Effects of *Bacillus coagulans* GBI-30, 6086 probiotic added to extruded pet food on apparent nutrient digestibility, stool quality, intestinal health indicators, and fecal microbiota of healthy adult dogs

by

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B.S., California State Polytechnic University, Pomona, 2009
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2021

Abstract

Bacillus coagulans GBI-30, 6086 is a spore-forming bacterium reported to survive environmental stresses, heat processing, and extreme-pH conditions, which are characteristics that support its utility in commercial food applications. Extrusion is the most widely used method to produce commercial dog foods, however no previous studies have examined the survivability of this strain through extrusion or its efficacy with regards to gastrointestinal health of dogs. Thus, the objectives were to determine the viability of *Bacillus coagulans* GBI-30, 6086 through extrusion cooking and drying, and its effects on nutrient utilization, intestinal health, and the fecal microbiota of healthy adult dogs. Two experiments were conducted to determine the reduction in viability of the organism at graded flour inoculation doses (0, 6.2, 6.7, and 7.3 $\log_{10}$ CFU/g) through extrusion with varying levels of extruder water inputs (10, 12, and 20 kg/h), extruder screw speeds (400, 500, and 600 rpm), and dryer settings (49 °C for 10 min; 107 °C for 16 min; and 66 °C for 46 min). The low SME extrusion conditions (in-barrel moisture of 35%, extruder screw speed of 400 rpm, and specific mechanical energy of 129 kJ/kg) resulted in the greatest retention ($P<0.05$), with a mean $\log_{10}$ reduction of viable spores of 0.44, 2.15, and 2.67 for the low, moderate, and severe extrusion conditions, respectively. Viability of spores subjected to three dryer conditions were observed to be similar across all treatments. To evaluate the effects of *Bacillus coagulans* on nutrient utilization, stool quality, and intestinal health indicators in dogs, extruded diets containing graded levels of probiotic 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 ten individually housed adult Beagle dogs (7 castrated males, 3 spayed females) of similar age (5.75 ± 0.23 years) and body weight (12.3 ± 1.5 kg). 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 9 $\log_{10}$ CFU/d with increases ($P < 0.05$) observed in gross energy and organic matter digestibility compared to the negative control. No significant changes were observed in food intake or fecal scores, moisture content, pH, ammonia, short-chain fatty acids, or branched-chain fatty acids for the probiotic-containing treatments compared to a non-probiotic control. To evaluate the effects of *Bacillus coagulans* on the fecal microbiota of dogs, fresh fecal samples were analyzed by 16S rRNA gene pyrosequencing. Firmicutes comprised the greatest proportion of observational taxonomic units (mean 81.2% ± 5), followed by Actinobacteria (mean 9.9% ± 4.4), Bacteroidetes (mean 4.5% ± 1.7), Proteobacteria (mean 1.3% ± 0.7), and Fusobacteria (mean 1.1% ± 0.6). No evidence of a shift in predominant phyla, class, family, or genus taxonomic levels were found apart from the *Bacillus* genus, which was observed to have a greater relative abundance (P=0.0189) in the low probiotic coating (5.92 x 10^5 CFU/g) and high probiotic coating (6.84 x 10^6 CFU/g) treatment groups compared to the extruded probiotic (1.06 x 10^4 CFU/g) and non-probiotic control treatments. Alpha-diversity indices (Richness, Chao1, ACE, Shannon, Simpson, Inverse Simpson, and Fisher) were similar for all treatments. Beta-diversity metrics (principal coordinate analysis) did not provide evidence of clustering for UniFrac distances among treatment groups. Overall, *Bacillus coagulans* GBI-30, 6086 retained varying degrees of viability during extrusion and drying of pet food. As a functional probiotic, our data supports an improvement in nutrient utilization and maintenance of stool quality, intestinal health indicators, and fecal microbiota in healthy adult dogs at a dose of 9 $\log_{10}$ CFU/day.

Keywords: *Bacillus coagulans*; Probiotics; Extrusion; Dogs; Nutrient utilization; Intestinal health; Fecal microbiome

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Acknowledgements

I would like to express my sincere appreciation for all who had a role in the completion of this dissertation:

The pet research coordination team at Kerry, Inc., for their collaboration and sponsoring the projects;

My employer, Michael Landa with Nulo, Inc., for providing the opportunity for continuous employment, guidance and professional development over the years, and financial support towards my PhD;

My graduate program advisor, Dr. Greg Aldrich, for inspiring me to reach my full potential as a researcher, pet food industry leader, and product developer;

My committee members, Drs. Alavi, Paulk, and Nagaraja for generously offering their time, expertise, and constructive guidance throughout the preparation and review of this document;

Eric Michael, for generously sharing his knowledge in extrusion processing and for the successful production of diets uses in these projects;

Dr. Li (and graduate students) with the Grain Science Protein Chemistry lab, for generously sharing the use of equipment to complete colorimetric assays;

Dr. Hardwidge, Michael Hays, and Dr. Palinski (and graduate students) with the Veterinary Diagnostic Lab for their collaboration and analysis of the fecal microbiome work;

The Grain Science and Industry staff and faculty for their support in processing numerous supplies order requests with precision and speed;

My lab mates Isabella Corsato-Alvarenga, Amanda Dainton, Ryan Lierz, Dalton Holt, Krystina Lema, Aiswariya Deliephan, Janak Dhakal, Julia Guazzelli-Pezzali, Renan Donadelli, Megan Haverkamp, Samuel Kiprotich, Logan Kilburn, Alaina Mooney, and others for sharing their friendship, knowledge, and support with projects and sample analysis;

My fiancé David Mendoza, for offering unconditional support and unlimited patience as I pursued this achievement;

My family and friends, for supporting my goals and recognizing the enormity of personal and professional investments this accomplishment required.

Chapter 1 - Literature Review

Introduction

Recent U.S. pet ownership statistics estimate that two thirds of U.S. households own at least one pet, accounting for nearly 85 million homes (APPA, 2020). Collectively in 2019, Americans invested $95.7 billion in their pets by purchasing pet foods, veterinary care, supplies, and non-medical pet care services, a clear indication that pets have become highly valued members of society. Over the past two centuries, the societal role of dogs has evolved from predominantly labor (i.e., guardianship, transportation, herding, and hunting), to a range of special operations (i.e., rescue, police, and military), therapeutic care (i.e., disease detection, assisting the sensory impaired, emotional support), and general companionship, deepening the reaches of the human-animal bond and a rising anthropomorphic view of companion animals (Bradshaw and Casey, 2007). Considering their increasing prominence in American lives, many pets today are viewed as members of the family and as such are being fed and nurtured with the goal of improving their wellness, longevity, and quality of life instead of solely production and performance.

A shift in feeding strategy for companion animals is perhaps most evident in the emerging market of functional foods and treats, which are foods considered to offer a positive health outcome that extends beyond providing essential nutrients (Hasler, 2002). Functional ingredients may include plant extracts, fibers with varying degrees of fermentability, joint supplements, non-essential nutrients, or microorganism and yeast-derived products, which can add value to pet foods by serving a preventative or therapeutic role (Di Cerbo et al., 2017). Among these, direct-fed microbials (DFM) (commonly referred to as “probiotics”) have been used for centuries to ferment staple human food products such as yogurt, cheese, wine, and bread and have only recently been embraced as health-promoting supplements (Gasbarrini et al., 2016). The efficacy of probiotics in

pets is a relatively new area of research, and innovations in the form of new application strategies, unique probiotic strain selection, and substantiating the potential health benefits is necessary to ensure the efficacy of products containing these beneficial microorganisms. The objective of this chapter is to provide a thorough review of the research that has been conducted with the use of probiotics in pet food products, with a focus on the use of *Bacillus* probiotics in the dog.

