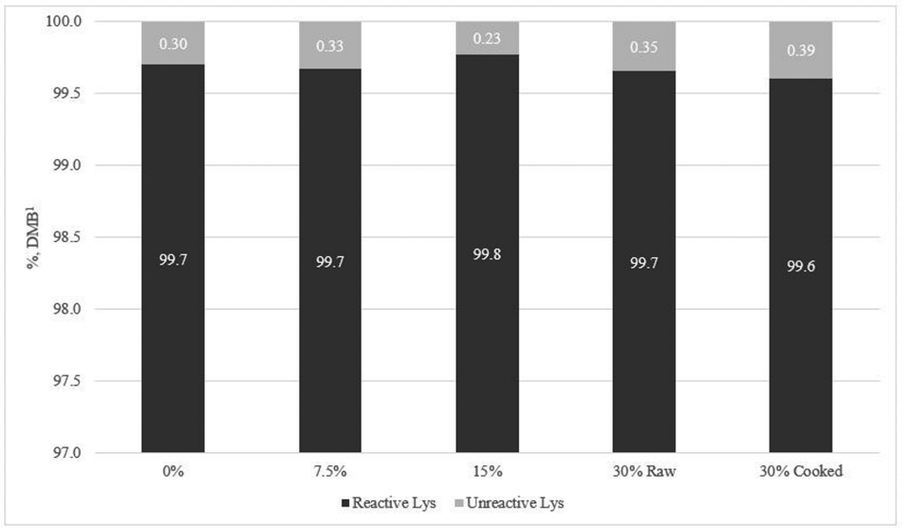
	Anti-nutritional factors		Oligosaccharides (%, DMB[1])		
Diet stage of processing	Trypsin inhibitor (TIU/g)	Urease activity(Net pH Increase)	Raffinose	Stachyose	Verbascose
Base mix					
0% GB	0	0	0.02	0.01	0
7.5% GB	0	0.03	0.05	0.08	0
15% GB	351.5	0.03	0.10	0.12	0
30% raw GB	1,970.1	0.02	0.19	0.31	0
30% cooked GB	336.9	0.02	0.22	0.37	0
After preconditioner					
0% GB	0	0.05	0.03	0.03	0
7.5% GB	0	0	0.05	0.04	0
15% GB	148.1	0	0.06	0.06	0
30% raw GB	1,118.4	0	0.17	0.11	0.01
30% cooked GB	0	0	0.20	0.31	0
After extruder					
0% GB	0	0	0.02	0.01	0
7.5% GB	0	0	0.08	0.13	0
15% GB	0	0	0.14	0.26	0
30% raw GB	644.9	0	0.22	0.49	0.01
30% cooked GB	1,157.1	0	0.21	0.37	0
Final diet					
0% GB	682.9	0	0.03	0.02	0
7.5% GB	0	0	0.07	0.10	0
15% GB	267.3	0	0.08	0.17	0
30% raw GB	0	0.03	0.18	0.46	0.01
30% cooked GB	0	0.03	0.18	0.34	0

[1]DMB, dry matter basis.



[1] DMB = Dry matter basis
ABBR: HMF = Hydroxymethylfurfural, FL = Fructoselysine, FS = Furosine, CML = Carboxymethyllysine
Statistical analysis could not performed due to use of technical replicate

Figure 1. Presence of Maillard reaction production feline diets containing graded inclusion levels of garbanzo beans. [1]DMB, dry matter basis. HMF, hydroxymethylfurfural; FL, fructoselysine; FS, furosine; CML, carboxymethyllysine. Statistical analysis could not be performed due to the use of technical replicate.



[1] DMB = Dry matter basis

Figure 2. Reactive lysine content of feline diets containing graded inclusion levels of garbanzo beans. [1]DMB, dry matter basis.

Serum metabolites (Table 8) were all within range with the exception of phosphorus and glucose, which were above reference ranges for all dietary treatments. The increased glucose levels are attributed to the side effect of the drug used to sedate the cats for blood collections. The reason for the increased serum concentration of phosphorus is not known, but it has been observed consistently in this cat colony over time. The analyzed CBC were evaluated and deemed normal for healthy adult cats (data not shown).

Fecal microbiota

The microbial composition at the phylum level is shown in Table 9. The most abundant phyla included Firmicutes (ranging from 52% of the sequences for cats fed 30% raw GB to 62% for cats fed 0% GB), Bacteroidota (ranging from 24.2% for 0% GB to 27.7% for 30% raw GB), and Actinobacteria (ranging from 4.5% for 0% GB to 12.3% for 7.5% GB). A greater relative abundance ($P < 0.05$) was observed in cats fed 0% GB for Firmicutes (62.1%) but no differences ($P > 0.05$) were observed among treatments for the Bacteroidota phyla. Proteobacteria corresponded to 7.4% or less of the sequences among dietary treatments. All GB-containing diets had a greater relative abundance ($P < 0.05$) of Proteobacteria than cats fed 0% GB. Campilobacterota comprised approximately 1% or less of the sequences among dietary treatments.

