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Evaluation of graded levels of *Bacillus coagulans* GBI-30, 6086 on apparent nutrient digestibility, stool quality, and intestinal health indicators in healthy adult dogs

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Abstract

Bacillus coagulans GBI-30, 6086 is a commercially available spore-forming non-toxigenic microorganism approved for use in dog foods with high resiliency to stresses associated with commercial manufacturing. The objectives of this research were to examine the effect of *B. coagulans* on stool quality, nutrient digestibility, and intestinal health markers in healthy adult dogs. Extruded diets containing graded levels of *B. coagulans* applied either to the base ration before extrusion or to the exterior of the kibble as a topical coating after extrusion were randomly assigned to 10 individually housed adult beagle dogs (7 castrated males and 3 spayed females) of similar age (5.75 ± 0.23 yr) and body weight (12.3 ± 1.5 kg). The study was designed as a 5 × 5 replicated Latin square with 16-d adaptation followed by 5-d total fecal collection for each period. Five dietary treatments were formulated to deliver a dose of 0-, 6-, 7-, 8-, and 9-$\log_{10}$ colony-forming units (**CFU**) per dog per day for the control (**CON**), extruded *B. coagulans* (**PEX**), and low, moderate, and high *B. coagulans* coating levels (**PCL**, **PCM**, and **PCH**), respectively. Food-grade TiO_2 was added to all diets at a level of 0.4% to serve as an indigestible dietary marker for digestibility calculations. Data were analyzed using a mixed model through SAS (version 9.4, SAS Institute, Inc., Cary, NC) with treatment as a fixed effect and room (i.e., replicate), period, and dog(room) as random effects. Apparent total tract digestibility of organic matter, crude protein, crude fat, and gross energy calculated by the marker method were numerically greatest for dogs fed the 9-$\log_{10}$ dose treatment with increases ($P < 0.05$) observed in gross energy and organic matter digestibility compared with the negative control. No significant differences were observed in food intake, stool quality, fecal pH, fecal ammonia, fecal short-chain fatty acids, or branched-chain fatty acids for the extruded *B. coagulans* treatment (PEX) or the coated *B. coagulans* treatments (PCL, PCM, and PCH) compared with CON. These results suggest that *B. coagulans* has a favorable impact on nutrient digestibility and no apparent adverse effects when added to extruded diets at a daily intake level of up to 9-$\log_{10}$ CFU in healthy adult dogs.

Key words: canine, direct-fed microbials, gastrointestinal health

Abbreviations

ATTD	apparent total tract digestibility
BCFA	branched-chain fatty acids
BCS	body condition score
BW	body weight
CFU	colony-forming units
DFM	direct-fed microbials
ME	metabolizable energy
NFE	nitrogen-free extract
SCFA	short-chain fatty acids
TFC	total fecal collection

Introduction

Functional pet foods, such as those containing direct-fed microbials (**DFM**), are considered a key growth driver in the US$36.9 billion market of dog and cat foods in the United States (Di Cerbo et al., 2017; APPA, 2020). DFM products are purported to contain live (viable) microorganisms (bacteria and/or yeast) (FDA, 1995). Foods containing DFM are considered to be "functional" in that they offer enhanced health benefits beyond supplying essential nutrients when consumed on a regular basis (Hasler, 2000). In companion animal nutrition, DFM provide an opportunity to modify a pet's intestinal microbiota by introducing exogenous bacteria into the intestinal lumen with the goal of manipulating fecal consistency (German et al., 2010), improving intestinal health (Chrzastowska et al., 2009; Herstad et al., 2010), and modulating the immune system (Lee et al., 2003; Gonçalves et al., 2007; Jones and Versalovic, 2009; Pagnini et al., 2010). Researchers have also demonstrated that DFM may improve growth performance and nutrient digestibility in animals. This latter characteristic is largely attributed to the activities of microbial enzymes in the intestinal lumen, including α-amylase, α-galactosidases, cellulase, protease, and lipase (Keating et al., 1998; Tzortzis et al., 2004; Yu et al., 2008; Bajagai et al., 2016). The most widely used DFM for companion animals include non-sporulating lactic acid bacteria such as *Lactobacillus* spp., *Bifidobacteria* spp., and *Enterococcus* spp. (Jugan et al., 2017). These microorganisms have well-documented health-promoting potential. However, their survival during commercial processing, storage, and gastrointestinal transit is generally very poor (Weese and Arroyo, 2003; Champagne et al., 2005; Tripathi and Giri, 2014). Consequently, spore-forming strains such as members of the *Bacillus* genus have been explored as DFM candidates for food applications due to their enhanced tolerance to harsh environments associated with commercial processing and within the gastrointestinal tract (Cutting, 2011; Elshaghabee et al., 2017).

In one of the earliest reports of the use of *Bacillus* DFM for dogs, Biourge et al. (1998) observed that supplementing healthy adult German Shorthaired Pointer and German Shepherd dogs with *Bacillus cereus* CIP 5832 at a dose of 7.5×10^8 colony-forming units (**CFU**)·d^{-1} resulted in a slight improvement to the digestibility of dry matter, protein, lipid, and metabolizable energy (**ME**), although the differences were not significant compared with a non-DFM control. Recently, Schauf et al. (2019) evaluated *Bacillus subtilis* C-3102 supplemented in the diets of adult Beagle dogs at a dose of 3.47×10^8 CFU·d^{-1} and observed higher apparent digestibility of crude fat and nitrogen-free extract as well as a trend toward higher dry matter and organic matter digestibility compared with non-DFM-treated diets. Bastos et al. (2020) did not find improvements to nutrient digestibility in dogs in response to supplementation with *B. subtilis* and *Bacillus licheniformis* at a dose of 7.47×10^6 CFU·d^{-1}; however, improvements in fecal scores and a reduction in fecal biogenic amines were observed. These investigations demonstrate variability in the effectiveness of *Bacillus* DFM for improving nutrient digestibility and highlight the importance of identifying the minimal effective dosage of novel DFM strains.

Of interest in this research area is *Bacillus coagulans* GBI-30, 6086, a novel DFM that has been identified as having several properties that support its utility in thermally processed foods (Hyronimus et al., 2000; Keller et al., 2010; Konuray and Erginkaya, 2018). *Bacillus coagulans* is also reported to have proteolytic, amylytic, and lipolytic activity and thus has the potential to contribute to the digestion of nutrients (Keating et al., 1998; Kumar et al., 2005; Prihanto et al., 2013; Reyes-Mendez et al., 2015). However, the efficacy of isolated *B. coagulans* in the diets of dogs with regard to gastrointestinal health has not previously been reported in the peer-reviewed literature. We hypothesized that supplementation with a sufficient dose of *B. coagulans* would enhance apparent nutrient digestibility and positively influence the intestinal environment of dogs. Therefore, the objective of the current study was to evaluate the effects of graded doses of *B. coagulans* GBI-30, 6086 on nutrient digestibility and intestinal health indicators (stool quality, defecation frequency, fecal pH, and microbial fermentative metabolites, including short-chain fatty acids [**SCFA**], branched-chain fatty acids [**BCFA**], and ammonia) of healthy adult Beagle dogs.

Materials and Methods

The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee under protocol #4097 and the Institutional Biosafety Committee under protocol #1187 at Kansas State University (Manhattan, KS).

Experimental diets

A grain-free high-protein pet food ration was formulated to be nutritionally adequate for adult dogs (AAFCO, 2020a; Table 1). Five experimental diets were developed to contain no DFM (**CON**) or graded levels of *B. coagulans* blended into the base ration before extrusion (**PEX**) or as a topical coating to the exterior of the kibble at low (**PCL**), moderate (**PCM**), and high (**PCH**) concentrations. The DFM levels in the experimental treatments were selected to achieve a minimum of one $\log_{10}$ separation between doses with 0, 10^6, 10^7, 10^8, and 10^9 CFU consumed per dog per day (Table 2). Food-grade TiO_2 (FD&C Kowet High Purity Grade Titanium Dioxide; Sensient, St. Louis, MO) was included in the diets at a level of 0.4% as an indigestible marker to be used for digestibility calculations.