Historical Highlights of Probiotics

Probiotics have been present in food since early human civilization. It is presumed that our knowledge of bacteria in our food began when instances of spoilage and poisoning were encountered as early as 8,000-10,000 years ago (Gogineni, 2013). It wasn't until the mid-nineteenth century, however, that Louis Pasteur made the scientific community aware of acid-forming microorganisms and their role in the souring of milk and fermentation of wine (Berche, 2012). This discovery prompted a succession of experiments aimed at identifying other microorganisms and uncovering their invisible but significant role in our food system (Figure 1.1). Nearly a half-century later in 1907, Nobel prize-winning scientist, Elie Metchnikoff, proposed that lactic acid bacteria in fermented milk were responsible for certain health benefits, particularly in delaying the onset of aging (Metchnikoff, 1908). This came about from observing Bulgarian centenarians, who consumed the curdled milk ("yogurt") regularly. In one of his books, "The Prolongation of Life," Metchnikoff proposed that *Lactobacillus* might have a part in counteracting the putrefactive waste products of metabolism that contributed to disease and symptoms of aging, and thus the notion of consuming certain bacteria for promoting health was born. This intriguing theory inspired researchers over the next several decades to turn their focus to the health-promoting mechanisms behind the consumption of microorganisms.

Besides *Lactobacillus*, bacterial spore-formers were also discovered in the same time period. In 1876, Ferdinand Cohn recognized and named the bacterium *Bacillus subtilis* and shortly after Robert Koch described the life cycle of *Bacillus anthrax* (Drews, 2000). *Bacillus coagulans* (originally named *Lactobacillus sporogenes*) was later described by the Iowa Agricultural Experiment station in curdled milk, and the organism was successfully isolated in 1932 (Hammer, 1915; Sarles and Hammer, 1932). The unique sporulated condition of *Bacillus* microorganisms was credited with allowing them to survive in the environment as well as endure certain industrial processes such as the vacuum drying of evaporated milk. This provided early evidence that sporulated bacteria have the potential to survive an industrial food production process.

At the turn of the 21st century, the passing of the Dietary Supplement Health and Education Act of 1994 led to exponential growth in the sales of products marketed as probiotics for humans (DSHEA, 1994). The global market of probiotic-fortified foods is expected to grow from $48 billion to $94 billion with a 7.9% compound annual growth rate between the years 2020 – 2027 (Fortune Business Insights, 2020). This surge in interest in functional foods for humans inspired similar developments in the pet food industry, although far less research is available for the use of probiotics for dogs. For example, the PubMed open-access database returns >20,000 publications for "human" and "probiotic" between 1990 – 2021, whereas <250 publications are returned for "dog" and "probiotic" (Figure 1.2). Of these, less than 10 studies have evaluated the use of *Bacillus* spp. probiotics in dogs, with no studies reporting on the use of *Bacillus coagulans* specifically. Despite the small body of research available relative to that of humans, probiotics are still promoted for dogs in pet supplements, foods, and treats, and have garnered some support by veterinarians for use in clinical practice (Wynn, 2009; Jugan et al., 2017; Schmitz, 2021). This rapidly growing market warrants a closer evaluation of novel probiotic strains, their viability

through processing, as well as their ability to deliver similar health benefits as has been observed in humans.

Definitions and Regulatory Status

The term "probiotic" is derived from the Latin preposition "pro," which means "before, in front of" and the Greek word "biōtikós" meaning "of life" (Merriam-Webster, 2021). Over the last several decades, the definition of probiotics has been refined to incorporate various aspects of a probiotic's intended use and benefits (Table 1.1). The term "probiotic" is often used interchangeably with "direct-fed microbial" when referring to pet foods. However, the most current definition, and that which is used as the context for this publication, is "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014).

The criteria for receiving approval as an acceptable probiotic strain in animal feeds involves a framework for verifying the ingredient's compositional analysis, toxicological potential, and evaluation of animal exposure with a focus on potential adverse health effects (Bajagai et al., 2016). The Food and Drug Administration's Center for Veterinary Medicine along with the Association of American Feed Control Officials (AAFCO) first issued a list of bacterial and yeast organisms for use in animal feeds in 1989 that has been revised over the years to include new organisms based on available research mainly in swine and poultry. Today, there are 41 non-toxigenic bacteriological species that have been deemed safe for use in companion animals (AAFCO, 2020). These microorganisms can be further classified based on physiological characteristics such as the structure of their cell wall, oxygen tolerance, and whether or not they are spore-forming **(**Table 1.2). Which traits these microorganisms share in common, and which

make them unique, are important for the assessment of their potential use in specific food applications.

Strain Selection Criteria

In addition to meeting safety and regulatory guidelines, in general a probiotic candidate should have some degree of resistance to acid and bile salts, which are two principal chemical stressors that will be encountered in the gastrointestinal tract (Conway, 1996; Tuomola et al., 2001; Shewale et al., 2014). The canine digestive system has evolved with mechanisms to effectively inactivate pathogenic microorganisms and extract nutrients from a broad assortment of ingested materials. Comprehensive reviews of canine gastrointestinal tract physiology are available, and serve as a useful reference for identifying the conditions that would exert the most stress on a potential probiotic microorganism (i.e., lowest gastric pH, and longest gastric and upper intestinal transit times; Kararli, 1995; Smeets-Peeters et al., 1998). For example, conditions mimicking gastric transit (1 h at pH 2.0), small intestinal transit (4 h at pH 6.80), and colonic transit (6 – 10 h at pH 5.6 – 6.9), with simultaneous exposure to other relevant biochemical components (i.e., digestive enzymes and bile salts) have been used in the development of *in vitro* canine gastrointestinal models (Smeets-Peters, 2000; (Duysburgh et al., 2020). These conditions could also be applied for the screening of microorganisms intended for use in the diets of dogs.

In addition, any strains intended for application in commercially-processed foods pet foods should exhibit high resiliency to process-related stresses, such as heat, prolonged shelf-life, and chemical composition of the food itself (i.e., matrix acidity, oxygen presence, water activity, or presence of microbial inhibitors; (Tripathi and Giri, 2014). For pet owners, feeding probiotics as part of a food offers the convenience of daily administration to a pet while increasing the perceived value of the product compared to conventional foods (Urala et al., 2011). However, when

probiotics are selected without consideration for these characteristics, the resilience of individual strains in commercial food applications is still open to question. In a study investigating the probiotic integrity of pet foods obtained from the marketplace, 53% of the sampled commercial pet foods were found to be severely inadequate with respect to strain identity and colony-forming unit guarantees on pet food labels (Weese and Arroyo, 2003). This highlights a need for validation of probiotic strains to ensure viability of probiotics at the time of consumption by the animal.