The microbial composition at the family level is shown in Table 9, identifying approximately 40 different families. The relative abundance at the family level varied among different treatments. The most abundant families included Prevotellaceae (ranging from 10.6% for 0% GB to 15.1% for 30% cooked GB) and Lachnospiraceae (ranging from 21.7% for 30% raw GB to 27.8% for 0% GB). In the Actinobacteria phyla, Bifidobacteriaceae was the most abundant family, followed by Coriobacteriaceae. The microbial composition

Table 6. Apparent total tract macronutrient and energy digestibility by cats fed diets containing graded inclusions of garbanzo beans (GB)

	Dietary treatment					
Nutrient digestibility	0% GB	7.5% GB	15% GB	30% raw GB	30% cooked GB	SEM[1]
Dry matter, %	82.8[a]	83.7[a]	81.5[a]	81.4[a]	77.3[b]	0.986
Organic matter, %	87.6[a]	88.6[a]	86.0[a]	86.4[a]	81.7[b]	0.779
Crude protein, %	83.9[ab]	86.2[a]	82.3[b]	84.5[ab]	81.6[b]	0.839
Acid hydrolyzed fat, %	93.6[a]	93.3[a]	92.8[a]	92.4[ab]	91.5[b]	0.399
Total dietary fiber, %	53.7[a]	44.4[b]	44.2[bc]	45.7[a]	35.0[c]	2.641
Digestible energy, kcal/g, DMB[2]	4.73[a]	4.67[ab]	4.61[bc]	4.58[bc]	4.49[c]	0.035
Metabolizable energy, kcal/g	2.49	2.19	2.46	2.69	2.67	0.199

[1]DMB, dry matter basis.
[2]SEM, standard error of the mean.
[a,b,c] Means within a row with different superscript letters are different ($P < 0.05$).

Table 7. Fecal fermentative end-product concentrations for cats fed diets containing graded levels of GB

	Dietary treatment					
Item (DMB[1])	0% GB	7.5% GB	15% GB	30% raw GB	30% cooked GB	SEM[2]
pH	6.68	6.44	6.47	6.59	6.28	0.112
Ammonia, μmol/g	108.5[a]	74.8[b]	94.3[ab]	102.3[ab]	106.6[ab]	8.271
SCFA[3], μmol/g						
Acetate	251.5[c]	308.4[bc]	345.4[abc]	373.6[ab]	434.6[a]	35.995
Propionate	74.9[d]	121.9[c]	133.6[bc]	173.1[ab]	206.8[a]	12.601
Butyrate	35.2	43.6	48.9	44.1	41.1	4.789
Total SCFA	361.6[b]	474.1[b]	527.9[ab]	590.7[ab]	682.4[a]	50.570
BCFA[3], μmol/g						
Isobutyrate	6.9	5.5	6.5	6.7	5.8	0.651
Isovalerate	9.7	6.5	8.7	9.0	7.6	0.996
Valerate	14.5[a]	11.6[ab]	13.5[ab]	11.5[ab]	8.6[b]	1.422
Total BCFA	31.0	23.6	28.7	27.2	22.1	2.677
Phenols/indoles, μmol/g						
Phenols	0.04[b]	0.04[b]	0.12[ab]	0.26[ab]	0.32[a]	0.062
Indoles	3.1[a]	1.3[b]	1.6[b]	1.4[b]	1.4[b]	0.166
Total phenol/indoles	3.2[a]	1.3[b]	1.7[b]	1.7[b]	1.7[b]	0.179

[1]DMB, dry matter basis.
[2]SEM, standard error of the mean.
[3]SCFA, short-chain fatty acids; BCFA, branched-chain fatty acids.
[a,b]Means within a row with different superscript letters are different ($P < 0.05$).

at the genus level is shown in Table 10. Over 70 different genera were identified. Of these, 29 were considered significant ($P < 0.05$) among treatments. *Fusobacteriaceae* was greater ($P < 0.05$) in cats fed 0% GB (4%) than the GB-containing diets (1.2% to 2.3%). *Megamonas* was greater ($P < 0.05$) in cats fed the GB-containing diets (ranging from 4.2% for 7.5% GB to 5.9% for 30% cooked GB) than in cats fed 0% GB (1.1%). However, due to the complexity of the hindgut microbial composition, the changes in microbial diversity for each dietary treatment were also determined.

There were no differences in α-diversity measured by Faith's phylogenetic diversity (Figure 3) among treatments, indicating that species evenness within a sample was not affected by treatment. The β-diversity was determined based on weighted UniFrac analysis (Figure 4A). Fecal microbial community abundance was similar between cats fed 7.5% and 15% GB. Cats fed 0% GB differed from all other diets ($P < 0.05$). Cats fed 30% raw and 30% cooked GB differed from cats fed 7.5% GB ($P < 0.05$). The β-diversity based on unweighted UniFrac analysis (Figure 4B) showed that fecal microbial community abundance was similar between the GB-containing diets. Cats fed 0% GB differed from all other diets ($P < 0.05$).

Discussion

Diet, food intake, and fecal characteristics

All five diets were formulated to target similar nutrient and ingredient composition, with the exception of the graded inclusion of GB, which were included at the expense of poultry by-product meal and rice. Although statistically different, the difference in food intake (DMB) observed in cats fed the diets containing GB were small and approximately 2 g on an as-is basis and 3 g on a DMB, on average. Therefore, all diets were considered to be well-accepted and did not result in inadequate daily food intake or body weight loss during the study.

The large differences in fecal output on an as-is basis are indicative of the substantial water-holding capacity of GB. Legumes and pulses typically contain approximately 30% dietary fiber (McCrory et al., 2010) which are responsible for the water-holding capacity of the GB included in these diets. Although significantly different, fecal scores were within the range considered to be ideal (2 to 3).

Table 8. Serum metabolites for cats fed diets containing graded levels of garbanzo beans (GB)

