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

| Abbreviation | Description |
|--------------|-------------|
| 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; Goncalves 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-spore-forming 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 $7.5 \times 10^6$ 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 $3.47 \times 10^3$ 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 $7.47 \times 10^3$ 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, amylolytic, 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 $1.5 \times 10^{10}$ 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   |
| Beef pulp, % | 3.00   |
| Digest flavoring, % | 1.00   |
| Potassium chloride, % | 0.50   |
| Salt, % | 0.50   |
| Dicalcium phosphate, % | 0.50   |
| Titanium dioxide<sup>1</sup>, % | 0.40   |
| D-Methionine, % | 0.25   |
| Choline chloride, % | 0.20   |
| Fish oil, % | 0.20   |
| Vitamin premix<sup>2</sup>, % | 0.15   |
| Trace mineral premix<sup>1</sup>, % | 0.10   |
| Natural antioxidant, % | 0.07   |
| B. coagulans (15B CFU·g<sup>−1</sup>)<sup>4</sup> | *     |

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<sup>3</sup>, kcal·kg<sup>−1</sup> | 3.671  |

<sup>1</sup>Food-grade TiO<sub>2</sub> was used as an indigestible marker for digestibility calculations.

<sup>2</sup>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 D<sub>3</sub> supplement, biotin, vitamin B12 supplement, and folic acid.

<sup>3</sup>Trace mineral premix: zinc proteinate, calcium carbonate, zinc sulfate, iron proteinate, ferrous sulfate, copper proteinate, copper sulfate, manganese proteinate, sunflower oil, sodium selenite, manganese oxide, calcium iodate, and ethylenediamine dihydroiodide.

<sup>4</sup>ME of diets was calculated using modified Atwater factors of 3.5, 3.5, and 8.5 kcal·g<sup>−1</sup> for energy from crude protein, NFE, and crude fat, respectively (NRC, 2006).

<sup>5</sup>CFU counts analyzed in extruded, dried, and coated diets at time of feeding.

Analyzed CFU·g<sup>−1</sup> in diet<sup>4</sup>

| Treatment | 10<sup>5</sup> | 10<sup>6</sup> | 10<sup>7</sup> | 10<sup>8</sup> | 10<sup>9</sup> |
|-----------|---------------|---------------|---------------|---------------|--------------|
| CON       | 2.32×10<sup>4</sup> | 6.93×10<sup>5</sup> | 6.14×10<sup>6</sup> | 1.37×10<sup>7</sup> | 6.84×10<sup>8</sup> |
| PEX       | 3.71×10<sup>5</sup> | 6.93×10<sup>5</sup> | 6.14×10<sup>6</sup> | 1.37×10<sup>7</sup> | 6.84×10<sup>8</sup> |
| PCL       | 4.58×10<sup>5</sup> | 6.93×10<sup>5</sup> | 6.14×10<sup>6</sup> | 1.37×10<sup>7</sup> | 6.84×10<sup>8</sup> |
| PCM       | 3.71×10<sup>5</sup> | 6.93×10<sup>5</sup> | 6.14×10<sup>6</sup> | 1.37×10<sup>7</sup> | 6.84×10<sup>8</sup> |
| PCH       | 3.71×10<sup>5</sup> | 6.93×10<sup>5</sup> | 6.14×10<sup>6</sup> | 1.37×10<sup>7</sup> | 6.84×10<sup>8</sup> |

<sup>1</sup>CON, control; PEX, DFM applied before extrusion; PCM, DFM applied as coating at moderate dose; PCH, DFM applied as coating at high dose.

<sup>2</sup>Formula inclusion as percent of batch weight with 6 g·kg<sup>−1</sup> of 15 billion CFU·g<sup>−1</sup> (Kerry, Inc., Beloit, WI, USA).

<sup>3</sup>Bacillus coagulans CFU counts analyzed in base ration before extrusion.

<sup>4</sup>Bacillus coagulans CFU counts analyzed in extruded, dried, and coated diets at time of feeding.