When an organism can be guaranteed safe delivery to the gut, the metabolic activities of a bacteria are strain-specific. All species of bacteria do not favor the same metabolic pathways, nor do different strains within a species (Marteau, 2011). *Enterococcus*, *Lactobacillus* and *Bifidobacterium* are the most commonly used probiotics for animals, which produce lactic acid as a primary end product. Traditionally, lactic acid producing bacterial strains are Gram-positive, anaerobes or facultative anaerobes, and non-spore-forming (Song and Ibrahim, 2012). These strains also produce other substances such as hydrogen peroxide and bacteriocins which can influence the host microbiota (Holzapfel et al., 2001). The health benefits conferred to dogs have been summarized in several recent reviews, and include improvements to stool quality and mixed effects on apparent total tract digestibility, microbial fermentation end products, as well as immune system responses (Vester and Fahey Jr., 2010; Vester Boler and Fahey Jr., 2012; Baffoni, 2018). However, as vegetative cells intended for food applications, they are more susceptible to injury and death from the stresses associated with cooking and gastrointestinal transit. The survival of these microorganisms may be enhanced by the use of cell protection technologies, such as microencapsulation (Terpou et al., 2019). This is a growing area of research that is critical for the future of functional foods incorporating non-sporulating probiotics.

Spore-Forming Probiotics

Many bacterial species have the ability to cope with rapidly changing and sometimes hostile conditions to protect themselves (Pedraza-Reyes et al., 2012). One of the most effective adaptations is forming spores in response to a nutrient-deficient environment, low water activity, or unfavorable temperatures or pH (Checinska et al., 2015). While in a sporulated condition, microorganisms regress to a state of dormancy characterized by low metabolic and respiratory activity (Nicholson et al., 2000; Errington, 2003). Gram-positive bacteria, such as *Clostridia* and *Bacillus* species, can form thick protective barriers within the bacterial cell. The main layers of the spore include the core, peptidoglycan-rich germ cell wall and cortex, proteinaceous coats, and exosporium (Figure 1.3). Environmental sensing mechanisms allow the spore to germinate when favorable growth conditions are detected, such as the activation of nutrient and non-nutrient receptors located on the outer spore membrane (Paidhungat and Setlow, 2000; Cabrera-Martinez et al., 2003). A metabolically dormant microorganism can be advantageous with regard to survival in prepared foods due to an increased tolerance to processing conditions and shelf-life during storage (Konuray and Erginkaya, 2018). In addition, spores exhibit higher thermo-tolerance compared to vegetative cells and persist under conditions of low pH and in the presence of external proteases (Mckenney et al., 2013). Once the bacteria reach a suitable environment, the spores will initiate the germination process and be restored to a metabolically active state (Setlow, 2014).

Bacillus spp. is a sporulating genus that has been evaluated in the diets of calves, broilers, and piglets over the past decade (Ripamonti et al., 2009; Meng et al., 2010; Zhou et al., 2010). Key findings of these works include validating spore survival through the ruminant digestive tract, improvements to growth performance, and increases in apparent total tract digestibility. There are no documented reports of *Bacillus coagulans* in the diets of companion animals, despite this strain

being included on the approved microorganisms list. Even so, products containing *Bacillus coagulans* are available nationally in stores for consumers to purchase. *Bacillus coagulans* GBI-30, 6086 is a lactic-acid producing, Gram-positive, spore-forming rod-shaped bacterium that is microaerophilic. This strain was developed by researchers at Ganeden Biotech (now a subsidiary of Kerry, Inc., Beloit, WI), under U.S. Patent No. 7,713,726. It was granted generally recognized as safe (GRAS) status in 2012 and became the first probiotic strain to receive a published monograph in the Food Chemical Codex (USP Monograph FCC 10). The isolate name GBI-30, 6086 signifies an optimal growth temperature of 30 °C with an American Type Culture Collection designation number of PTA-6086. The spores of this strain are resistant to temperatures of up to 90 °C, able to germinate in the body while resisting damage by gastric acids and bile salts as determined by both *in vitro* and *in vivo* evaluations (Fitzpatrick et al., 2011; Honda et al., 2011; Keller et al., 2019). In addition, the safety of this strain with regard to toxigenic and genomic properties is well-established (Endres et al., 2009; Endres et al., 2011; Orrù et al., 2014; Salvetti et al., 2016). Thus, making this strain a compelling candidate for incorporation into pet food products.

Application of Probiotics in Extruded Pet Foods

Once a desired strain is selected, probiotics have several hurdles to overcome before they can confer a benefit to the animal (Figure 1.4). For probiotics incorporated into food products, one of the most intense stressors is thermal processing. The vast majority of pet foods are cooked to some degree or commercially sterilized to extend shelf-life and reduce the risk of pathogenic microorganisms or their toxins from enduring in the finished, ready-to-feed product. This practice is enforced by federal regulations, such as the Food Safety Modernization Act (FSMA, 2011) and the FDA's zero-tolerance policy for pet foods contaminated with Salmonella (FDA, 2013). As

such, process controls are vigilantly developed as part of food safety plans to ensure pathogenic species are effectively inactivated (FDA, 2017).

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). Depending on the organism and intensity of heat treatment, the action of heat may lead to one or more of these events, and the ultimate goal is to render pathogenic cells injured beyond repair. Spore-forming microorganisms are reported to exhibit greater wet-heat resistance compared to vegetative cells (Kort et al. 2005). The mechanisms controlling heat resistance of spores have not been fully elucidated. However, known heat resistance factors include the accumulation of divalent cations such as Ca^{2+} and the dehydrated state of the spore core (Setlow, 2014). Dipicolinic acid (DPA) also serves an important role by chelating the cations, which helps maintain a low moisture environment and high mineral density in the center of the core (Beaman and Gerhardt, 1986). Microorganisms which possess genes encoding for DPA during the sporulation process tend to show increased heat resistance (Kort et al., 2005).

Extrusion cooking is the most widely used technology in the commercial production of pet foods today, representing the largest category of pet food in terms of market share. Extruded pet foods are nutrient-dense, highly palatable, shelf-stable, and can be produced in a continuous process with high throughput. Extrusion is a high-temperature, short-time, high-shear process in which pre-conditioned raw materials are conveyed by a rotating screw through a barrel and forced through a small opening (a die) that results in vapor flash-off and expansion of the exiting product. Extruders are available as single- or double-screw configurations, and there are a variety of screw elements that can be combined to create a customizable screw profile in a given system.

Throughout the conveying process, thermal energy (usually in the form of steam injected at the pre-conditioning step) and mechanical energy (generated by shear forces from the rotating screws contacting the material) cause the temperature inside the barrel to rise, which allows for the gelatinization of starch, cooking of the material, and serves as a key step in the destruction of spoilage and pathogenic microorganisms that may have been carried in with the raw materials (Alonso et al., 2000). It has been demonstrated that the ratio of specific thermal energy to specific mechanical energy applied to the food mass during extrusion influences the structural characteristics of pet food kibble (Monti et al., 2016; Pacheco et al., 2018). While thermal destruction of pathogens and surrogate microorganisms has been extensively studied, less is known about the effects of specific mechanical energy on microbes. It is possible that extrusion may influence microbial survival differently than other food processes.