Raw materials for the base ration were purchased from and blended by a commercial mill (Fairview Mills, Bern, KS) with particle size reduced via hammer mill to pass through a 2-mm screen. *Bacillus coagulans* GBI-30, 6086 was obtained from the ingredient manufacturer (Kerry, Inc., Beloit, WI, USA) in powdered form at a concentration of 1.5×10^{10} CFU·g^{-1}. For treatment PEX, *B. coagulans* was blended into the base ration in a series of 1:5 ratio dilutions for 5 min in a paddle mixer until a minimum of 9 kg mixing batch was reached, and the inoculated ration was incorporated into a 227-kg batch and

Table 1. Ingredient composition and proximate analysis (as-is basis) of a grain-free pet food formula produced to evaluate the effect of *Bacillus coagulans* in an extruded dog kibble application

Formulation	Amount
Ingredients	
Chicken meal, %	34.64
Peas, dehydrated, %	20.00
Sweet potatoes, flaked, %	20.00
Chicken fat, %	8.50
Tapioca flour, %	5.00
Pea protein, %	5.00
Beet pulp, %	3.00
Digest flavoring, %	1.00
Potassium chloride, %	0.50
Salt, %	0.50
Dicalcium phosphate, %	0.50
Titanium dioxide[1], %	0.40
DL-Methionine, %	0.25
Choline chloride, %	0.20
Fish oil, %	0.20
Vitamin premix[2], %	0.15
Trace mineral premix[3], %	0.10
Natural antioxidant, %	0.07
B. coagulans (15B CFU·g^{-1})	*
Analyzed nutrient composition	
Moisture, %	4.92
Crude protein, %	34.90
Crude fat, %	15.60
Crude fiber, %	3.28
Ash, %	9.21
Nitrogen-free extract (NFE), %	32.09
ME[4], kcal·kg^{-1}	3,671

[1]Food-grade TiO_2 was used as an indigestible marker for digestibility calculations.
[2]Vitamin premix: pea fiber, calcium carbonate, vitamin E supplement, niacin supplement, thiamine mononitrate, D-calcium pantothenate, vitamin A supplement, sunflower oil, pyridoxine hydrochloride, riboflavin supplement, vitamin D3 supplement, biotin, vitamin B12 supplement, and folic acid.
[3]Trace mineral premix: zinc proteinate, calcium carbonate, zinc sulfate, iron proteinate, ferrous sulfate, copper proteinate, copper sulfate, manganese proteinate, sunflower oil, sodium selenite, manganous oxide, calcium iodate, and ethylenediamine dihydroiodide.
[4]ME of diets was calculated using modified Atwater factors of 3.5, 3.5, and 8.5 kcal/g for energy from crude protein, NFE, and crude fat, respectively (NRC, 2006).
*Each experimental diet contained differing levels of *B. coagulans* applied as reported in Table 2.

blended in a double-ribbon mixer for 5 min. The remaining four treatments were produced without DFM in the base ration before extrusion.

Diet production was carried out at the Bioprocessing Industrial and Value-Added Products (BIVAP) facility at Kansas State University. The dry ingredient blends were passed through a gravimetric feed hopper into a differential diameter cylinder preconditioner (Wenger Manufacturing Inc., Sabetha, KS). The preconditioned material was fed into a pilot-scale single-screw extruder (Single Screw X-20, Wenger Manufacturing Inc., Sabetha, KS). The extruder shaft speed, operational torque, steam flow, water flow, knife speed, and extruder zone temperatures were kept constant during the processing of all treatments and were recorded from the control panel output. Extruded kibbles were transported pneumatically from the extruder exit into a three-pass horizontal wire belt dryer (Wenger Manufacturing Inc., Model 4800, Sabetha, KS). The product was dried at 110 °C for 8 min and 12 min of retention time for the first and second conveyor passes, respectively, followed by 10 min for a third pass in the ambient cooler.

Coating of all diets was completed in the Pet Food Processing Laboratory at Kansas State University. Dried kibbles were sprayed with liquified chicken fat (American Dehydrated Foods, Inc., Springfield, MO) to reach a level of 8% of the batch by weight in a rotating barrel mixer. Following the application of chicken fat, flavor digest (AFB International, St. Charles, MO) was sifted onto the rotating kibbles over a 5-min period at a level of 1% of the batch by weight. For treatments PCL, PCM, and PCH, the flavor digest was inoculated with *B. coagulans* by blending the DFM powder with the flavor digest in a paddle mixer for 5 min 1 wk prior to coating. The coating sequence proceeded from CON, PEX, PCL, PCM, and PCH with a cleanout procedure utilized to minimize carry-over between treatments. Coated diets were packaged in multiwall bulk kraft paper bags with a polyethylene interior liner and stored in an indoor temperature-controlled location for the duration of the study.

Proximate analysis of the diets was completed at a commercial laboratory (Midwest Laboratories, Omaha, NE) to validate nutritional composition and estimate caloric density before initiating the animal feeding study. Enumeration of viable *B. coagulans* CFU was performed in triplicate at the Pet Food Microbiology & Toxicology Laboratory at Kansas State University following the procedures described in USP Monograph FCC 10, First Supplement for *B. coagulans* GBI-30, 6086 with modifications made to accommodate analysis of 50 g kibble samples.

Table 2. Application method and concentration of *Bacillus coagulans* in five experimental diet treatments (as-is basis)

	Treatment[1]				
Bacillus coagulans treatment	CON	PEX	PCL	PCM	PCH
Application method	None	Base ration	Coating	Coating	Coating
Formula inclusion[2], %	0.00	0.03	0.0002	0.002	0.02
Analyzed CFU·g^{-1} in ration[3]	0.00	4.58×10^6	0.00	0.00	0.00
Analyzed CFU·g^{-1} in diet[4]	0.00	1.06×10^4	5.92×10^4	6.86×10^5	6.84×10^6
Dose (CFU·dog^{-1}·d^{-1})[5]	0.00	2.12×10^6	1.18×10^7	1.37×10^8	1.37×10^9

[1]CON, control; PEX, DFM applied before extrusion; DFM applied as coating at low dose; PCM, DFM applied as coating at moderate dose; PCH, DFM applied as coating at high dose.
[2]Formula inclusion as percent of batch weight with *B. coagulans* added as a powder with 15 billion CFU·g^{-1} (Kerry, Inc., Beloit, WI, USA).
[3]*Bacillus coagulans* CFU counts analyzed in base ration before extrusion.
[4]*Bacillus coagulans* CFU counts analyzed in extruded, dried, and coated diets at time of feeding.
[5]Based on an expected average daily food intake of 200 g·dog^{-1}·d^{-1}

Animal feeding

The feeding trial was conducted at the Kansas State University Large Animal Research Center where 10 healthy adult Beagle dogs (3 spayed females and 7 castrated males) of similar age (5.75 ± 0.23 yr), body weight (**BW**; 12.3 ± 1.5 kg), and body condition score (**BCS**; 6.3 ± 1.2 on a 9-point scale, with 1 being very thin, 4 to 5 being ideal, and 9 being excessively obese; Laflamme, 1997) were individually housed in metabolic pens (1.83 × 1.20 m) equipped with an acrylic-coated mesh floor to allow for the separation of urine and feces. The animals were maintained as five dogs per room in a temperature-controlled (23 °C) modular building with automatic light timers set to 16:8 (L:D) h for each 24 h cycle. Food allowance was controlled by pre-weighing portions for each animal and feeding twice daily (at 0800 and 1700 hours) in equal portions at each meal. Orts were removed and weighed after 30 min of feeding. Initial food quantities on day 0 were determined by weighing the dogs and calculating the daily ME requirement for inactive lab kennel dogs ($95 \times BW_{kg}^{0.75}$; NRC, 2006). Throughout the study, BW was recorded weekly and caloric portioning was adjusted ± 5% for the subsequent week to maintain BW. BCS was recorded on the first and final day of the experiment. Water was provided for ad libitum consumption.

Sample collection

The study was conducted as a 5 × 5 replicated Latin square consisting of five periods with 16 d of acclimation to the diet followed by 5 d of total fecal collection (**TFC**) for a total duration of 105 d. Random assignment of experimental treatments to each of the 10 dogs was carried out with the aid of a Balanced Latin Square Designer Excel spreadsheet-based program (Kim and Stein, 2009). After the 16 d of acclimation, fecal samples were collected three times daily and scored on a 5-point scale wherein: 1 = liquid stool; 2 = soft consistency, unformed stool; 3 = very moist stool that retains shape; 4 = well-formed stool that does not leave residue when picked up; and 5 = very hard, dry pellets that crumble when pressed. A fecal score of 3.5 was considered ideal. After scoring, feces were collected in individual Whirl-pak bags, weighed, and stored frozen at –20 °C pending further analysis. During each 5-d collection period, one fresh fecal sample from each dog was immediately collected (within 15 min of excretion) and measured for pH by inserting a calibrated glass-electrode pH probe (FC240B, Hanna Instruments, Smithfield, RI) directly into the sample in triplicate. Six 2-g aliquots of the fresh sample were transferred into plastic microcentrifuge tubes and stored at –80 °C for pending analysis of SCFA, BCFA, and ammonia. After each collection period, bagged feces were thawed at room temperature, pooled by dog, and dried in a forced-air oven at 55 °C for up to 48 h, turning every 8 to 12 h. Diets and partially dried fecal samples were ground using a fixed blade laboratory mill (Retsch, type ZM200, Haan, Germany) fitted with a 0.5-mm screen and stored in lidded glass jars in preparation for chemical analysis.

Chemical analysis

All chemical analysis was performed in duplicate unless otherwise specified. The ground diets and partially dried feces were analyzed for dry matter, organic matter, and ash according to methods of the Association of Official Analytical Chemists (AOAC, 2019; methods 934.01 and 942.05). Crude protein content of the samples was determined by the Dumas combustion method (AOAC 990.03) using a nitrogen analyzer (FP928, LECO Corporation, Saint Joseph, MI). Crude fat was determined by acid hydrolysis (AOAC 954.02). Gross energy was determined by bomb calorimetry (Parr 6200 Calorimeter, Parr Instrument Company, Moline, IL). The titanium content in the samples was determined according to the colorimetric method described by Myers et al. (2004).