Item	Reference range[1]	Dietary treatment					SEM[2]
		0% GB	7.5% GB	15% GB	30% raw GB	30% cooked GB	
Creatinine, mg/dL	0.4–1.6	1.6	1.6	1.6	1.6	1.6	0.059
BUN[3], mg/dL	18–38	25.4	23.8	24.2	23.3	23.7	0.629
Total protein, g/dL	5.8–8	6.4	6.5	6.3	6.4	6.4	0.097
Albumin, g/dL	2.8–4.1	3.4	3.4	3.4	3.5	3.5	0.047
Globulin, g/dL	2.6–5.1	3.1	3	2.9	3.1	3.1	0.1013
Ca, mg/dL	8.8–10.2	9.6	9.7	9.7	9.6	9.6	0.111
P, mg/dL	3.2–5.3	6.2	6.2	6.3	6.3	6.3	0.125
Na, mmol/L	145–157	149.6	150.7	150.2	150	150.9	0.407
K, mmol/L	3.6–5.3	4.3	4.3	4.3	4.3	4.4	0.082
Na:K ratio	28–36	34.7	35.2	35	35	34.6	0.735
Cl, mmol/L	109–126	115.9	116.4	116.1	116.1	116.6	0.553
Glucose, mg/dL	60–122	110.5	122.8	127.7	104.1	106.2	10.357
Total bilirubin, mg/dL	0–0.3	0.2	0.1	0.2	0.1	0.1	0.015
Cholesterol, mg/dL	66–160	143.0	132.8	130.9	127.0	122.4	11.220
Triglycerides, mg/dL	21–166	34.4	33.1	32.5	31.6	33.3	2.362
Bicarbonate, mmol/L	12–21	19.2	18.8	19.1	19.5	19.8	0.389

[1]Reference ranges were provided by the University of Illinois Veterinary Diagnostics Laboratory.
[2]SEM, standard error of the means.
[3]BUN, blood urea nitrogen.

Table 9. Relative abundance of bacterial phyla and families of cats fed diets containing graded levels of garbanzo beans (GB)

Phylum, % sequences	Family	Dietary treatment					SEM[1]
		0% GB	7.5% GB	15% GB	30% raw GB	30% cooked GB	
Actinobacteria		4.5^{c}	12.3^{a}	10.1^{ab}	9.9^{ab}	7.7^{bc}	0.995
	Bifidobacteriaceae	0.8^{c}	6.5^{a}	5.7^{ab}	4.4^{ab}	3.1^{bc}	0.840
	Atopobiaceae	0.1^{b}	0.1^{b}	0.1^{b}	0.4^{a}	0.1^{b}	0.057
	Coriobacteriaceae	3.3^{b}	5.4^{a}	4.1^{ab}	4.8^{a}	4.3^{ab}	0.388
Bacteroidota		24.2	24.4	26.1	27.7	26.4	1.568
	Prevotellaceae	10.6^{b}	12.3^{ab}	14.0^{ab}	14.8^{ab}	15.1^{a}	1.257
	Tannerellaceae	1.7^{a}	1.1^{a}	1.4^{ab}	1.1^{a}	1.1^{a}	0.138
Campilobacterota		0.4^{b}	0.9^{a}	0.8^{ab}	0.7^{ab}	0.8^{ab}	0.138
	Campylobacteraceae	0.4^{b}	0.9^{a}	0.8^{ab}	0.7^{ab}	0.8^{ab}	0.138
Firmicutes		62.1^{a}	53.8^{b}	53.3^{b}	52.0^{b}	55.1^{b}	1.756
	Erysipelotrichaceae	4.1^{a}	1.7^{b}	1.5^{b}	1.8^{b}	1.7^{b}	0.257
	Clostridia UCG014	2.2^{ab}	1.7^{ab}	2.1^{ab}	2.4^{a}	1.2^{b}	0.334
	Lachnospiraceae	27.8^{a}	23.8^{ab}	22.5^{ab}	21.7^{b}	23.4^{ab}	1.365
	Ruminococcaceae	8.3^{a}	5.6^{b}	6.1^{b}	6.1^{b}	6.8^{ab}	0.501
	Anaerovoracaceae	2.7^{a}	1.1^{b}	1.4^{b}	1.2^{b}	1.3^{b}	0.284
	Peptostreptococcaceae	3.4^{a}	2.7^{ab}	2.3^{bc}	1.6^{c}	1.9^{bc}	0.244
	Acidominococcaceae	1.1^{ab}	1.8^{ab}	1.2^{ab}	0.9^{b}	2.1^{a}	0.259
Fusobacteriota		4.0^{a}	1.4^{b}	1.6^{b}	1.2^{b}	2.3^{b}	0.381
	Fusobacteriaceae	4.0^{a}	1.4^{b}	1.6^{b}	1.2^{b}	2.3^{b}	0.381
Proteobacteria		3.6^{b}	6.5^{a}	7.4^{a}	7.4^{a}	7.2^{a}	0.742
	Succinivibrionaceae	1.3^{b}	4.1^{a}	4.7^{a}	5.1^{a}	4.6^{a}	0.637

[1]SEM, standard error of the mean.
[a,b,c] Means within a row with different superscript letters are different ($P < 0.05$).

Maillard reaction products

Maillard reactions are nonenzymatic browning reactions that occur spontaneously between a reducing sugar, such as glucose, and a free amino group in a protein when exposed to prolonged heat. Although Maillard reactions are important for flavor development, Maillard reactions can be detrimental to protein quality if allowed to progress to advanced stages (Seiquer et al., 2006; Lee et al., 2017). Lysine is subjected to Maillard reactions due to its free epsilon amino group, causing it to be easily bound in Maillard reactions resulting in reduced lysine availability (Hemmler et al., 2018). As the first- or second-limiting amino acid in most commercially available cat foods, decreased lysine availability can negatively impact the overall protein quality of the diet (van Rooijen et al., 2013).