Okelo et al. (2008) and colleagues proposed that thermophilic organisms, such as *Bacillus* spp., were better suited for process validation studies since they would exhibit more thermotolerance and therefore be a reliable indicator for developing processes to achieve sterilization. They designed an experiment wherein different settings for the extruder barrel exit temperature, mash feed moisture content, and barrel retention time were combined to create 15 process combinations in order to compare the suitability of *Bacillus thermophilus* as a surrogate organism for Salmonella during single screw extrusion of animal feed. The results of the study indicated no survival of Salmonella when the feed was extruded at 24.5% moisture content, 3 s retention time, and 82 °C or higher die temperature. On the other hand, *B. stearothermophilus*, a spore-former, was detectable at all processing conditions in the range of moisture from 24.5 – 34.5%, retention times of 3 – 11 s, and extruder die temperatures of 77 – 100 °C). This study demonstrates the potential for sporulated microorganisms to survive extrusion, while also allowing

for destruction of pathogenic cells. Additional studies evaluating microorganisms of sporulating and non-sporulating taxa are summarized in Table 1.3.

Mechanisms of Action for Gastrointestinal Health

When probiotic viability can be ensured at the time of consumption of the pet, there is potential to positively influence the host's gastrointestinal health. For example, certain probiotics may be able to enhance the nutrient digestibility of a diet. As opportunistic scavengers, dogs have evolved with the omnivorous ability to digest and extract nutrients from a wide range of ingredients. In a single meal, a dog consumes a combination plant- and animal-derived materials, bringing with them starches, fibers, lipids, and proteins which require the necessary enzymes to convert polymers into molecules that can be absorbed and utilized by the animal. The catabolic events occurring along the gastrointestinal tract are shared by both host and microbial enzymatic activities. The role of probiotic microorganisms in improving nutrient digestibility for a host is attributed largely to the introduction of microbial enzymes into the intestinal lumen, including α-amylase, α-galactosidases cellulase, protease, and lipase (Lee et al., 2008; Bajagai et al., 2016; Latorre et al., 2016; Danilova and Sharipova, 2020). Considering that sporulated microorganisms are metabolically inactive at the time of ingestion, understanding the lifecycle of *Bacillus* spp. as they transit the gastrointestinal tract is valuable for understanding the extent of their ability to aid in nutrient utilization for an animal.

Oral and Gastric Transit

Commercial dog food diets typically contain a mixture of large polymers including carbohydrates (glycogen, starch, sugars, and fibers), lipids (triglycerides of fats and oils and cholesterols), and proteins (in-tact proteins, protein-complexes, peptides, and/or anhydrous amino

acids; Roberts et al., 2018). In the mouth of the dog, saliva is a complex fluid released from the salivary glands present in the oral cavity containing electrolytes (i.e., sodium, potassium, chloride), bicarbonate, mucin, and salivary alpha-amylase (Iacopetti et al., 2017). Canine saliva has a pH range between 7.2 - 8.1, and the average oral residence time of food is between 1 – 10 s (Kararli, 1995; Smeets-Peeters et al., 1998; Duysburgh et al., 2020). In *in vitro* experiments, sporulated probiotics remain unaffected by the conditions in the mouth and pass through to the stomach with little to no loss of viability (Dassi et al., 2018; Majeed et al., 2019).

Dogs have a glandular stomach with specialized cardiac, gastric and pyloric mucosal regions lined with columnar epithelial cells (Kararli, 1995). The cardiac region contains primarily mucus-secreting neck cells, which are important in producing mucus to protect the lining of the stomach from the highly acidic environment. The gastric region of the stomach contains HCl-secreting parietal cells and pepsinogen-secreting chief cells. HCl is extremely acidic (pH < 2) and helps to begin the process of protein denaturation, making protein molecules more accessible for proteolytic enzymes. Pepsinogen, a proenzyme secreted by chief cells, becomes activated to pepsin by the gastric acids and begins breaking the peptide bonds of large polypeptide chains. In the dog, the gastric region makes up a significant portion of the stomach, which allows for a higher secretion of HCl relative to the stomach size in comparison to other monogastric species (Kararli, 1995; Meyer et al., 1999).

In addition to priming larger macromolecules for subsequent enzymatic digestion, the low pH of gastric acid paired with proteolysis by pepsin is the most effective defense mechanism dogs have against ingested microorganisms (Martinsen et al., 2019). At neutral pH, disruption of the outer membrane of vegetative cells is weak, but at acidic pH, the lipid component of bacterial membranes is disturbed, causing bacterial lysis (Guan and Liu, 2020). The killing of vegetative

cells is also related to time of exposure to these harsh conditions, with the time being required to kill more than 90% of bacteria at pH 2.0 being less than 30 minutes (Giannella et al., 1972). The gastric residence time in dogs ranges between 0.5 - 2 h, depending on factors such as the meal size, meal composition, and meal particle size (Itoh et al., 1986; Weber et al., 2001; Rolfe et al., 2002; Martinez and Papich, 2009; Duysburgh et al., 2020). This creates a formidable environment for vegetative cells.

Spores on the other hand are remarkably resistant to acid hydrolysis (Clavel et al., 2004; Ceuppens et al., 2012a). The outermost layer of the spore consists of proteins, lipids, and carbohydrates (primarily peptidoglycans) that make the spore insoluble (Terry et al., 2017). The inner membrane is also highly impermeable, preventing both hydrophobic and hydrophilic molecules from penetrating the core of the cell (Mckenney et al., 2013). One of the key triggers for germination of spores is exposure to low pH, which aids in the activation of spores before reaching the small intestine. This is suspected to be the result of changes in spore protein structure that stimulate the nutrient germination receptors (Luu et al., 2015).

Small Intestinal Transit

The small intestine is comprised of the duodenum, jejunum, and ileum. Chyme from the stomach is passed in controlled amounts through the pyloric sphincter into the duodenum, triggering the release of pancreatic proenzymes, bile, and bicarbonate. Bile salts represent a second major barrier to incoming microorganisms due to the unfolding and aggregation of cytosolic proteins (Cremers et al., 2014). In addition, bile salts can chelate iron and calcium and cause oxidative damage to the DNA of bacteria, which can inhibit cell growth cause cell death (Ceuppens et al., 2012b; Urdaneta and Casadesús, 2017). Due to the bactericidal nature of these compounds, criteria for screening potential probiotics commonly includes an assessment of bile

tolerance (Tuomola et al., 2001; Mbareche et al., 2020). Spore-forming lactic acid bacteria appear to have a high survival capacity to bile salts *in vitro*, with *Bacillus coagulans* exhibiting greater tolerance compared to other *Bacillus* strains (Hyronimus et al., 2000).

Upon survival through the chemical stressors in the duodenum, spores will initiate germination in the presence of germination triggers, such as nutrients (i.e., sugars, amino acids, or minerals) or non-nutrients (i.e., lysozyme, salts, high pressure, or DPA; Setlow, 2003; Swick et al., 2016). The presence of nutrients coupled with favorable conditions such as a more neutral pH and low microbial competition provide a less hostile environment vegetative cells (Tam et al., 2006). When the nutrient germination receptors are activated, the core of the spore releases calcium ions and DPA. This allows water to enter the core and activate spore enzymes that begin hydrolyzing the spore cortex. The event of cortex hydrolyses is irreversible, committing the cell to germination (Yi and Setlow, 2010). Under *in vitro* conditions, *Bacillus* spores have been determined to germinate within 60 minutes (Latorre et al., 2016), with 90% of germination occurring before the terminal ileum (Keller et al., 2019). It is in the small intestine that probiotics have the greatest opportunity to contribute to nutrient digestibility. This is achieved through the secretion of exoenzymes, as well as stimulating host digestive enzyme activities throughout the jejunum (Yang et al., 2005; Wang and Ji, 2019).