Two methods were utilized to estimate apparent total tract nutrient digestibility. The TFC method is widely used in animal nutrition research and requires the collection of all fecal material excreted by the animal. However, due to instances of occasional coprophagic behavior by the dogs and loss of sample residue during daily pen sanitation, this method may lead to an overestimation of apparent total tract nutrient digestibility compared with the use of an indigestible dietary marker (Alvarenga et al., 2019). Apparent total tract digestibility (**ATTD**) of dry matter, organic matter, crude protein, crude fat, ash, and gross energy was calculated according to the TFC (NRC, 2006) and marker methods (AAFCO, 2020b):

TFC method:

$$\text{Nutrient Digestibility, \%} = \frac{\text{nutrient consumed } (g \cdot d^{-1}) - \text{nutrient excreted } (g \cdot d^{-1})}{\text{nutrient consumed } (g \cdot d^{-1})} \times 100\% \quad (1)$$

Marker method:

$$\text{Nutrient Digestibility, \%} = 1 - \frac{\%\ \text{Nutrient in Feces} \times \%\ TiO_2 \text{ in Food}}{\%\ \text{Nutrient in Food} \times \%\ TiO_2 \text{ in Feces}} \times 100\% \quad (2)$$

Ammonia concentration in the fresh fecal samples was determined according to the colorimetric method described by Chaney and Marbach (1962). Fecal SCFA and BCFA contents were determined by gas-liquid chromatography (Erwin et al., 1961) using a capillary column (15 m × 0.35 mm internal diameter; 0.5 µm film thickness; Nukol column, Sulpeco, Bellefonte, PA; 7890A GC System, Agilent Technologies, Santa Clara, CA). The system was equipped using hydrogen as a carrier gas with a flow rate of 3.5 mL·min^{-1} and utilizing a 10:1 split ratio injector with injection size of 1 µL. A flame ionization detector was configured with nitrogen as the makeup gas with a flow rate of 25 mL·min^{-1} to clarify peak resolution. The detector and injector temperatures were set at 300 °C, and the initial oven temperature was set to 70 °C with a ramp rate of 20 °C·min^{-1} to 190 °C for a total run time of 20 min. The peak area of chromatograms was analyzed using integrative software (Agilent OpenLAB CDS version A.01.04, Agilent Technologies, Santa Clara, CA). SCFA (acetate, propionate, and butyrate) and BCFA (isobutyrate, valerate, and isovalerate) were quantified by comparing the sample peak area to a known standard of 10 mM concentration (Volatile Free Acid Mix, Sigma-Aldrich, St. Louis, MO) and correcting for fecal DM content.

Statistical analysis

Digestibility and intestinal health indicator data (fecal score, defecation frequency, fecal moisture content, fecal dry matter content, fecal pH, SCFA, BCFA, and ammonia) were analyzed using the MIXED procedure in SAS (version 9.4, SAS Institute, Inc., Cary, NC) with diet as a fixed effect and room (i.e., replicate), period, and dog(room) as random effects. Differences of least square means were assessed using the Tukey's post hoc test for multiple comparisons. Results were considered significant at $P < 0.05$ and trends were considered at $0.05 < P \leq 0.10$.

Results and Discussion

Dietary treatments

Bacillus coagulans was not detected in the control diet (indicated by the absence of colony growth) and reached the intended concentrations in the inoculated diets (Table 2). The diet was comprised of animal- and plant-origin ingredients selected to replicate a grain-free (containing peas, sweet potatoes, and tapioca) high-protein (>30% crude protein on an as-is basis) formula representative of products currently in the marketplace. Products of this design are frequently positioned at a higher cost to consumers, who view functional additives such as DFM as a valuable component of their pet's diet. Part of the working definition of DFM is based on the presence of an adequate number of viable cells necessary to impart a benefit to the host. However, the amount needed to produce observable effects may vary depending on the microorganism, processing method, mode of delivery, and the specific metabolic activities occurring (Minelli and Benini, 2008). The suggested dosage of *B. coagulans* GBI-30, 6086 for humans is between 1.0×10^8 and 3.0×10^9 CFU·d^{-1} with a no-observed-adverse-effect level of 1.36×10^{11} CFU·BW$_{kg}^{-1}$·d^{-1} (Endres et al., 2009, 2011). Studies with *Bacillus* spp. in dogs have investigated doses in the range of 6 to 9 log$_{10}$ CFU·d^{-1} (Schauf et al., 2019; Bastos et al., 2020), so our treatments were designed accordingly to deliver graded doses that spanned the range reported in the literature. Because the addition of functional additives such as DFM adds cost to products, identifying the minimum effective dose for a given strain is desirable.

The DFM application methods we selected were aimed at representing commercially relevant practices used in the pet food industry. Adding DFM as a topical coating to the exterior of the pet food kibble or treats has been used as a strategy to circumvent high-temperature processing and improve the viability of DFM microorganisms in shelf-stable cereal products (Biourge et al., 1998; González-Forte et al., 2014; Rodrigues et al., 2020). The application of liquified chicken fat before the flavor digest promotes greater adhesion of powder particles to the surface of the kibbles (Stemler, 2003). For treatments PCL, PCM, and PCH, pre-blending the DFM powder with the flavor digest for 5 min before the coating was used to facilitate the uniform distribution of *B. coagulans* throughout the diets (Alyami et al., 2017).

In addition to post-process applications, there is mounting evidence that thermally inactivated cells may still impart health benefits to the host (Hasegawa et al., 1994; Jensen et al., 2017; Piqué et al., 2019) and so incorporating DFM into the base ration before extrusion is becoming increasingly common. For treatment PEX, a 0.03% (w/w) inclusion of the DFM in the formula resulted in 4.58×10^6 CFU·g^{-1} of *B. coagulans* in the base ration before extrusion, and a final count of 1.06×10^4 CFU·g^{-1} remaining post-extrusion, drying, and coating, indicating a 2.6 log$_{10}$ loss in viability during processing. Although PEX was designated as the treatment with the lowest viable CFU count, the number of total cells (a mixture of viable and thermally inactivated spores) was comparable to PCH with 6.84×10^6 CFU·g^{-1}. To our knowledge, ours is the first study to evaluate both a heat-processed and direct coating application of DFM to dogs.

Animals

All 10 dogs remained healthy throughout the study as confirmed by veterinary staff. The mean BW of the dogs was 12.3 kg (range: 10.8 to 13.8 kg) at day 0 and 13.5 kg (range: 11.4 to 15.6 kg) at day 105. A paired *t*-test indicated that the average weight increase was significant (1.2 kg; $P = 0.0009$), while mean BCS remained the same (6.3 ± 1.2; $P = 0.1679$). Although food allowance was adjusted weekly to maintain BW, the weight gain observed might have been due to the use of an activity factor of 95 for ME calculations (NRC, 2006), which may have overestimated their energy expenditure compared with research dogs housed in kennels configured with exercise runs. Nevertheless, the dogs consumed a similar amount of food across all treatments with a mean consumption of 197 ± 4 g·d^{-1} ($P = 0.1364$; Table 3). Because the DFM dosage level was developed based on an expected 200 g·d^{-1} intake, this indicates that at least 98.5% of the target dose of *B. coagulans* CFU was consumed for each DFM-containing treatment. No differences in food intake were expected because the dietary treatments only differed with respect to the DFM application method and dose.

Fecal characteristics

Wet fecal output (range: 113 to 126 g·d^{-1}; $P = 0.1356$), fecal moisture content (range: 69.7% to 70.25%; $P = 0.6415$), defecations per day (range: 1.98 to 2.18; $P = 0.3041$), and fecal score (range: 3.68 to 3.77; $P = 0.5507$) did not differ between treatments (Table 3). In humans, supplementation with *B. coagulans* has demonstrated increased intestinal peristalsis and improved fecal scores in subjects with functional constipation (Minamida et al., 2015), as well as improved bowel movement frequency, shape, and color (Ara et al., 2002) when administered at a level 10^8 CFU·d^{-1}. Animal studies with *B. coagulans* have primarily focused on the prevention and treatment of the gastrointestinal tract disorders such as colitis and diarrhea (Sauter et al., 2006; Fitzpatrick et al., 2012; Paap et al., 2016; Wu et al., 2018). Because this cohort of dogs consisted of healthy adults with no prior history of intestinal disease, diarrhea, or constipation, it was expected

Table 3. Food intake and stool quality parameters of dogs fed diets with differing levels of *Bacillus coagulans*

	Treatment[1]						
Parameter	CON	PEX	PCL	PCM	PCH	SEM	P-value[2]
Food intake, g·d^{-1}	189.23	198.82	200.91	197.96	197.40	6.857	0.1364
Wet fecal output, g·d^{-1}	112.60	119.72	126.34	114.25	116.72	6.529	0.1356
Fecal moisture, %	70.25	69.98	70.30	69.71	70.19	0.645	0.6415
Fecal dry matter, %	29.75	30.02	29.70	30.29	29.81	0.645	0.6415
Defecations per day	2.00	2.12	2.18	2.02	1.98	0.116	0.3041
Fecal score	3.70	3.71	3.75	3.77	3.68	0.054	0.5507

[1]CON, control; PEX, DFM applied before extrusion; DFM applied as coating at low dose; PCM, DFM applied as coating at moderate dose; PCH, DFM applied as coating at high dose.
[2]P-value represents type 3 test of fixed effects for diet.

that stool quality, moisture content, and defecation frequency would be maintained throughout the study. These results are supported by the findings of other DFM strains fed to healthy adult dogs, with no observed changes compared with a non-supplemented control (Biourge et al., 1998; González-Ortiz et al., 2013; Kumar et al., 2016; Schauf et al., 2019).