A common marker of advanced stages of Maillard reactions is the production of HMF, as it is a common intermediate product (Surh and Tannenbaum, 1994; van Rooijen et al., 2014a). The greater concentration of HMF observed in the 30% cooked GB could

Table 10. Relative abundance of bacterial genera of cats fed diets containing graded levels of garbanzo beans (GB)

		Dietary treatment					
Phylum, % sequences	Genus	0% GB	7.5% GB	15% GB	30% raw GB	30% cooked GB	SEM[1]
Actinobacteria	*Bifidobacterium*	0.8^{c}	6.5^{a}	5.7^{ab}	4.4^{ab}	3.1^{bc}	0.840
	Libanicoccus	0.03^{b}	0.1^{b}	0.1^{b}	0.3^{a}	0.1^{b}	0.050
	Collinsella	3.3^{b}	5.4^{a}	4.1^{ab}	4.8^{ab}	4.3^{ab}	0.389
Bacteroidetes	*Paraprevotella*	0.02^{b}	0.02^{b}	0.08^{ab}	0.2^{a}	0.03^{b}	0.033
	Parabacteroides	1.7^{a}	1.1^{b}	1.4^{ab}	1.1^{b}	1.1^{b}	0.138
Fusobacteria	*Fusobacterium*	4.0^{a}	1.4^{b}	1.6^{b}	1.2^{b}	2.3^{b}	0.381
Proteobacteria	*Succinivibrio*	1.3^{b}	4.1^{a}	4.7^{a}	5.1^{a}	4.6^{a}	0.634
	Parasutterella	0.7^{a}	0.4^{ab}	0.5^{ab}	0.1^{b}	0.5^{ab}	0.128
Campilobacterota	*Campylobacter*	0.4^{b}	0.9^{a}	0.8^{ab}	0.7^{ab}	0.8^{ab}	0.138
Firmicutes	*Erysipelatoclostridium*	0.7^{a}	0.4^{ab}	0.2^{b}	0.3^{b}	0.3^{b}	0.084
	Holdemanella	1.9^{a}	1.1^{b}	1.0^{b}	1.1^{b}	1.3^{b}	0.161
	Solobacterium	1.2^{a}	0.5^{b}	0.4^{b}	0.6^{b}	0.4^{b}	0.118
	Clostridia UCG014	2.2^{ab}	1.7^{ab}	2.1^{ab}	2.4^{a}	1.2^{b}	0.333
	Anaerostignum	0.04^{b}	0.4^{a}	0.5^{a}	0.6^{a}	0.4^{a}	0.099
	Lachnoclostridium	3.6^{a}	2.9^{ab}	2.3^{b}	2.6^{ab}	2.0^{b}	0.355
	Sellimonas	0.8^{a}	0.5^{ab}	0.1^{b}	0.7^{ab}	0.5^{ab}	0.172
	Tyzzerella	1.1^{a}	0.1^{b}	0.2^{b}	0.05^{b}	0.0^{b}	0.095
	Ruminococcus Gnavus Group	1.2^{b}	2.1^{ab}	1.6^{ab}	1.7^{ab}	2.6^{a}	0.291
	Ruminococcus Torques Group	1.2^{a}	0.5^{ab}	0.7^{ab}	0.3^{b}	0.6^{ab}	0.233
	Intestinimonas	0.9^{a}	0.7^{ab}	0.7^{ab}	0.6^{ab}	0.4^{b}	0.126
	Oscillibacter	0.4^{b}	0.6^{ab}	0.8^{a}	0.9^{a}	0.5^{b}	0.102
	Candidatus Soleaferrea	0.3^{a}	0.2^{ab}	0.3^{ab}	0.2^{ab}	0.1^{b}	0.053
	Faecalibacterium	2.6^{a}	0.8^{b}	1.1^{b}	0.9^{b}	1.9^{ab}	0.341
	Eubacterium Nodatum Group	1.1^{a}	0.4^{b}	0.6^{ab}	0.5^{b}	0.6^{ab}	0.121
	Peptoclostridium	3.0^{a}	2.5^{ab}	2.1^{ab}	1.5^{b}	1.6^{b}	0.243
	Phascolarctobacterium	1.1^{ab}	1.8^{ab}	1.2^{ab}	0.9^{b}	2.1^{a}	0.259
	Megamonas	1.1^{b}	4.2^{a}	4.7^{a}	4.6^{a}	5.9^{a}	0.603
	Allisonella	0.5^{b}	1.0^{ab}	1.2^{a}	1.0^{a}	0.9^{ab}	0.128

[1]SEM, standard error of the mean.
[a,b,c]Means within a row with different superscript letters are different ($P < 0.05$).

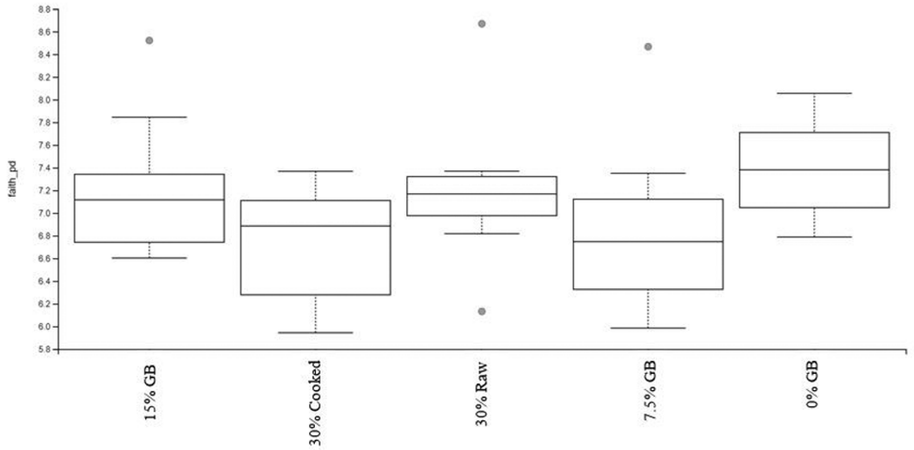


Figure 3. Fecal microbial alpha-diversity analysis determined by Faith's phylogenetic diversity of cats fed diets containing graded levels of garbanzo beans (GB).

be attributed to the additional processing step of the GB prior to extrusion. van Rooijen et al. (2014a) estimated that a 70 kg adult human has an average daily intake of 0.28 mg HMF/kg $BW^{0.75}$ compared with the intake of a 4 kg adult cat, fed a commercial extruded diet, at 10.9 mg HMF/kg $BW^{0.75}$ per day. Because cats consistently consume heat-processed diets, such as extruded kibble, they also consume greater concentrations of MRP compared with humans, as demonstrated by the 38-fold difference between the human and feline diets. Additionally, many ingredients included in pet foods, such as the cooked GB used in the current study, are exposed to heat processing prior to the extrusion, which can increase the formation of MRP in the overall diet.