Large Intestinal Transit

The large intestine is comprised of the cecum, colon, rectum, and anus, and serves as the primary sites for the absorption of water and electrolytes. Digestate transiting beyond the ileum towards the colon encounters the ileocecal junction. The cecum of dogs is small diverticulum of the proximal colon, making up only 1% of the relative capacity of the dog's gastrointestinal tract (Kararli, 1995; Washabau, 2013). Here, a small amount of fermentation of fiber occurs by the

microorganisms residing within the cecum. As the digestate progresses along the colon, the density of microorganisms increases exponentially. In dogs, the colon comprises 13% of the relative capacity of the digestive tract (Kararli, 1995) and harbors between 10^9 and 10^{11} CFU/g of microorganisms within the luminal content (Mentula et al., 2005; Suchodolski, 2011).

Despite the efficiency of host digestion, fibrous and nitrogen-containing compounds that escaped digestion in the small intestine along with endogenous secretions become substrates for the intestinal microbiota. The principal sources of carbon and energy for bacteria growing in the large intestine are resistant starches, plant cell wall polysaccharides, and various proteins, peptides and low-molecular-weight carbohydrates (i.e., FOS and MOS) (Cummings and Macfarlane, 1991). Diet composition, such as changes in protein or fiber levels and sources, can significantly alter the fermentative end products produced by host microbiota (Wernimont et al., 2020). Generally, the major end products of microbial fermentation in the large intestine are organic acids, vitamins, volatile compounds, and amines (Wolin, 1981; Wong et al., 2006). The shift in production of these compounds is driven by several factors, especially the substrates available to the microbial community. Short chain fatty acids are mainly produced during the breakdown of carbohydrates by intestinal bacteria and include predominantly acetate, propionate, and butyrate, which are normally present in molar ratios of 4:2:1 to 10:3:1 in dog feces (Middelbos et al., 2007; Barry et al., 2009; Kröger et al., 2017). Acetate is produced by reductive methylation of CO_2, which is a metabolic pathway common to several bacterial groups (Morrison and Preston, 2016). Propionate, butyrate and lactate production are more strain- and substrate-specific. For instance, propionate can be produced through three biochemical pathways, depending on the type of propionic bacteria and substrate being metabolized (Reichardt et al., 2014). Fermentation of resistant starch and soluble fibers is associated with increased butyrate production, which mainly occurs by the

condensation and reduction of acetyl-CoA (Vital et al., 2014; Peixoto et al., 2018). Fermentation of protein can yield branched-chain fatty acids, ammonia, biogenic amines (i.e., putrescine, cadaverine, histamine), indoles, phenols, sulphides and thiols. With the exception of branched-chain fatty acids, an excess of these metabolites has been implicated in gastrointestinal disorders (Richardson et al., 2013; Diether and Willing, 2019; Vierbaum and Zentek, 2019).

A benefit of SCFA production for the host includes recovering energy that would otherwise be lost (Bergman, 1990). For example, as much as 90% of butyrate produced in the gut is metabolized as an energy source for the colonocytes (Hamer et al., 2008). Propionate, once absorbed, is metabolized in the liver where it serves as a precursor for gluconeogenesis, and acetate is mainly taken up by peripheral tissues and used by adipocytes for lipogenesis (Sukkar et al., 2019). SCFA can influence the luminal environment by decreasing pH, which can inhibit the growth of potentially pathogenic species while stimulating growth of more acid-tolerate species (Sun and O'Riordan, 2013). In addition, a reduction of the end products from protein fermentation is viewed as promoting a healthier environment in the gut. These and other fermentation products that are not absorbed along the intestine are excreted in the feces. Thus, measurement of SCFA and other fermentation metabolites in feces are an indicator which that can provide information about changes in fermentation activities that are occurring due to the introduction of a probiotic supplement into a dog's diet.

Feces

Feces voided from the animal represent a conglomeration of water, undigested organic and inorganic residues, bacterial biomass, fermentation end products, as well bile salts, cholesterol, host cells, mucous, and volatile compounds (Rose et al., 2015). The analysis of feces has contributed greatly to our understanding of the gastrointestinal tract. One of the most apparent

indicators of gastrointestinal health is the texture and form of the feces an animal produces. A number of scoring systems have been developed as a research tool that can be used to subjectively evaluate the appearance of a fecal sample on a defined rating scale (Meyer et al., 1999; Sokolow et al., 2005; Allenspach et al., 2007; Grellet et al., 2012). The available scales differ with respect to breadth (ranging between 5 – 10 numerical values) and direction (i.e., high or low values may have different interpretations). However, all scales designate extreme ratings as poor quality (i.e., diarrhea or constipation). In contrast, more centrally allocated scores are considered ideal. In evaluating the fecal quality of dogs, the notion of "ideal" feces form takes into consideration both the comfort of the animal to pass the stool (i.e., not too dry and firm) as well as the ability of a pet owner to sanitarily dispose of feces without leaving behind a large amount of residue.

Fecal scoring is correlated to fecal moisture levels, wherein higher fecal moisture corresponds with softer feces (Grellet et al., 2016). Along the large intestine, dry matter content increases with a concomitant decrease in moisture as a result of the net absorption of water (Washabau, 2013). The moisture content of the feces of healthy dogs ranges between 60 – 80%. The moisture content of feces may be higher for feces voided at later times of day compared to the early morning for dogs feed a canned food diet (79% versus 71%; Hill et al., 2011). There may also be differences in fecal moisture content of dogs of different body sizes, with large breed dogs producing poorer fecal consistency and higher moisture content due, in part, to a prolonged colonic transit time compared to medium and small breed dogs (Rolfe et al., 2002; Weber et al., 2003; Hernot et al., 2005; Weber et al., 2017). In addition, fiber level and sources may also influence fecal moisture content but not necessarily fecal scores due to differences in water-binding capacity of different fibers (Kienzle et al., 2006).

Supplementation with probiotics in dogs has been observed to reduce fecal moisture content and improve fecal scores in puppies, healthy adult dogs, as well as dogs affected by acute gastroenteritis (Felix et al., 2010; Herstad et al., 2010; Paap et al., 2016; Schauf et al., 2019). This effect may be related to the increase in fatty acids produced by microbial fermentation, promoting water absorption in the colon. However, if very high levels of fermentation occur, this can result in the opposite effect. Jeong et al. (2017) observed a 17% increase in fecal moisture content in mice supplemented with *Lactobacillus kefiranofaciens*. This was attributed to the bowel-stimulating properties of the lactic and acetic acids contributed by the probiotic. Thus, the changes observed after administration of a probiotic may be considered beneficial or detrimental depending on the animal, the level of fermentation occurring, and the fecal quality before supplementation.