Apparent total tract digestibility

The TFC data did not reveal differences in the ATTD of dry matter (range: 80.3% to 81.5%; $P = 0.3023$), organic matter (range: 84.8% to 86.1%; $P = 0.1656$), crude protein (range: 83.1% to 84.1%; $P = 0.3620$), or gross energy (range: 82.7% to 83.8%; $P = 0.1938$) between treatments (Table 4). However, ash digestibility varied widely across treatments (range: 31.6% to 45.5%; $P < 0.0001$) with PCH being significantly greater than PEX, PCL, and PCM but not different than CON. A trend was also observed in crude fat digestibility ($P = 0.0793$), with PEX lower compared with CON, but not different from PCL, PCM, or PCH. In general, the TiO_2 marker method produced digestibility data that were numerically lower than those obtained from the TFC method (Table 4). However, evidence of improvement to digestibility of dry matter (an increase of 2.73%; $P = 0.0044$), organic matter (an increase of 2.21%; $P = 0.0122$), and gross energy (an increase of 2.02%; $P = 0.0003$) was found as well as a trend (increase of 1.96%; $P = 0.0743$) in crude protein digestibility for PCH compared with CON. However, Tukey's post hoc test revealed that the protein digestibility means did not differ significantly.

Few studies have investigated the effects of DFM on the metabolism of minerals or trace elements or on bone health in dogs, but a 2.53% and 10.06% improvement in crude ash digestibility was reported in growing small breed and large breed puppies, respectively, supplemented with *Enterococcus faecium* at a dose of 5×10^8 CFU·dog^{-1}·d^{-1} (Gabinaitis et al., 2013). There is some evidence that the metabolites of microbial metabolism may influence bone accretion and increase the solubility of minerals by reducing luminal pH via SCFA production (Scholz-Ahrens et al., 2007; Sjögren et al., 2012; Mccabe et al., 2013). However, mineral absorption is controlled by a tightly regulated endocrine pathway (Kastenmayer et al., 2002), and differences in ash digestibility estimates are more likely to be related to the individual animal's mineral metabolism rather than a direct effect of the DFM supplementation.

The mode of action of *B. coagulans* supplementation that has been proposed on nutrient utilization includes the secretion of enzymes that promote the digestion of protein, carbohydrates, and lipids (α- and β-galactosidase, α-amylase, protease, and lipase; Cao et al., 2020). In addition to stability through production stresses and gastric transit, studies evaluating the activities of this strain using in vitro gastrointestinal models have reported improvements in the digestion of milk protein and lactose (Maathuis et al., 2010), plant proteins (Keller et al., 2017), and galactooligosaccharides in legumes and root vegetables (Nam et al., 2014). Germination of *B. coagulans* spores is stimulated by exposure to favorable conditions, including the presence of nutrient triggers (sugars, purine nucleosides, and amino acids; Casula and Cutting, 2002; Bressuire-Isoard et al., 2018), with up to 93% germination found in the upper small intestine in an in vitro model (Keller et al., 2019). This process is facilitated by heat activation after ingestion and subsequent release of enzymes that degrade the spore's protein-rich peptidoglycan outer coat (Setlow, 2014). In the process, nearby peptides are liberated into amino acids that can be absorbed by the host or utilized for energy by nearby microorganisms (Jäger et al., 2018). During proliferation, vegetative cells can then act directly on a variety of food substrates during luminal transit (Rowland et al., 2018).

In our study, we observed a small but significant improvement in apparent nutrient digestibility. It is possible that the limited magnitude of improvement to nutrient digestibility by DFM is because the exogenous enzymes introduced into the gut represent only a small fraction of the host-associated pancreatic enzymes. For example, the protease activity of *B. coagulans* PSB-07 is known to depend on intrinsic and extrinsic factors (i.e., temperature, pH, and carbon and nitrogen substrates), ranging from approximately 100 to 760 units/mL (Olajuyigbe and Ehiosun, 2013). In comparison, protease activity by the dog pancreas appears to adapt with the diet composition, ranging from 22,300 to 28,100 units/g of dietary protein (Behrman and Kare, 1969). There is also general agreement that DFM effects in vivo are species and often strain specific (Rowland et al., 2010). Pasupathy et al. (2001) reported an increase in daily weight gain in growing puppies when supplemented with *Lactobacillus*

Table 4. ATTD of dogs fed diets with differing levels of *Bacillus coagulans* estimated by TFC and TiO_2 as dietary marker methods

	Treatment[1]						
Nutrient	CON	PEX	PCL	PCM	PCH	SEM	P-value[2]
TFC method							
Dry matter, %	81.21	80.78	80.28	81.51	81.30	0.565	0.3023
Organic matter, %	85.36	85.36	84.78	86.06	85.41	0.476	0.1656
Crude protein, %	83.54	83.13	83.13	84.10	83.15	0.521	0.3620
Crude fat, %	92.55^{x}	90.74^{y}	91.71^{xy}	91.97^{xy}	91.87^{xy}	1.862	0.0783
Ash, %	41.67^{ab}	40.43^{ab}	38.79^{b}	31.60^{c}	45.05^{a}	1.527	<0.0001
Gross energy, %	83.81	82.84	83.41	82.66	83.54	0.476	0.1938
Marker method							
Dry matter, %	79.04^{b}	79.45^{b}	78.65^{b}	78.75^{b}	81.77^{a}	0.718	0.0034
Organic matter, %	83.67^{b}	84.36^{ab}	83.51^{b}	84.01^{ab}	85.79^{a}	0.553	0.0101
Crude protein, %	81.64	81.95	81.70	81.77	83.60	0.547	0.0743
Crude fat, %	91.69	90.28	90.96	90.85	91.69	2.097	0.1981
Ash, %	34.94^{b}	36.23^{b}	33.76^{b}	31.06^{c}	46.31^{a}	2.506	<0.0001
Gross energy, %	81.94^{ab}	81.66^{b}	82.03^{ab}	80.08^{b}	83.96^{a}	0.595	0.0002

[1]CON, control; PEX, DFM applied before extrusion; DFM applied as coating at low dose; PCM, DFM applied as coating at moderate dose; PCH, DFM applied as coating at high dose.
[2]P-value represents type 3 test of fixed effects for diet.
[a–c]Means within a row with different superscripts differ ($P < 0.05$).
[x,y]Means within a row with different superscripts differ ($P < 0.10$).

acidophilus, and Tyagi et al. (2014) observed that supplementing Labrador puppies with *Lactobacillus sporogenes* tended to increase the apparent dry matter digestibility, organic matter digestibility, and BW gain ($g \cdot d^{-1}$). Likewise, Gabinaitis et al. (2013) observed different levels of digestibility improvements in dry matter, organic matter, and crude fiber, between small, medium, and large breed puppies supplemented with *E. faecium* at a level of 5×10^8 CFU·d^{-1}. However, Sun et al. (2019) reported no difference in ATTD when supplementing dogs with *Weissella cibaria* at levels up to 1.5×10^{11} CFU·d^{-1} compared with a non-DFM control. These inconsistent results may be explained, in part, by the doses, dietary substrates, age of the animals, and length of administration in each experiment.

Fecal pH, SCFA, BCFA, and ammonia concentrations

In the current study, fecal pH (range: 5.33 to 5.49; $P = 0.4402$), fecal ammonia concentration (range: 94 to 107 µmol·g DM^{-1}; $P = 0.8414$), total SCFA (range: 171 to 197 µmol·g DM^{-1}; $P = 0.7924$), and total BCFA (range: 9 to 12 µmol·g DM^{-1}; $P = 0.5766$) were not different among treatments (Table 5). The relative proportions of acetate (range: 52.2% to 54.0%; $P = 0.6637$), propionate (range: 36.9% to 37.8%; $P = 0.9212$), butyrate (range: 9.1% to 10.7%; $P = 0.2327$), isovalerate (range: 44.7% to 52.2%; $P = 0.1199$), isobutyrate (range: 32.3% to 36.5%; $P = 0.2216$), and valerate (range: 14.8% to 23.0%; $P = 0.1224$) were also not different among treatments.