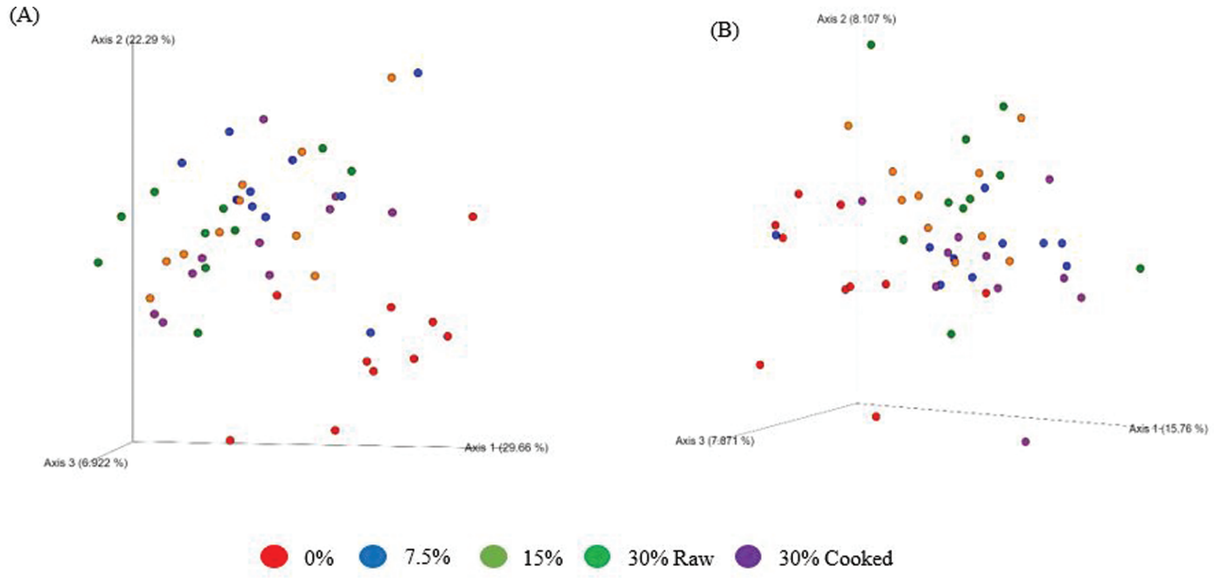


Figure 4. Principal coordinated plots of weighted (A) and unweighted (B) UniFrac distances of fecal microbial communities of cats fed diets containing graded levels of garbanzo beans (GB).

The FL content of the diet can be indirectly measured through the analysis of FS. The acid hydrolysis process causes FL to be chemically converted to FS, regenerated lysine, and pyridosine (Krause et al., 2003). The formation of FS is assumed to be formed at a constant yield of about 30% to 34% (Krause et al., 2003), allowing for the formation of FL to be calculated. Similar to other MRP, the formation of FL can vary depending on diet format and ingredient composition (Chiang, 1983; van Rooijen et al., 2014a). van Rooijen et al. (2014a) analyzed how different diet formats (extruded and canned) influenced the formation of FL in adult feline diets. The FL contents averaged 0.73 g/kg (DMB) for extruded diets and 4.30 g/kg for canned diets. The average FS contents of the diets were 15.9 g/kg (DMB) in extruded diets and 34.1 g/kg in canned diets (van Rooijen et al., 2014a). A different study measured the FS content of a single extruded dog diet to be 0.9 mg/g (as-is) but increased to 1.5 mg/g after 12 wk of storage at 22.2 °C or 3.2 mg/g after 12 wk of storage at 37.8 °C (Chiang, 1983). Although the current study measured fewer FL and FS than these previous studies, this could be due to the differences in extrusion parameters, water content of the diet, and storage conditions of the diet.

Reactive lysine, or the lysine available for protein synthesis, can be calculated using the FS procedure (Pahm et al., 2008). This calculation relies on the assumption that the same FS content in milk (32% of all Amadori compounds formed) is consistent for all ingredients (Bujard et al., 1978; Pahm et al., 2008). However, it has been well-established that differences in processing conditions can influence the amount of available lysine in a diet. A previous study has measured the reactive to total lysine content of extruded commercial canine maintenance and growth diets to be 0.85 and 0.75, respectively (Williams et al., 2006). Similarly, van Rooijen et al. (2014b) analyzed the reactive to total lysine ratio in commercially available extruded, canned, and pelleted canine diets to be 0.90, 0.98, and 0.84, respectively.

Advanced glycation end-products (AGE) have been associated with negative health effects in humans, rats, and dogs, such as increased inflammatory and oxidative stress or insulin resistance (Ames, 2008; Teodorowicz et al., 2018; Prosser et al., 2019; Palaseweenun et al., 2021). The formation of CML in foods is often used as a marker for AGE formation and, therefore, is commonly measured in human foods as a method to help reduce the formation of AGE (Prosser et al., 2019). van Rooijen et al. (2014a) measured the CML concentration to be 13.3 mg/kg (DMB) in extruded adult feline diets. In canned adult feline diets, the CML concentration was 41.6 mg/kg (van Rooijen et al., 2014a), which were significantly greater than concentrations observed in this study. In addition, concentrations of MRP reported herein are within range or below the concentration of these compounds present in foods for human nutrition. For example, FL concentration in extruded soybean (140 °C for 20 to 30 s) was approximately 67 µg/g (Zilić et al., 2014); HMF concentration of natural or roasted coffee was 110 and 1,734 mg/kg, respectively (Arribas-Lorenzo and Morales, 2010), and mean CML concentrations in meat and fish, dairy products, cereals, and fruits and vegetables were 45, 5,143, 281, and 27 mg/kg protein, respectively (Hull et al., 2012).