Defecation frequency is another commonly used indicator of gastrointestinal health. The number of times a pet voluntarily voids feces each day not only provides information about their gastrointestinal motility but can also impact the pet owner's care regimen for the pet depending on the pet's living arrangement (i.e., indoor, outdoor, or both). Particularly in urban living conditions, fewer defecations per day is viewed as a sign of high digestive efficiency and results in less pet waste to manage. An increase in defecations per day may be beneficial in pets prone to constipation, which is described as the absent, infrequent, or difficult defecation associated with retention of feces in the colon and rectum (Rossi et al., 2018).

Several mechanisms have been proposed for the action of probiotics on intestinal motility (Dimidi et al., 2017). These include enhanced colonic contractility by directly stimulating of smooth muscle cells (Bär et al., 2009), promoting cyclic initiation and aboral propagation of migrating myoelectric complex (Husebye et al., 1994), and ultimately shortening intestinal transit time (Miller and Ouwehand, 2013; Chandrasekharan et al., 2019). No studies have investigated

the effect of *Bacillus* probiotics on the gastrointestinal motility of dogs, although several studies have demonstrated a positive effect in this area for humans. For example, supplementation with *Bacillus coagulans* significantly increased fecal mass, improved sensation of evacuation, increased defecation frequency, and decreased abdominal pain and discomfort during defecation in human patients experiencing constipation (Minamida et al., 2015; Chang et al., 2020; Madempudi et al., 2020).

Apparent Total Tract Nutrient Digestibility

In addition to serving as a non-invasive tool to gather the information previously discussed, the total collection of feces also allows for the measurement of apparent total tract digestibility (ATTD). By this method, the difference between the quantity of nutrients consumed and that remaining in the feces is used to compute the nutrient disappearance, represented as the digestible fraction (NRC, 2006). There are a few drawbacks to relying on total fecal collection for digestibility measurements, including that this sampling method is time- and labor-intensive. In addition, sample loss is highly probable due to occasional coprophagic behavior by kenneled dogs. A more precise method for estimating ATTD which overcomes these issues is possible with the aid of indigestible markers (i.e., TiO_2), which are consumed at a known concentration in the food. The ratio of the marker in the diet or feces relative to the nutrient of interest is used to compute the nutrient disappearance, usually reported as a percent digestibility (AAFCO, 2020). This approach has several advantages, including that it allows for subsampling rather than total fecal collection. Estimating digestibility by analysis of feces is considered "apparent" in that it does not separate non-dietary fractions (i.e., the host endogenous losses, or bacterial biomass) making this method inherently biased towards underestimating true digestibility. Methods are available for estimating true digestibility by collecting the luminal contents at the terminal ileum through a surgically

placed canula. Since the small intestine is the primary site of mammalian enzymatic digestion, it is useful for determining the digestibility of nutrients by the host animal. This method, however, does not take into account the many transformations occurring from the actions of microbes on the substrates as they transit through the large bowel.

Researchers have observed mixed effects of probiotics on the apparent digestibility of dry matter, organic matter, energy, crude protein, and crude fiber in dogs, despite their ability to secrete exoenzymes while transiting the gut. Supplementation with *Lactobacillus acidophilus, Lactobacillus sporogenes,* and *Enterococcus faecium* has been reported to improve daily weight gain and apparent digestibility of dry matter, organic matter, or crude fiber in growing puppies (Pasupathy et al., 2001; Gabinaitis et al., 2013; Tyagi et al., 2014). In adult dogs, Biourge et al. (1998) reported that apparent nutrient digestibility tended to improve with higher levels of supplementation with *Bacillus* CIP 5832. However, their results were not significantly different from a non-probiotic control. Similarly, Sun et al. (2019) investigated the effects of *Weissella cibaria* in adult dogs and reported no differences in apparent dry matter digestibility. Schauf et al. (2019) evaluated a commercial strain of *Bacillus subtilis* supplemented in the diets of adult Beagles and reported higher apparent digestibility of fat and nitrogen-free extract compared to non-probiotic treated diets. Whereas, dry matter and crude protein digestibility tended to increase in adult dogs supplemented with *L. acidophilus* in combination with a prebiotic fiber for 28 days in the study by Swanson et al. (2002). The findings from other experiments evaluating *Bacillus* organisms are summarized in Table. 1.4. The variability in nutrient digestibility enhancing effects supports the need for substantiation of the effects of novel probiotics strains in dogs and with different dietary substrates.

Microbiota Modulation

The earliest publications characterizing the diversity of the canine microbiome date back more than a century (Torrey, 1919; Davis et al., 1977), although these initial observations were limited by culture-dependent and microscopic techniques. With rapidly advancing molecular sequencing technologies, we are now able to study characteristics such as which organisms are present in the gut, which are most abundant, and how these attributes may be contributing to states of health and disease. In addition to simply characterizing the microbiome, there is also interest in modifying the gut microbiota towards what is considered a "healthy" composition (i.e., increase in lactic-acid producing bacteria or decrease in potentially pathogenic bacteria). Much of our understanding of what constitutes a healthy canine microbiome comes from studying the microbial richness and diversity in healthy dogs. This has been the focus of several recent and extensive reviews (Deng and Swanson, 2015; Blake and Suchodolski, 2016; Baffoni, 2018; Barko et al., 2018; Huang et al., 2020; Pilla and Suchodolski, 2020). The general consensus is that a healthy microbiome is characterized by a high level of species diversity and richness, and sustaining these attributes is beneficial to the animal's health. Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria make up >99% of the bacterial phyla in the colon and feces of dogs (Deng and Swanson, 2015). When the balance of the resident microbiota is disturbed, this can lead to a state of dysbiosis characterized by poor microbial diversity, a reduction of health-promoting microorganisms, and an increase in the relative abundance of pathogenic species (Schmitz and Suchodolski, 2016; AlShawaqfeh et al., 2017; Brüssow, 2020)).

While diet has been established as having a major impact on the composition and activities of the microorganisms in the gut, probiotics also have a role in the manipulation of the microbiome through direct and indirect mechanisms. On one hand, probiotics may contribute to the growth of

certain bacteria by supplying readily usable metabolites through the breakdown of complex nutrients. On the other hand, they can inhibit the growth of pathogenic bacteria by competing for nutrients and adhesion sites, production of organic acids and antimicrobial substances, and stimulation of intestinal immune response. For example, O'Mahony et al. (2009) observed a reduction in total fecal Clostridia counts (mainly *Clostridium difficile*) in healthy adult dogs fed a commercial dry food supplemented with 1.5 x 10^9 CFU/d of *Bifidobacterium animalis* AHC7. Xu et al. (2019) observed an increase in beneficial bacteria (including some *Lactobacillus* spp. and *Faecalibacterium prausnitzii*) and a decreased in potentially harmful bacteria (including *Escherichia coli* and *Sutterella stercoricanisin*) after supplementing dogs with a multi-strain probiotic (containing *Lactobacillus casei* Zhang, *Lactobacillus plantarum* P-8, and *Bifdobacterium animalis* subsp. lactis V9. Serum) for 60 d at a dose ranging between 8 x 10^9 – 2 x 10^9 CFU/d. González-Ortiz et al. (2013) reported a decrease in pathogenic Clostridia in the feces of dogs supplemented with *Bacillus amyloliquefaciens* CECT 5940 and *Enterococcus faecium* CECT 4515. In recent work, de Lima et al. (2020) found that supplementing dogs with *Bacillus subtilis* C-3102 presented a greater abundance of bacterial groups, which are considered to be beneficial for gut health, such as *Bacteroides*, *Faecalibacterium*, and *Allobaculum* when compared to a control group. In contrast to these findings, Pilla et al. (2019) reported that supplementing dogs with *Enterococcus faecium* NCIMB 10415 4b1707 at a dose of 1 x 10^9 CFU/d did not significantly alter the fecal microbiota richness or diversity. Notwithstanding the fact that so few studies have evaluated *Bacillus* spp. probiotics on the canine fecal microbiota to date, overall, these trends suggest that probiotic bacteria administered at a sufficiently high dose, can lead to increases in health-promoting bacterial species, as well as a decrease in potentially pathogenic bacteria.