<sup>5</sup>Based on an expected average daily food intake of 200 g·dog<sup>−1</sup>·d<sup>−1</sup>
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 weighed by the dogs and calculating the daily ME requirement for inactive lab kennel dogs (95 × BW^{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 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 35 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 coprophagous 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:

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

(1)

Marker method:

\[
\text{Nutrient Digestibility, } \% = 1 - \frac{\% \text{ Nutrient in Feces} \times \% \text{ TiO}_2 \text{ in Food}}{\% \text{ Nutrient in Food} \times \% \text{ TiO}_2 \text{ in Feces}} \times 100\%
\]

(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 ≤ 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 \(1.0 \times 10^8\) and \(3.0 \times 10^9\) CFU·d\(^{-1}\) with a no-observed-adverse-effect level of \(1.36 \times 10^8\) CFU·BW\(^{-1}·d^{-1}\) (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 liquefied 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 formulation resulted in \(4.58 \times 10^5\) CFU·g\(^{-1}\) of B. coagulans in the base ration before extrusion, and a final count of \(1.06 \times 10^6\) 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 \(6.84 \times 10^6\) 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; \(P = 0.1364\); Table 3) 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

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

| Parameter                      | Treatment¹ | SEM  | P-value² |
|-------------------------------|------------|------|----------|
|                               | CON        | PEX  | PCL      | PCM      | PCH      |        |          |
| 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   |

¹CON, control; PEX, DFM applied before extrusion; PCM, DFM applied as coating at moderate dose; PCH, DFM applied as coating at high dose.

²P-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 = 0.1658), 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 TiO2 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 on or 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 TiO2 as dietary marker methods

| Nutrient               | TFC method | Marker method |
|------------------------|------------|--------------|
|                        | CON       | PEX          | PCL          | PCM       | PCH       | SEM        | P-value |
| 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      | 0.1565   |
| Crude protein, %       | 83.54     | 83.13        | 83.13        | 84.10     | 83.15     | 0.521      | 0.3620   |
| Crude fat, %           | 92.55a    | 90.74ab      | 91.71a       | 91.97a    | 91.87a    | 1.862      | 0.0783   |
| Ash, %                 | 41.67ab   | 40.43ab      | 38.79b       | 31.60b    | 45.05b    | 1.527      | <0.0001 |
| Gross energy, %        | 83.81     | 82.84        | 83.41        | 82.66     | 83.54     | 0.476      | 0.1938   |

- **CON, control; PEX, DFM applied before extrusion; PCL, DFM applied as coating at low dose; PCM, DFM applied as coating at moderate dose; PCH, DFM applied as coating at high dose.**

1 CON, control; PEX, DFM applied before extrusion; 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.

3a Means within a row with different superscripts differ (P < 0.05).

3b Means within a row with different superscripts differ (P < 0.10).
acido philus, 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 d⁻¹). Likewise, Gabi naitis 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 × 10⁸ 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 × 10¹¹ 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), iso butyrate (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; Scharff 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, mannanooligosaccharides, 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., 2015; Patra, 2011; Strompfová et al., 2013; Markowiak and Sliżewska, 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 × 10⁷ 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 |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Parameter       | CON             | PEX             | PCL             | PCM             | PCH             | SEM             | P-value⁴       |
| Fecal pH        | 5.49            | 5.36            | 5.44            | 5.41            | 5.33            | 0.088           | 0.4402         |
| Fecal NH₃ μmol·g⁻¹ DM feces | 99.99          | 105.49          | 107.12          | 104.61          | 94.30           | 9.496           | 0.8414         |
| Total SCFA 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 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         |
| Isovalerate, %  | 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         |

¹CON, 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.
²Total SCFA (acetate + propionate + butyrate); individual SCFA is expressed as a percent of total SCFA.
³Total BCFA (isobutyrate + isovalerate + valerate); individual BCFA is expressed as a percent of total BCFA.
⁴P-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 (Okele 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 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 average 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 defections 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.

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