Anti-nutritional factors and oligosaccharides

Trypsin inhibitors are an important anti-nutritional factor that impede the function of trypsin, an important protease in the small intestine. Two classes of trypsin inhibitors have been identified (i.e., Kunitz or Bowman–Birk families) that cause a reduction of protein digestion and absorption in animals fed legumes (Khattab and Arntfield, 2009; Batt et al., 2015). GB contain mainly the Bowman–Birk family of trypsin inhibitors (Srinivasan et al., 2005). However, the addition of heat to these ingredients have demonstrated either a reduction or complete elimination of trypsin inhibitors (Kozlowska et al., 1980; Khattab and Arntfield, 2009). In diets containing high concentrations of GB, the reduction of anti-nutritional factors by heat processing helps to increase protein digestion and increase palatability (Hamid et al., 2017). The trypsin inhibitor content of whole raw GB has been reported to be 14.2 TIU/mg but decreases to 12.9 TIU/mg after soaking and 2.3 TIU/mg after cooking (Shi et al., 2017).

Urease activity is also often used as an indirect marker of the presence of anti-nutritional factors due to its correlation

with heat processing. Urease is responsible for the hydrolysis of urea to ammonia and carbon dioxide (Sirko and Brodzik, 2000). The basic pH of ammonia allows urease activity to be measured through increases in pH (Baker and Mustakas, 1973). Urease activity of ≤ 0.05 Δ pH units is indicative of over-processing, while activity ≥ 0.25 Δ pH units is indicative of under-processing (White et al., 2000; Silva et al., 2013). However, this range was based on the processing of soybeans and may not be as applicable in companion animal nutrition, where many ingredients have been processed prior to inclusion into the diet matrix. Additionally, urease is more susceptible to heat treatment than trypsin inhibitors (Purushotham et al., 2007) and activity would be easily eliminated or reduced with the inclusion of pre-processed ingredients. In the current study, the Δ pH of the diets would be reflective of the extrusion process. However, the high acceptability and digestibility of the diets by the cats, the minimal formation of MRP, and the extrusion parameters do not corroborate that the diets were over-processed. Few studies have analyzed the urease activity in extruded companion animal diets. One previous study measured similar urease activities in extruded canine diets containing traditional or defatted soybean meal or micronized, toasted, or raw soybeans (Félix et al., 2013). The analyzed urease activities in the diets ranged from 0 to 0.04 Δ pH units post-extrusion (Félix et al., 2013), which is similar to the values analyzed in the current study. A different study measured the urease activity of extruded canine diets containing 30% poultry by-product meal or 30% soybean meal. Similar urease activities to the current study were measured in the poultry by-product meal diet with a Δ pH of 0.05. Slightly greater Δ pH was measured at 0.08 for the extruded soybean meal-containing diet (Tortola et al., 2013).

Lastly, as with other pulse varieties, GB have been associated with the negative effects in the gastrointestinal tracts of animals, such as increased gas production (Abdel-Gawad, 1993; Rupérez, 1998). The gas accumulates due to the rapid fermentation of α-galactooligosaccharides (i.e., raffinose, stachyose, and verbascose) in the hind gut and causes gastrointestinal discomfort (Sosulski et al., 1982; Rupérez, 1998). A bacterial enzyme, α-galactosidase, breaks down oligosaccharides to D-galactose and sucrose in the hindgut and is commonly found in bacterial species belonging to the *Megamonas* genus (Polansky et al., 2015). In the current study, cats fed GB-containing diets had increased relative abundance of *Megamonas* compared to 0% GB, indicating these animals' improved ability to ferment oligosaccharides. In raw cultivars, Desi GB have been shown to have an average of 1.34% of stachyose and 0.50% of raffinose. Similarly, Kabuli types have an average of 1.16% of stachyose and 0.46% of raffinose (Singh et al., 1982). Geographical location and growing conditions can influence the oligosaccharide concentration, as well. A different study measured the oligosaccharide content of Kabuli types to be 2.5% stachyose and 1.2% raffinose, while Desi types had 0.8% raffinose and 2.4% stachyose (Bampidis and Christodoulou, 2011).

The negative effects associated with α-galactooligosaccharides can be alleviated with heat treatment (Kelkar et al., 2012), although results have proven to be inconsistent (Ai et al., 2016). Several factors, such as cultivar (either Desi or Kabuli types), could influence the variable response of the oligosaccharides to heat treatment. Similarly, pre-processing methods, such as soaking or pre-cooking of GB, could aid in decreasing the oligosaccharide content compared with raw ingredients (Kelkar et al., 2012). Additional heat processing (i.e., extrusion), temperature, and duration of heat exposure can impact the ability of the oligosaccharides to be reduced (Berrios et al., 2010; Ai et al., 2016). One study measured the effects of boiling, autoclaving, and microwaving on the oligosaccharide content in GB. The stachyose concentration in raw GB was 2.6% but decreased to 1.5% when boiled, autoclaved, or microwaved. The raffinose concentration was 14.5% but decreased to 7.6% when boiled, 8.1% when autoclaved, and 7.1% when microwaved (Bampidis and Christodoulou, 2011). The cooked GB in the current study were presoaked in water and heat-dried prior to inclusion in the diet. The 30% cooked GB diet had similar oligosaccharide content to the 30% raw GB diet so the duration or temperature of the processing may have been insufficient for decreasing oligosaccharide content.