Many of the beneficial effects associated with DFM in companion animal diets are attributed to the production of microbial fermentation products. *Bacillus* spp., like other lactic acid bacteria, are thought to contribute to intestinal health by fermentative activities in the colon, including the production of SCFA, such as acetate, propionate, and butyrate (Wong et al., 2006), reduction of ammonia concentrations (Ara et al., 2002), and reduction in luminal pH, which aids in the competitive inhibition of pathogenic microorganisms residing within the intestinal tract (Topping and Clifton, 2001). The substrates available to bacteria influence the metabolic end products that are generated. For example, carbohydrate fermentation yields SCFA including acetate, propionate, and butyrate which can reduce the pH of the lumen (Wong et al., 2006), whereas protein fermentation yields the production of BCFA and ammonia (Herrin, 1940; Nery et al., 2012). Ammonia accumulation in the intestine has been shown to shorten the life of colonocytes (Lin and Visek, 1991) and has cytotoxic properties (Fung et al., 2013). Thus, an increase in SCFA and a decrease in pH, BCFA, and ammonia could be interpreted as a positive effect on intestinal health (Verbeke et al., 2015). However, we failed to observe any changes in these intestinal health indicators in the present study. Our results agree with previous studies with dogs, which have reported no changes in fecal pH after supplementation with other *Bacillus* organisms (Felix et al., 2010; Schauf et al., 2019). Felix et al. (2010) reasoned that changes in fecal pH may be difficult to detect when supplementing with *B. subtilis* compared with *Lactobacillus* spp. that produce a greater level of lactic acid. Among *Bacillus* strains, it has been reported that *B. coagulans* tends to have improved lactic acid production efficiency compared with *B. thermoamylovorans*, *B. licheniformis*, and *B. subtilis* in batch fermentation models (Poudel et al., 2016); however, most of the investigations have focused on the fermentation of a purified substrate (i.e., glucose) rather than a complex matrix such as pet food. In our study, the experimental diet contained a mixture of animal- and plant-origin materials, including 20% legume seeds. These are proportionately higher in oligosaccharides, including raffinose, stachyose, and verbascose, compared with cereal grains and tubers (Henry and Saini, 1989; Le Blay et al., 2003; Han and Baik, 2006). By supplying a high concentration of fermentable substrate, the fermentation activity of the resident microbiota may have overwhelmed the changes contributed by the DFM (Gänzle and Follador, 2012). Similar studies evaluating fermentative metabolites in dogs supplemented with inulin, fructooligosaccharides, mannanoligosaccharides, and xylooligosaccharides (Strickling et al., 2000; Flickinger et al., 2003; Barry et al., 2009) or mixtures of prebiotics (i.e., fermentable substrates) and DFM ("synbiotics") have demonstrated the ability to reduce fecal pH and ammonia and increase fecal SCFA (Swanson et al., 2002; Gagné et al., 2013; Patra, 2011; Strompfová et al., 2013; Markowiak and Ślizewska, 2018). Further investigation could be conducted in a low-oligosaccharide formula to determine if the DFM fermentation activity of *B. coagulans* may be detected.

DFM application methods

Overall, we did not find evidence to support that adding *B. coagulans* to the diet before extrusion improved nutrient digestibility or the intestinal health indicators measured in this study at a level of 1.06×10^4 CFU·g^{-1} compared with the non-DFM control. Adding DFM into a pre-blend before extrusion may offer manufacturing advantages, such as simplified raw material logistics, optimized mixing uniformity, and less need

Table 5. Fecal chemical analysis of dogs fed diets with differing levels of *Bacillus coagulans*

	Treatment[1]						
Parameter	CON	PEX	PCL	PCM	PCH	SEM	P-value[4]
Fecal pH	5.49	5.36	5.44	5.41	5.33	0.088	0.4402
Fecal NH_3, µmol·g^{-1} DM feces	99.99	105.49	107.12	104.61	94.30	9.496	0.8414
Total SCFA[2], µmol·g^{-1} DM feces	171.28	183.64	197.20	179.36	192.22	21.685	0.7924
Acetate, %	52.24	54.04	53.10	53.31	53.16	1.272	0.6637
Propionate, %	37.07	36.91	37.75	36.91	37.61	1.404	0.9212
Butyrate, %	10.69	9.05	9.16	9.78	9.23	0.788	0.2327
Total BCFA[3], µmol·g^{-1} DM feces	11.05	9.02	9.48	12.09	9.61	1.912	0.5766
Isovalerate, %	47.97	52.24	49.53	44.74	46.79	2.039	0.1199
Isobutyrate, %	33.93	32.44	35.67	32.27	36.52	1.630	0.2216
Valerate, %	18.10	15.33	14.81	22.98	16.68	2.442	0.1224

[1]CON, control; PEX, DFM applied before extrusion; DFM applied as coating at low dose; PCM, DFM applied as coating at moderate dose; PCH, DFM applied as coating at high dose.
[2]Total SCFA (acetate + propionate + butyrate); individual SCFA is expressed as a percent of total SCFA.
[3]Total BCFA (isobutyrate + isovalerate + valerate); individual BCFA is expressed as a percent of total BCFA.
[4]P-value represents type 3 test of fixed effects for diet.

for specialized coating processes. However, it would seem counterproductive to intentionally subject a DFM to a process that has been validated for microbial load reduction to improve food safety (Okelo et al., 2006; Bianchini et al., 2012). There are several mechanisms that have been proposed for the action of heat on vegetative cells, including damaging the outer cellular membrane and peptidoglycan wall, loss of cytoplasmic membrane integrity, and the denaturation of cellular organelles, RNA, DNA, and enzymes (Cebrián et al., 2017). Sporulated microorganisms are also susceptible to injury by heating, though the degree of heat resistance depends on several factors, including time and temperature of cooking, initial count of the spores, how the strains are isolated and prepared, and the composition of the matrix the spores are heated in (Likimani and Sofos, 1990; Li et al., 1993). Similar to our study, Biourge et al. (1998) evaluated sporulated *B. cereus* survival through extrusion and found a loss of greater than 99% of the initial CFU when incorporated into the food matrix prior to extrusion and up to 46% loss when applied as an exterior coating and stored for 12 mo. Depending on the microorganisms of concern and intensity of heat treatment, the goal is to render pathogenic cells injured beyond repair while preserving the viability of DFM. This application method requires an overage of CFU to be supplied in the base ration to account for processing losses, which may increase the cost of the formula. This also highlights the importance of validating CFU counts for different process conditions, DFM strains, and diet compositions.

For the coated treatments, our results support an improvement in dry matter, organic matter, and ash digestibility for dogs fed PCH compared with CON. These differences were not seen for PCL and PCM, however, which suggests that the minimum effective dose of *B. coagulans* for improving ATTD was 1.3×10^9 CFU·d^{-1}. It is possible that the difference in results observed between the extruded and coating application methods is related to the low number of viable cells in PEX, which was lower than PCL, PCM, and PCH. It stands to reason that vegetative cells must be present and active in the lumen of the gut in sufficient numbers to impart measurable changes in the digestion of organic material and production of fermentation products. Thus, it cannot be ruled out that applying *B. coagulans* at a higher dose before extrusion would not incite similar changes as coating the kibble with an equivalent number of CFU.

Conclusions

In summary, the current study provides evidence that supplementation with *B. coagulans* GBI-30, 6086 improved dry matter, organic matter, and gross energy digestibility of an extruded pet food. The dose at which significant positive treatment effects were observed in healthy adult dogs was 1.3×10^9 CFU consumed daily, with no adverse effects observed for fecal score, fecal moisture content, or number of defecations per day. Contrary to expectations, no differences were observed in fecal pH or concentration of ammonia, SCFA, or BCFA. This could possibly be due to the highest dose in this study not being sufficient to produce a measurable effect or may be related to the diet composition. Regarding application methods, subjecting the DFM to thermal treatment through extrusion and drying did not appear advantageous to the gastrointestinal health parameters measured in this study compared with a non-DFM control, whereas post-process application by coating at the highest dose yielded positive results.

It should also be pointed out that our research has two limitations. The first is in the comparison of extruded and coated DFM treatments, the dose of viable cells was lowest for the extruded treatment. Future research could evaluate a higher initial dose for an extruded treatment in comparison to an equivalent DFM level by coating. A second limitation is that we only investigated ATTD and intestinal health indicators in healthy adult dogs over a 21-d period. Consequently, these findings do not allow for extrapolation to other populations, such as growing puppies, aging dogs, or dogs with gastrointestinal disease.

Despite these limitations, we have demonstrated that *B. coagulans* GBI-30, 6086 has a promising role as a functional additive in extruded dog foods. Future investigations will be necessary to explore its utility in diets for other companion animal species as well as in alternative food formats.

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Conflict of interest statement

H.L.A. declares that she was directly employed (part time) for the duration of this study by Nulo, Inc. (Austin, TX), a company that develops and markets foods and treats for dogs and cats.

Literature Cited

AAFCO (Association of American Feed Control Officials). 2020a. AAFCO dog food nutrient profiles based on dry matter. In: *2020 Official publication*. Champaign (IL): Association of American Feed Control Officials Inc.; p. 158–159.

AAFCO (Association of American Feed Control Officials). 2020b. AAFCO dog and cat food metabolizable energy protocols. In: *2020 Official publication*. Champaign (IL): Association of American Feed Control Officials Inc.; p. 199–201.

Alvarenga, I. C., C. G. Aldrich, and Z. Ou. 2019. Comparison of four digestibility markers to estimate fecal output of dogs. *J. Anim. Sci.* **97**:1036–1041. doi:10.1093/jas/skz020

Alyami, H., E. Dahmash, J. Bowen, and A. R. Mohammed. 2017. An investigation into the effects of excipient particle size, blending techniques and processing parameters on the homogeneity and content uniformity of a blend containing low-dose model drug. *PLoS One*. **12**:e0178772. doi:10.1371/journal.pone.0178772

American Pet Products Association (APPA). 2020. Pet industry market size & ownership statistics. Stamford (CT): American Pet Products Association. Available from http://www.americanpetproducts.org/press_industrytrends.asp [accessed March 5, 2021].

AOAC. 2019. *Official methods of analysis*. 21st ed. Arlington (VA): Association of Official Analytical Chemists.

Ara, K., S. Meguro, T. Hase, I. Tokimitsu, K. Otsuji, S. Kawai, S. Ito, and H. Iino. 2002. Effect of spore-bearing lactic acid-forming bacteria (*Bacillus coagulans* SANK 70258) administration on the intestinal environment, defecation frequency, fecal characteristics and dermal characteristics in humans and rats. *Microb. Ecol. Health Dis.* **14**:4–13. doi:10.1080/089106002760002694

Bajagai, Y., A. Klieve, P. Dart, and W. Bryden. 2016. *Probiotics in animal nutrition – production, impact, and regulation*. Rome: FAO (Food and Agriculture Organization of the United Nations). Animal Production and Health Paper No.: 179.