Conclusion

There is increasing interest in the use of probiotics to improve the gastrointestinal health of pets due to their potential to influence on the host fecal parameters (stool quality and defecations per day), nutrient utilization, and fecal microbiota composition. Evaluation of the specific health effects must be conducted on the novels strains being fed commercially because the results reported for other strains and animal models may not be extrapolated directly for usage in dogs. It is evident that there is a gap in research supporting the use of *Bacillus* probiotics in commercial pet food applications and the efficacy of this organism with regards to the gastrointestinal health indices of healthy dogs.

Hypotheses

Considering that loss of viability has been reported for other sporulated microorganisms during extrusion, we hypothesized that higher specific mechanical energy applied during pet food extrusion and higher temperatures for drying would negatively affect the survival of *Bacillus coagulans GBI-30, 6086*. With regard to potential health benefits, *Bacillus coagulans* possesses similar physiological traits to other sporulated probiotics. We hypothesized that when administered at a sufficiently high dose, *Bacillus coagulans* GBI-30, 6086 would positively affect the apparent nutrient digestibility, stool quality, and fecal microbiota of healthy adult dogs.

Objectives

The overall objectives of the studies presented in this dissertation were:

To determine the survival *Bacillus coagulans* GBI-30, 6086 through pet food extrusion with varying levels of specific mechanical energy and drying conditions.

To evaluate the effects of *Bacillus coagulans* GBI-30, 6086 on gastrointestinal health indicators in healthy adult dogs (stool quality, apparent total tract nutrient digestibility, and fermentation metabolites).

To characterize the fecal microbiota in healthy adult dogs supplemented with graded doses of *Bacillus coagulans* GBI-30, 6086.

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Chapter 1 Figures

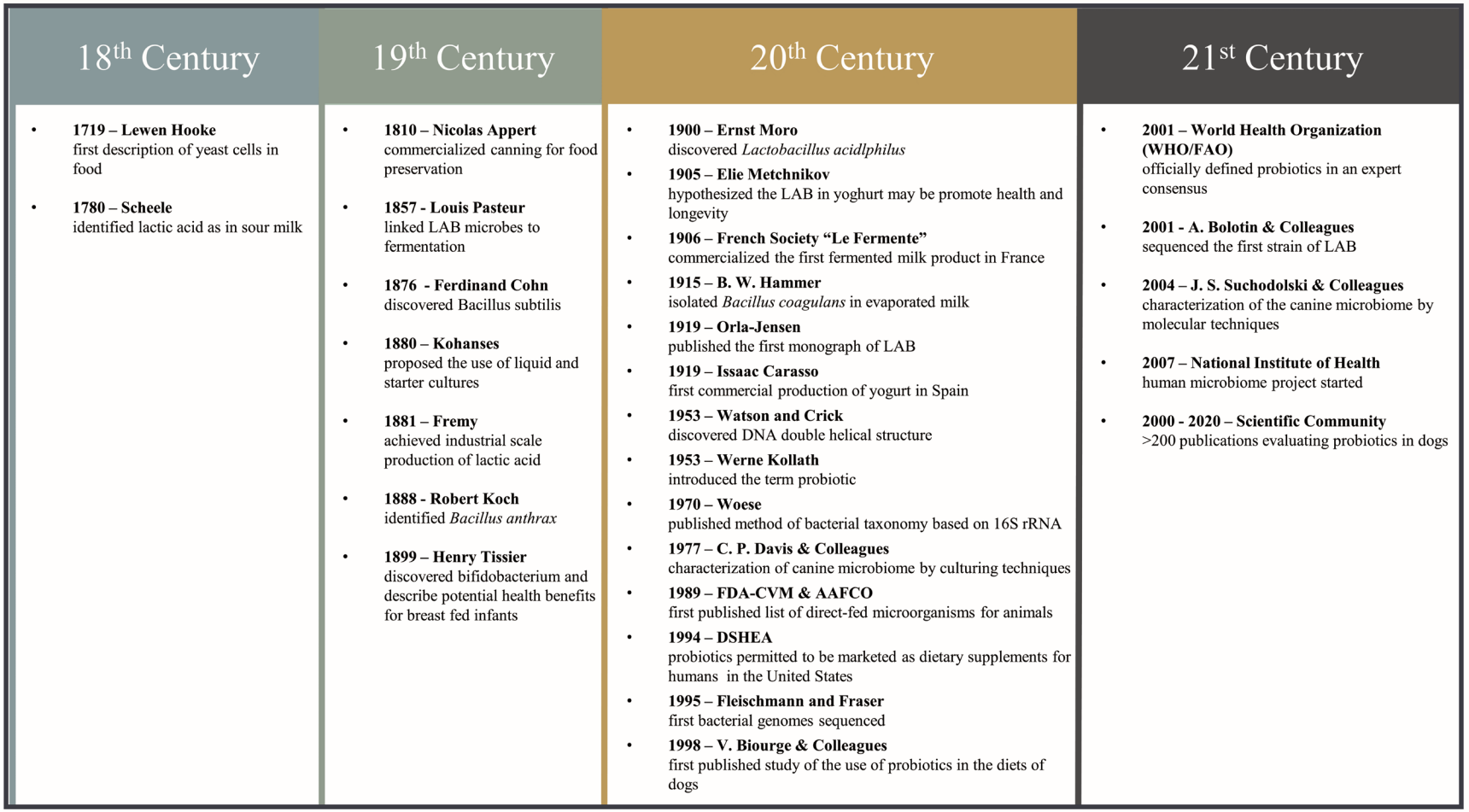

Figure 1.1. Scientific landmarks in the history of probiotics and microbiome research in dogs (References: Jay, 2005; Gogineni, 2013; Land et al., 2015; Ozen and Dinleyici, 2015; Gasbarrini et al., 2016; Huang et al., 2020).