Apparent total tract macronutrient and energy digestibility

The dietary treatments in the current study were considered to be well-digested by the cats, as the digestibility of the nutrients ranged from 77% to 93% digestible, with the exception of TDF digestibility, due to fiber's passage through the small intestine and fermentation in the large intestine. In companion animal nutrition, it is becoming increasingly common that pulses, such as GB, are used as alternative protein sources to animal-based proteins and in grain-free diets. Previous evaluations of GB in companion animal studies have corroborated that GB are valuable, highly digestible ingredients in pet foods. In dogs, a diet containing 12% GB and lentils resulted in DM ATTD of 90%. Additionally, the ATTD of OM and CP were approximately 92% (Cargo-Froom et al., 2019). In general, plant-based protein sources are more widely accepted in canine diets, as dogs are classified as omnivores. In contrast, as obligate carnivores, the use of plant-based ingredients in feline diets is often questioned by consumers (Prantil et al., 2018). However, as observed in the current study and in previous studies, plant-based ingredients can be valuable inclusions in feline diets. In fact, Golder et al. (2020) calculated the true protein digestibility of cats fed dry diets containing vegetable protein source (i.e., rice proteins, corn proteins, and soy proteins) was greater (average 95%) than it was for dogs (average 89%). A previous study assessing the effect of legumes on nutrient digestibility in cats demonstrated similar results to the current study. Cats fed 67% lentils had ATTD of DM, OM, and CP of 77%, 79%, and 81%, respectively (de-Oliveira et al., 2008). Similarly, cats fed 65% peas had apparent total tract DM, OM, and CP digestibilities of 76%, 79%, and 82%, respectively (de-Oliveira et al., 2008).

In the current study, it was hypothesized that the diet containing the cooked GB would have greater nutrient digestibility values, as pre-cooking would potentially soften the GB seed, aiding in the milling and extrusion processes. Based on the MRP data presented in this study, it is possible that the increased HMF, FS, and FL compared with other dietary treatments may be an indication that the heat associated with the processing of cooked GB impacted protein ATTD. Additional protein complexes with fat and starch, as reviewed in Wang et al. (2020), caused by pre-processing the GB may also negatively impact digestibility. Although the results in this study were unexpected, previous studies have also measured decreased nutrient digestibility of heat-processed GB. Although in vivo studies assessing the use of GB in feline diets are few, in vitro studies have analyzed the digestibility of raw and cooked GB. Rehman and Shah (2005) found that the protein digestibility of raw GB was 36% which increased to 73% after cooking at 121 °C for 10 min. However, the protein digestibility decreased after cooking for 20 min (69%), 40 min (67%), 60 min (66%), or

90 min (65%). Additionally, when the temperature was raised to 128 °C and cooked for 20 min, the protein digestibility of the GB was 65% (Rehman and Shah, 2005). Therefore, temperature and duration of pre-processing of GB, in addition to the extrusion process, are important considerations for inclusion of GB in diets.

Fecal fermentative end-products

SCFAs (i.e., acetate, propionate, and butyrate) are produced as a result of the degradation of fibrous material by saccharolytic bacteria (Morrison and Preston, 2016). In the current study, the fecal concentrations of SCFA increased with corresponding increased inclusion levels of GB. Sandri et al. (2019) reported the total SCFA fecal concentrations to be less than that of the current study for dogs fed 15% GB, pea flour, or a commercial diet. The dogs fed GB had fecal SCFA concentrations of 163 μmol/g compared with pea flour (179 μmol/g) and a commercial diet (182 μmol/g). The acetate, propionate, and butyrate concentrations for the 15% GB diet in Sandri et al. (2019) were 68, 47, and 15.2 μmol/g, respectively. The greater SCFA concentrations measured in the current study could be due to the fragments of GB that escaped grinding during diet manufacturing as particles were observed in the finished kibble and fecal samples of the cats. Larger fragments of GB that were ingested by the cats could lead to decreased ability of pancreatic enzymes to adequately digest, increased substrate passage into the hindgut, and therefore, increased exposure to saccharolytic fermentation compared with GB that were ground to a uniform particle size.

Typically, acetate is the most abundant SCFA produced and it is utilized in acetyl-coA formation, an important coenzyme in several metabolic processes, including the Krebs cycle (Zhang and Davies, 2016). Of the SCFA, acetate had the highest fecal concentrations in cats fed all five diets. Butyrate, the preferred energy source for colonocytes (den Besten et al., 2013), was similar for all dietary treatments. Studies analyzing the effects of raw or cooked plant-based protein sources in cats are minimal. However, Kerr et al. (2012) measured fecal SCFA concentrations in cats fed a commercial extruded diet, a raw beef-based diet, or a cooked beef-based diet. The total SCFA concentrations were similar among treatments (305 μmol/g extruded; 266 μmol/g raw beef-based; and 405 μmol/g cooked beef-based) but showed butyrate concentrations to be decreased in raw beef-based (21 μmol/g) and cooked beef-based (25 μmol/g) compared with the commercial diet at 38 μmol/g (Kerr et al., 2012).

Proteolytic fermentation takes place mainly in the distal large intestine, with BCFA as the primary end-products (Tiwari et al., 2019). In dogs fed 15% GB, Sandri et al. (2019) reported total fecal BCFA concentrations to be 13.1 μmol/g which was comprised of 3.8 μmol/g isobutyrate, 7.8 μmol/g isovalerate, and 1.5 μmol/g valerate. The greater fecal BCFA concentrations observed in the current study could be attributed to species differences or increased protein reaching the large intestine for fermentation. However, because fecal concentrations are not reflective of production rates of BCFA, it should be noted that results can be difficult to interpret (den Besten et al., 2013).