Barry, K. A., D. C. Hernot, I. S. Middelbos, C. Francis, B. Dunsford, K. S. Swanson, and G. C. Fahey Jr. 2009. Low-level fructan supplementation of dogs enhances nutrient digestion and modifies stool metabolite concentrations, but does not alter fecal microbiota populations. *J. Anim. Sci.* **87**:3244–3252. doi:10.2527/jas.2008-1659

Bastos, T. S., D. C. de Lima, C. M. M. Souza, A. Maiorka, S. G. de Oliveira, L. C. Bittencourt, and A. P. Félix. 2020. *Bacillus subtilis* and *Bacillus licheniformis* reduce faecal protein catabolites concentration and odour in dogs. *BMC Vet. Res.* **16**:116. doi:10.1186/s12917-020-02321-7

Behrman, H. R., and M. R. Kare. 1969. Adaptation of canine pancreatic enzymes to diet composition. *J. Physiol.* **205**:667–676. doi:10.1113/jphysiol.1969.sp008989

Bianchini, A., J. Stratton, S. Weier, T. Hartter, B. Plattner, G. Rokey, G. Hertzel, L. Gompa, B. Martinez, and A. M. Eskridge. 2012. Validation of extrusion as a killing step for *Enterococcus faecium* in a balanced carbohydrate-protein meal by using a response surface design. *J. Food Prot.* **75**:1646–1653. doi:10.4315/0362-028X.JFP-12-085

Biourge, V., C. Vallet, A. Levesque, R. Sergheraert, S. Chevalier, and J. L. Roberton. 1998. The use of probiotics in the diet of dogs. *J. Nutr.* **128**(12 Suppl):2730S–2732S. doi:10.1093/jn/128.12.2730S

Bressuire-Isoard, C., V. Broussolle, and F. Carlin. 2018. Sporulation environment influences spore properties in *Bacillus*: evidence and insights on underlying molecular and physiological mechanisms. *FEMS Microbiol. Rev.* **42**:614–626. doi:10.1093/femsre/fuy021

Cao, J., Z. Yu, W. Liu, J. Zhao, H. Zhang, Q. Zhai, and W. Chen. 2020. Probiotic characteristics of *Bacillus coagulans* and associated implications for human health and diseases. *J. Funct. Foods.* **64**:103643. doi:10.1016/j.jff.2019.103643

Casula, G., and S. M. Cutting. 2002. *Bacillus* probiotics: spore germination in the gastrointestinal tract. *Appl. Environ. Microbiol.* **68**:2344–2352. doi:10.1128/aem.68.5.2344-2352.2002

Cebrián, G., S. Condón, and P. Mañas. 2017. Physiology of the inactivation of vegetative bacteria by thermal treatments: mode of action, influence of environmental factors and inactivation kinetics. *Foods*. **6**:107. doi:10.3390/foods6120107

Champagne, C. P., N. J. Gardner, and D. Roy. 2005. Challenges in the addition of probiotic cultures to foods. *Crit. Rev. Food Sci. Nutr.* **45**:61–84. doi:10.1080/10408690590900144

Chaney, A. L., and E. P. Marbach. 1962. Modified reagents for determination of urea and ammonia. *Clin. Chem.* **8**:130–132. doi:10.1093/clinchem/8.2.130

Chrzastowska, M., M. Kander, and A. Depta. 2009. Prospects for the use of probiotic bacteria in the treatment of gastrointestinal diseases in dogs. *Pol. J. Vet. Sci.* **12**:279–284.

Cutting, S. M. 2011. *Bacillus* probiotics. *Food Microbiol.* **28**:214–220. doi:10.1016/j.fm.2010.03.007

Di Cerbo, A., J. C. Morales-Medina, B. Palmieri, F. Pezzuto, R. Cocco, G. Flores, and T. Iannitti. 2017. Functional foods in pet nutrition: focus on dogs and cats. *Res. Vet. Sci.* **112**:161–166. doi:10.1016/j.rvsc.2017.03.020

Elshaghabee, F. M. F., N. Rokana, R. D. Gulhane, C. Sharma, and H. Panwar. 2017. *Bacillus* as potential probiotics: status, concerns, and future perspectives. *Front. Microbiol.* **8**:1490. doi:10.3389/fmicb.2017.01490

Endres, J. R., A. Clewell, K. A. Jade, T. Farber, J. Hauswirth, and A. G. Schauss. 2009. Safety assessment of a proprietary preparation of a novel probiotic, *Bacillus coagulans*, as a food ingredient. *Food Chem. Toxicol.* **47**:1231–1238. doi:10.1016/j.fct.2009.02.018

Endres, J. R., I. Qureshi, T. Farber, J. Hauswirth, G. Hirka, I. Pasics, and A. G. Schauss. 2011. One-year chronic oral toxicity with combined reproduction toxicity study of a novel probiotic, *Bacillus coagulans*, as a food ingredient. *Food Chem. Toxicol.* **49**:1174–1182. doi:10.1016/j.fct.2011.02.012

Erwin, E. S., G. J. Marco, and E. M. Emery. 1961. Volatile fatty acid analyses of blood and rumen fluid by gas chromatography. *J. Dairy Sci.* **44**:1768–1771. doi:10.3168/jds.S0022-0302(61)89956-6

FDA. 1995. Compliance policy guide sec. 689.100 direct-fed microbial products. Available from https://www.fda.gov/regulatory-information/search-fda-guidance-documents/cpg-sec-689100-direct-fed-microbial-products [accessed March 5, 2021].

Felix, A. P., M. V. T. Netto, F. Y. Murakami, C. B. M. de Brito, S. G. de Oliveira, and A. Maiorka. 2010. Digestibility and fecal characteristics of dogs fed with *Bacillus subtilis* in diet. *Cienc. Rural.* **40**:2169–2173. doi:10.1590/S0103-84782010005000166

Fitzpatrick, L. R., J. S. Small, W. H. Greene, K. D. Karpa, S. Farmer, and D. Keller. 2012. *Bacillus coagulans* GBI-30, 6086 limits the recurrence of *Clostridium difficile*-induced colitis following vancomycin withdrawal in mice. *Gut Pathog.* **4**:13. doi:10.1186/1757-4749-4-13

Flickinger, E. A., E. M. Schreijen, A. R. Patil, H. S. Hussein, C. M. Grieshop, N. R. Merchen, and G. C. Fahey Jr. 2003. Nutrient digestibilities, microbial populations, and protein catabolites as affected by fructan supplementation of dog diets. *J. Anim. Sci.* **81**:2008–2018. doi:10.2527/2003.8182008x

Fung, K. Y., C. C. Ooi, M. H. Zucker, T. Lockett, D. B. Williams, L. J. Cosgrove, and D. L. Topping. 2013. Colorectal carcinogenesis: a cellular response to sustained risk environment. *Int. J. Mol. Sci.* **14**:13525–13541. doi:10.3390/ijms140713525

Gabinaitis, P., A. Januškevičius, and R. Stankevičius. 2013. Effect of probiotic preparations on the growth and assimilation of nutritive substances in different breeds of puppies. *Vet. ir Zootech.* **61**:14–21.

Gagné, J. W., J. J. Wakshlag, K. W. Simpson, S. E. Dowd, S. Latchman, D. A. Brown, K. Brown, K. S. Swanson, and G. C. Fahey Jr. 2013. Effects of a synbiotic on fecal quality, short-chain fatty acid concentrations, and the microbiome of healthy sled dogs. *BMC Vet. Res.* **9**:246. doi:10.1186/1746-6148-9-246

Gänzle, M. G., and R. Follador. 2012. Metabolism of oligosaccharides and starch in lactobacilli: a review. *Front. Microbiol.* **3**:1–15. doi:10.3389/fmicb.2012.00340

German, A. J., L. J. Halladay, and P. J. Noble. 2010. First-choice therapy for dogs presenting with diarrhoea in clinical practice. *Vet. Rec.* **167**:810–814. doi:10.1136/vr.c4090

Gonçalves, M., R. P. Maluta, F. Dahlke, A. Maiorka, and F. A. Ávila. 2007. Evaluation of immunostimulant capacity and stability of thermo stable probiotic used in the diet of dogs. *Arch. Vet. Sci.* **12**:25.

González-Forte, L., E. Bruno, and M. Martino. 2014. Application of coating on dog biscuits for extended survival of probiotic bacteria. *Anim. Feed Sci. Technol.* **195**:76–84. doi:10.1016/j.anifeedsci.2014.05.015

González-Ortiz, G., L. Castillejos, J. J. Mallo, M. Àngels Calvo-Torras, and M. Dolores Baucells. 2013. Effects of dietary supplementation of *Bacillus amyloliquefaciens* CECT 5940 and *Enterococcus faecium* CECT 4515 in adult healthy dogs. *Arch. Anim. Nutr.* **67**:406–415. doi:10.1080/1745039X.2013.830517

Han, I. H., and B. K. Baik. 2006. Oligosaccharide content and composition of legumes and their reduction by soaking, cooking, ultrasound, and high hydrostatic pressure. *Cereal Chem.* **83**:428–433. doi:10.1094/CC-83-0428

Hasegawa, T., Y. Ogura, T. Inomata, Y. Murakumo, T. Yamamoto, S. Abe, and H. Yamaguchi. 1994. Effect of orally administered heat-killed *Enterococcus faecalis* FK-23 preparation on leukopenia in mice treated with cyclophosphamide. *J. Vet. Med. Sci.* **56**:1203–1206. doi:10.1292/jvms.56.1203

Hasler, C. M.. 2000. Functional foods: benefits, concerns and challenges—a position paper from the American Council on Science and Health. *J. Nutr.* **132**:3772–3781. doi:10.1093/jn/132.12.3772

Henry, R. J., and H. Saini. 1989. Characterization of cereal sugars and oligosaccharides. *Cereal Chem*. **66**:362–365.
Herrin, R. C. 1940. The secretion of ammonia by the small intestine of the dog. *Am. J. Physiol. Content*. **129**:146–154. doi:10.1152/ajplegacy.1940.129.1.146
Herstad, H. K., B. B. Nesheim, T. L'Abée-Lund, S. Larsen, and E. Skancke. 2010. Effects of a probiotic intervention in acute canine gastroenteritis – a controlled clinical trial. *J. Small Anim. Pract*. **51**:34–38. doi:10.1111/j.1748-5827.2009.00853.x
Hyronimus, B., C. Le Marrec, A. H. Sassi, and A. Deschamps. 2000. Acid and bile tolerance of spore-forming lactic acid bacteria. *Int. J. Food Microbiol*. **61**:193–197. doi:10.1016/s0168-1605(00)00366-4
Jäger, R., M. Purpura, S. Farmer, H. A. Cash, and D. Keller. 2018. Probiotic *Bacillus coagulans* GBI-30, 6086 improves protein absorption and utilization. *Probiotics Antimicrob. Proteins* **10**:611–615. doi:10.1007/s12602-017-9354-y
Jensen, G. S., H. A. Cash, S. Farmer, and D. Keller. 2017. Inactivated probiotic *Bacillus coagulans* GBI-30 induces complex immune activating, anti-inflammatory, and regenerative markers in vitro. *J. Inflamm. Res*. **10**:107–117. doi:10.2147/JIR.S141660
Jones, S. E., and J. Versalovic. 2009. Probiotic *Lactobacillus reuteri* biofilms produce antimicrobial and anti-inflammatory factors. *BMC Microbiol*. **9**:35. doi:10.1186/1471-2180-9-35
Jugan, M. C., A. J. Rudinsky, V. J. Parker, and C. Gilor. 2017. Use of probiotics in small animal veterinary medicine. *J. Am. Vet. Med. Assoc*. **250**:519–528. doi:10.2460/javma.250.5.519
Kastenmayer, P., G. L. Czarnecki-Maulden, and W. King. 2002. Mineral and trace element absorption from dry dog food by dogs, determined using stable isotopes. *J. Nutr*. **132**(6 Suppl 2):1670S–1672S. doi:10.1093/jn/132.6.1670S
Keating, L., C. Kelly, and W. Fogarty. 1998. Mechanism of action and the substrate dependent pH maximum shift of the α-amylase of *Bacillus coagulans*. *Carbohydr. Res*. **309**:311–318. doi: 10.1016/S0008-6215(98)00143-8
Keller, D., S. Farmer, A. McCartney, and G. Gibson. 2010. *Bacillus coagulans* as a probiotic. *Food Sci. Technol. Bull. Funct. Foods* **7**:103–109. doi:10.1616/1476–2137.16015
Keller, D., R. Van Dinter, H. Cash, S. Farmer, and K. Venema. 2017. *Bacillus coagulans* GBI-30, 6086 increases plant protein digestion in a dynamic, computer-controlled in vitro model of the small intestine (TIM-1). *Benef. Microbes* **8**:491–496. doi:10.3920/BM2016.0196
Keller, D., S. Verbruggen, H. Cash, S. Farmer, and K. Venema. 2019. Spores of *Bacillus coagulans* GBI-30, 6086 show high germination, survival and enzyme activity in a dynamic, computer-controlled in vitro model of the gastrointestinal tract. *Benef. Microbes* **10**:77–87. doi:10.3920/BM2018.0037
Kim, B. G., and H. H. Stein. 2009. A spreadsheet program for making a balanced Latin Square design. *Rev. Colomb. de Cienc. Pecu*. **22**:591–596.
Konuray, G., and Z. Erginkaya. 2018. Potential use of *Bacillus coagulans* in the food industry. *Foods* **7**:92. doi:10.3390/foods7060092
Kumar, S., K. Kikon, A. Upadhyay, S. S. Kanwar, and R. Gupta. 2005. Production, purification, and characterization of lipase from thermophilic and alkaliphilic *Bacillus coagulans* BTS-3. *Protein Expr. Purif*. **41**:38–44. doi:10.1016/j.pep.2004.12.010
Kumar, S., A. K. Pattanaik, T. Jose, S. Sharma, and S. E. Jadhav. 2016. Temporal changes in the hindgut health markers of Labrador dogs in response to a canine-origin probiotic *Lactobacillus johnsonii*. *Anim. Nutr. Feed Technol*. **16**:251–270. doi:10.5958/0974-181X.2016.00022.6
Laflamme, D. 1997. Development and validation of a body condition score system for dogs. *Canine Pract*. **22**:10–15.
Le Blay, G. M., C. D. Michel, H. M. Blottière, and C. J. Cherbut. 2003. Raw potato starch and short-chain fructo-oligosaccharides affect the composition and metabolic activity of rat intestinal microbiota differently depending on the caecocolonic segment involved. *J. Appl. Microbiol*. **94**:312–320. doi:10.1046/j.1365-2672.2003.01836.x
Lee, Y. K., K. Y. Puong, A. C. Ouwehand, and S. Salminen. 2003. Displacement of bacterial pathogens from mucus and Caco-2 cell surface by lactobacilli. *J. Med. Microbiol*. **52**(Pt 10):925–930. doi:10.1099/jmm.0.05009-0
Li, Y., F. Hsieh, M. Fields, H. Huff, and S. Badding. 1993. Thermal inactivation and injury of *Clostridium sporogenes* spores during extrusion of mechanically deboned turkey mixed with white corn flour. *J. Food Process. Preserv*. **17**:391–403. doi:10.1111/j.1745-4549.1993.tb00739.x
Likimani, T. A., and J. N. Sofos. 1990. Bacterial spore injury during extrusion cooking of corn/soybean mixtures. *Int. J. Food Microbiol*. **11**:243–249. doi:10.1016/0168-1605(90)90017-y
Lin, H. C., and W. J. Visek. 1991. Colon mucosal cell damage by ammonia in rats. *J. Nutr*. **121**:887–893. doi:10.1093/jn/121.6.887
Maathuis, A. J., D. Keller, and S. Farmer. 2010. Survival and metabolic activity of the GanedenBC30 strain of *Bacillus coagulans* in a dynamic in vitro model of the stomach and small intestine. *Benef. Microbes* **1**:31–36. doi:10.3920/BM2009.0009
Markowiak, P., and K. Śliżewska. 2018. The role of probiotics, prebiotics and synbiotics in animal nutrition. *Gut Pathog*. **10**:21. doi:10.1186/s13099-018-0250-0
McCabe, L. R., R. Irwin, L. Schaefer, and R. A. Britton. 2013. Probiotic use decreases intestinal inflammation and increases bone density in healthy male but not female mice. *J. Cell. Physiol*. **228**:1793–1798. doi:10.1002/jcp.24340
Minamida, K., M. Nishimura, K. Miwa, and J. Nishihira. 2015. Effects of dietary fiber with *Bacillus coagulans* lilac-01 on bowel movement and fecal properties of healthy volunteers with a tendency for constipation. *Biosci. Biotechnol. Biochem*. **79**:300–306. doi:10.1080/09168451.2014.972331
Minelli, E. B., and A. Benini. 2008. Relationship between number of bacteria and their probiotic effects. *Microb. Ecol. Health Dis*. **20**:180–183. doi:10.1080/08910600802408095
Myers, W. D., P. A. Ludden, V. Nayigihugu, and B. W. Hess. 2004. Technical Note: A procedure for the preparation and quantitative analysis of samples for titanium dioxide. *J. Anim. Sci*. **82**:179–183. doi:10.2527/2004.821179x
Nam, K. H., M. S. Jang, H. Y. Park, and E. Koneva. 2014. Biochemical characterization of α-galactosidase-producing thermophilic *Bacillus coagulans* KM-1. *Korean J. Fish. Aquat. Sci*. **47**:516–521. doi:10.5657/kfas.2014.0516
Nery, J., R. Goudez, V. Biourge, C. Tournier, V. Leray, L. Martin, C. Thorin, P. Nguyen, and H. Dumon. 2012. Influence of dietary protein content and source on colonic fermentative activity in dogs differing in body size and digestive tolerance. *J. Anim. Sci*. **90**:2570–2580. doi:10.2527/jas.2011-4112
NRC. 2006. *Nutrient requirements of dogs and cats*. Washington (DC): National Academies Press.
Okelo, P. O., D. D. Wagner, L. E. Carr, F. W. Wheaton, L. W. Douglass, and S. W. Joseph. 2006. Optimization of extrusion conditions for elimination of mesophilic bacteria during thermal processing of animal feed mash. *Anim. Feed Sci. Technol*. **129**:116–137. doi:10.1016/j.anifeedsci.2005.12.011
Olajuyigbe, F., and K. Ehiosun. 2013. Production of thermostable and organic solvent tolerant alkaline protease from *Bacillus coagulans* PSB- 07 under different submerged fermentation conditions. *Afr. J. Biotechnol*. **12**:3341–3350. doi:10.5897/AJB2013.12219
Paap, P. M., J. H. Van der Laak, J. I. Smit, N. Nakamura, and A. C. Beynen. 2016. Administration of *Bacillus subtilis* C-3102 (Calsporin®) may improve feces consistency in dogs with chronic diarrhea. *Res. Opin. Anim. Vet. Sci*. **6**:256–260. doi:10.20490/roavs/16-043
Pagnini, C., R. Saeed, G. Bamias, K. O. Arseneau, T. T. Pizarro, and F. Cominelli. 2010. Probiotics promote gut health through stimulation of epithelial innate immunity. *Proc. Natl. Acad. Sci. U. S. A*. **107**:454–459. doi:10.1073/pnas.0910307107