Phenol and indoles are commonly referred to as putrefactive compounds, contributing to fecal odor. However, some physiological benefits have been associated with indole production in the body, such as increased mucosal barrier function and mucin production, as well as decreased production of proinflammatory cytokines (Bansal et al., 2010). Although studies analyzing plant ingredients in feline diets are scarce, Detweiler et al. (2019) analyzed the total phenol and indole fecal concentrations in cats fed extruded diets containing 14% soybean hulls. Similar to the current study, cats fed soybean hulls had total fecal phenol and indole concentrations of 1.9 μmol/g (Detweiler et al., 2019). Although unexpected, the GB-containing diets had decreased total phenol and indole fecal concentrations than the 0% GB diet, which would imply that greater inclusion levels of GB would correspond to decreased fecal odor.

Fecal microbiota

The gastrointestinal microbiota is a complex system and studies evaluating these microbial communities are rapidly expanding (Grześkowiak et al., 2015). The feline gastrointestinal microbiota has been shown to have active roles in energy metabolism, immune function, and neuro-behavioral development (Mondo et al., 2019), as well as maintaining the intestinal epithelial integrity and health (Moon et al., 2018). Microbial composition, as well as the microbial functions, can be modulated through diet (Moon et al., 2018).

The reported effects of GB on fecal microbial composition in companion animals are few. In dogs, Sandri et al. (2019) substituted an extruded commercial diet with raw meat complemented with either 15% GB flour or 15% pea flour to evaluate the effects of diet on the fecal microbiota in dogs. The major phyla observed were *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, *Proteobacteria*, and *Actinobacteria* in these dogs. Additionally, dogs fed 15% GB flour showed decreased relative abundance of *Prevotella* (15.6%), *Alloprevotella* (10.2%), *Erysipelotrichaceae* (5.0%), *Eubacterium* (3.3%), and *Sutterella* (1.3%) compared with the commercial diet (Sandri et al., 2019). The microbial composition at the phyla, family, and genera levels analyzed by Sandri et al. (2019) were similar to the current study. Additionally, a different study showed that fecal microbial compositions were altered in adult cats fed moderate or high protein contents (Lubbs et al., 2009). Cats fed 53% CP showed decreased *Bifidobacterium* genera and increased *Clostridium perfringens* than cats fed 34% CP (Lubbs et al., 2009).

A greater relative abundance of Proteobacteria were observed in cats fed the GB-containing diets than cats fed 0% GB, stemming from an increase in the genus *Succinivibrio*. Proteobacteria is often abundant in healthy dogs and cats, and especially in animals fed high-protein diets (Moon et al., 2018). However, a greater relative abundance of Proteobacteria has been associated with dysbiosis and gastrointestinal inflammatory diseases in dogs and cats (Suchodolski et al., 2012; Minamoto et al., 2015). Typically, the relative abundance of Proteobacteria accounts for less than 5% of the overall microbial composition in cats (Moon et al., 2018). Although the relative abundance of Proteobacteria was slightly greater than commonly observed, no detrimental effects were observed in fecal quality, digestibility, or overall health status of the cats throughout the study.

In the current study, a less relative abundance of Fusobacteria and *Fusobaceriaceae* was also observed in cats fed GB-containing diets compared with cats fed 0% GB. In humans, the presence of Fusobacteria has been associated with obesity (Andoh et al., 2016). In a trial comparing the intestinal microbiotas of lean or obese humans, Fusobacteria was only present in obese subjects, with a relative abundance of 1.9% ± 4.2%, compared with 0% in lean subjects (Andoh et al., 2016). However, in cats, a similar association between Fusobacteria and obesity is absent. A previous study has demonstrated that *Fusobacteriaceae* was decreased in obese cats than in lean cats, resulting in a –7.2 log change (Kieler et al., 2019).

Alternatively, cats fed GB-containing diets had increased relative abundances of *Bifidobacteriaceae* and *Coriobacteriaceae* within the Actinobacteria phyla compared with 0% GB, both

of which are generally recognized as beneficial to host health. *Bifidobacterium* are often associated with health effects, such as increased immune function and competitive exclusion of pathogenic intestinal bacteria in animals (O'Callaghan and van Sinderen, 2016). Similarly, *Coriobacteriaceae* has been associated with overall host health by decreasing blood glucose levels and increasing cholesterol absorption in mice (Claus et al., 2011). Bermingham et al. (2013) found that cats fed a moderate-protein high-carbohydrate extruded diet had a greater relative abundance of *Coriobacteriaceae* (16.5%) compared with cats fed a high-protein low-carbohydrate wet diet (0.13%). These findings corroborate the current study as GB contain a significant fraction of carbohydrates, with dry and cooked GB having been reported to have 63% and 27% carbohydrates, respectively (Wallace et al., 2016). Cats fed GB-containing diets also had an increased relative abundance of the family *Prevotellaceae* compared with 0% GB, a family which is also positively associated with healthy animals. In humans, the relative abundance of *Prevotella* is greater in people consuming vegetable-rich diets, as it is a primary fiber degrader (de Filippis et al., 2019). *Prevotella* sp. have been associated with beneficial health effects such as increased SCFA production, decreasing anti-inflammatory cytokines, and increasing glucose metabolism in humans (de Filippis et al., 2019). Based on the microbial composition, and because there were not detrimental effects seen in any of the cats, it can be concluded that the consumption of GB favors bacteria more strongly associated with healthy animals.

Conclusions

All dietary treatments were well accepted by cats. Based on the serum chemistry and CBC data, all cats remained healthy throughout the study with values in normal reference ranges for healthy adult cats. No negative effects were observed in fecal quality or fecal fermentative end-products for any test diet. Although unexpected, the cooked GB diet was less in ATTD of macronutrients than the diets containing raw GB. Regardless, all diets were well-digested for all macronutrients. It can be concluded from this study that the inclusion of up to 30% raw GB in extruded feline diets can successfully be fed to adult cats without compromising nutritional adequacy or gastrointestinal tolerance.

Conflict of interest statement

L.M.R., F.H., S.L. R.Z., B.R.S., and M.R.C.G. have no conflict of interest to declare. J.M.H. and G.M.D. are employed by ADM, company that supported this research.

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