Pasupathy, K., A. Sahoo, and N. N. Pathak. 2001. Effect of lactobacillus supplementation on growth and nutrient utilization in mongrel pups. *Arch. Tierernahr.* **55**:243–253. doi:10.1080/17450390109386195

Patra, A. K. 2011. Responses of feeding prebiotics on nutrient digestibility, faecal microbiota composition and short-chain fatty acid concentrations in dogs: a meta-analysis. *Animal* **5**:1743–1750. doi:10.1017/S1751731111000887

Piqué, N., M. Berlanga, and D. Miñana-Galbis. 2019. Health benefits of heat-killed (tyndalized) probiotics: an overview. *Int. J. Mol. Sci.* **20**:1–30. doi:10.3390/ijms20102534

Poudel, P., Y. Tashiro, and K. Sakai. 2016. New application of *Bacillus* strains for optically pure L-lactic acid production: general overview and future prospects. *Biosci. Biotechnol. Biochem.* **80**:642–654. doi:10.1080/09168451.2015.1095069

Prihanto, A., A. Darius, and M. Firdaus 2013. Proteolytic and fibrinolytic activities of halophilic lactic acid bacteria from two Indonesian fermented foods. *J. Microbiol. Biotechnol. Food Sci.* **2**:2291–2293.

Reyes-Mendez, A. I., C. Figueroa-Hernandez, G. Melgar-Lalanne, H. Hernandez-Sanchez, G. Dávila-Ortiz, and C. Jiménez-Martínez. 2015. Production of calcium-and iron-binding peptides by probiotic strains of *Bacillus subtilis*, *B. clausii* and *Bacillus coagulans* GBI-30. *Rev. Mex. Ing. Quim.* **14**:1–9.

Rodrigues, B. M., P. M. Olivo, M. P. Osmari, R. S. Vasconcellos, L. B. Ribeiro, F. I. Bankuti, and M. S. S. Pozza. 2020. Microencapsulation of probiotic strains by lyophilization is efficient in maintaining the viability of microorganisms and modulation of fecal microbiota in cats. *Int. J. Microbiol.* **2020**:1293481. doi:10.1155/2020/1293481

Rowland, I., L. Capurso, K. Collins, J. Cummings, N. Delzenne, O. Goulet, F. Guarner, P. Marteau, and R. Meier. 2010. Current level of consensus on probiotic science–report of an expert meeting–London, 23 November 2009. *Gut Microbes* **1**:436–439. doi:10.4161/gmic.1.6.13610

Rowland, I., G. Gibson, A. Heinken, K. Scott, J. Swann, I. Thiele, and K. Tuohy. 2018. Gut microbiota functions: metabolism of nutrients and other food components. *Eur. J. Nutr.* **57**:1–24. doi:10.1007/s00394-017-1445-8

Sauter, S. N., J. Benyacoub, K. Allenspach, F. Gaschen, E. Ontsouka, G. Reuteler, C. Cavadini, R. Knorr, and J. W. Blum. 2006. Effects of probiotic bacteria in dogs with food responsive diarrhoea treated with an elimination diet. *J. Anim. Physiol. Anim. Nutr. (Berl).* **90**:269–277. doi:10.1111/j.1439-0396.2005.00595.x

Schauf, S., N. Nakamura, and C. Castrillo. 2019. Effect of Calsporin® (*Bacillus subtilis* C-3102) addition to the diet on faecal quality and nutrient digestibility in healthy adult dogs. *J. Appl. Anim. Nutr.* **7**:1–9. doi:10.1017/jan.2019.2

Scholz-Ahrens, K. E., P. Ade, B. Marten, P. Weber, W. Timm, Y. Açil, C. C. Glüer, and J. Schrezenmeir. 2007. Prebiotics, probiotics, and synbiotics affect mineral absorption, bone mineral content, and bone structure. *J. Nutr.* **137**(3 Suppl 2):838S–846S. doi:10.1093/jn/137.3.838S

Setlow, P. 2014. Germination of spores of *Bacillus* species: what we know and do not know. *J. Bacteriol.* **196**:1297–1305. doi:10.1128/JB.01455-13

Sjögren, K., C. Engdahl, P. Henning, U. H. Lerner, V. Tremaroli, M. K. Lagerquist, F. Bäckhed, and C. Ohlsson. 2012. The gut microbiota regulates bone mass in mice. *J. Bone Miner. Res.* **27**:1357–1367. doi:10.1002/jbmr.1588

Stemler, T. 2003. Application systems. In: Kvamme, J. L., and T. D. Phillips, editors. *Petfood technology*. Mt. Morris (IL): Watt Publishing; p. 373.

Strickling, J. A., D. L. Harmon, K. A. Dawson, and K. L. Gross. 2000. Evaluation of oligosaccharide addition to dog diets: influences on nutrient digestion and microbial populations. *Anim. Feed Sci. Technol.* **86**:205–219. doi:10.1016/S0377-8401(00)00175-9

Strompfová, V., A. Lauková, and D. Cilik. 2013. Synbiotic administration of canine-derived strain *Lactobacillus fermentum* CCM 7421 and inulin to healthy dogs. *Can. J. Microbiol.* **59**:347–352. doi:10.1139/cjm-2012-0472

Sun, H. Y., K. P. Kim, C. H. Bae, A. J. Choi, H. D. Paik, and I. H. Kim. 2019. Evaluation of *Weissella cibaria* JW15 probiotic derived from fermented Korean vegetable product supplementation in diet on performance characteristics in adult beagle dog. *Animals* **9**:1–8. doi:10.3390/ani9080581

Swanson, K. S., C. M. Grieshop, E. A. Flickinger, L. L. Bauer, J. Chow, B. W. Wolf, K. A. Garleb, and G. C. Fahey Jr. 2002. Fructooligosaccharides and *Lactobacillus acidophilus* modify gut microbial populations, total tract nutrient digestibilities and fecal protein catabolite concentrations in healthy adult dogs. *J. Nutr.* **132**:3721–3731. doi:10.1093/jn/132.12.3721

Topping, D. L., and P. M. Clifton. 2001. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiol. Rev.* **81**:1031–1064. doi:10.1152/physrev.2001.81.3.1031

Tripathi, M. K., and S. K. Giri. 2014. Probiotic functional foods: survival of probiotics during processing and storage. *J. Funct. Foods* **9**:225–241. doi:10.1016/j.jff.2014.04.030

Tyagi, N., C. S. Ahuja, J. S. Lamba, M. Chandra, and S. S. Sikka. 2014. Effect of different probiotic supplements on the growth performance, nutrient digestibility and plasma metabolic profile of Labrador pups. *Indian J. Anim. Res.* **48**:362–365. doi:10.5958/0976-0555.2014.00456.7

Tzortzis, G., A. K. Goulas, M. L. Baillon, G. R. Gibson, and R. A. Rastall. 2004. In vitro evaluation of the fermentation properties of galactooligosaccharides synthesised by alpha-galactosidase from *Lactobacillus reuteri*. *Appl. Microbiol. Biotechnol.* **64**:106–111. doi:10.1007/s00253-003-1427-z

Verbeke, K. A., A. R. Boobis, A. Chiodini, C. A. Edwards, A. Franck, M. Kleerebezem, A. Nauta, J. Raes, E. A. van Tol, and K. M. Tuohy. 2015. Towards microbial fermentation metabolites as markers for health benefits of prebiotics. *Nutr. Res. Rev.* **28**:42–66. doi:10.1017/S0954422415000037

Weese, J. S., and L. Arroyo. 2003. Bacteriological evaluation of dog and cat diets that claim to contain probiotics. *Can. Vet. J.* **44**:212–216.

Wong, J. M., R. de Souza, C. W. Kendall, A. Emam, and D. J. Jenkins. 2006. Colonic health: fermentation and short chain fatty acids. *J. Clin. Gastroenterol.* **40**:235–243. doi:10.1097/00004836-200603000-00015

Wu, T., Y. Zhang, Y. Lv, P. Li, D. Yi, L. Wang, D. Zhao, H. Chen, J. Gong, and Y. Hou. 2018. Beneficial impact and molecular mechanism of *Bacillus coagulans* on piglets' intestine. *Int. J. Mol. Sci.* **19**:1–17. doi:10.3390/ijms19072084

Yu, H. F., A. N. Wang, X. J. Li, and S. Y. Qiao. 2008. Effect of viable *Lactobacillus fermentum* on the growth performance, nutrient digestibility and immunity of weaned pigs. *J. Anim. Feed Sci.* **17**:61–69. doi:10.22358/jafs/66470/2008