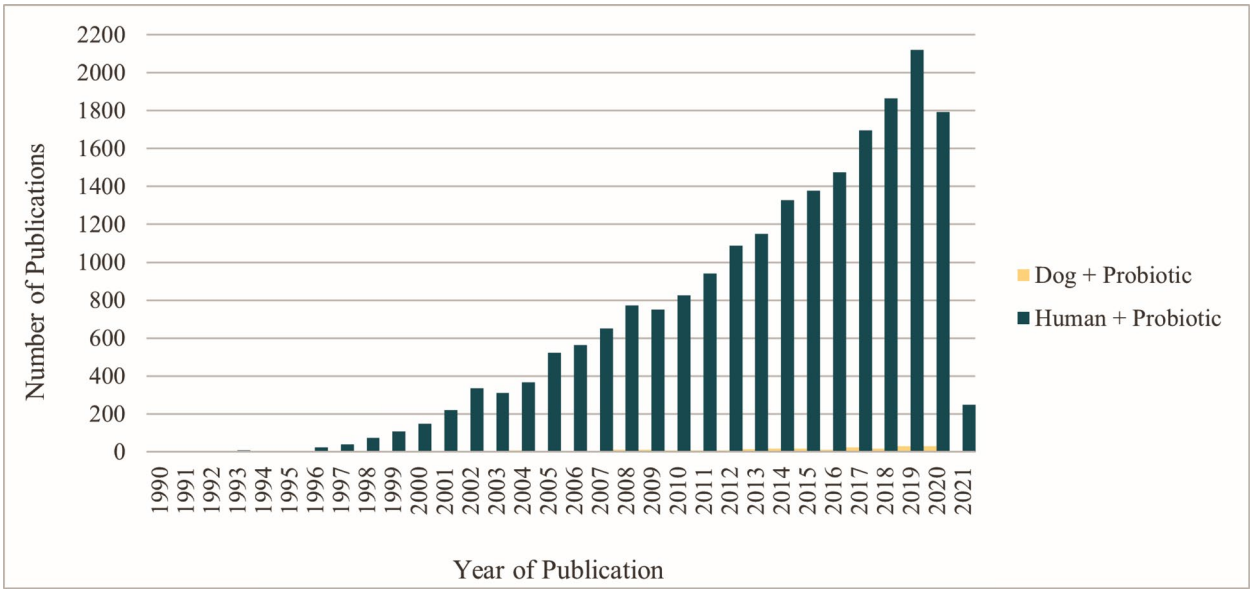

Figure 1.2. Number of research publications returned by the PubMed database for search terms "human" or "dog" and "probiotic" between 1990 – 2021. Data presented for 2021 represents year-to-date publication counts available as of March 2021.

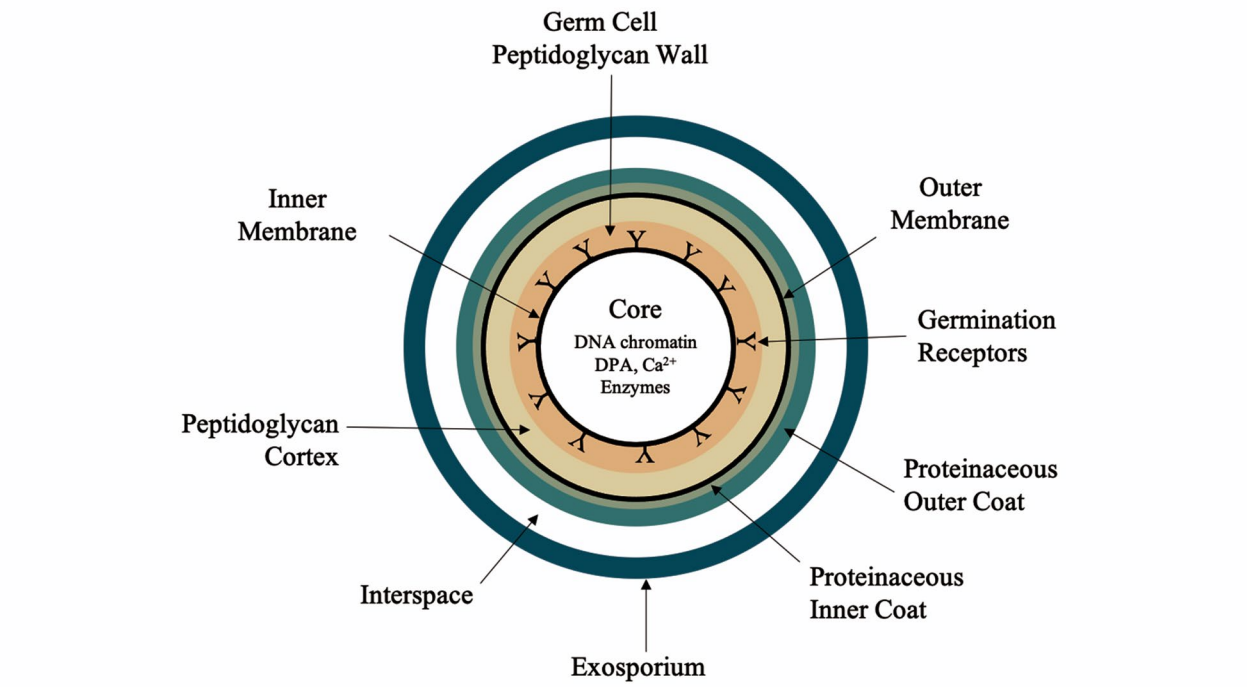

Figure 1.3. Crude rendering of a cross-section of *Bacillus* spore structures (adapted from Ohye and Murrel, 1962; Warth et al., 1963; Setlow, 2014; Bressuire-Isoard et al., 2018; Tehri et al., 2018; Garrison, 2019).

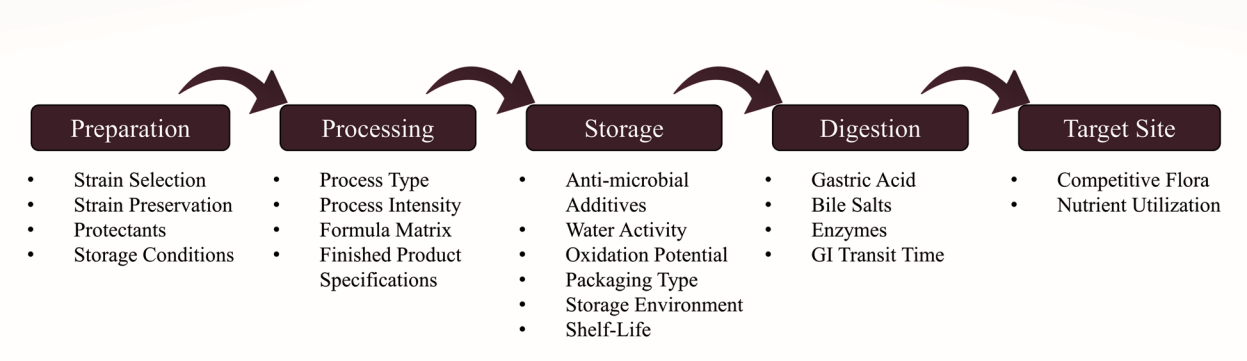

Figure 1.4. Diagram highlighting major hurdles probiotic microorganisms must overcome before reaching the target site of the canine intestine. Several variables are nested within each hurdle, adding to the complexity of factors that influence probiotic survival potential.

Chapter 1 Tables

Table 1.1. Published definitions of probiotics and direct-fed microbials

Term	Definition	Reference
Direct-fed microbials	Live microorganisms that, when provided in adequate amounts in the diet, can improve gut microbial balance; the anaerobic bacteria that are able to produce lactic acid and stimulate the growth of other organisms	Lilly and Stillwell (1965)
Probiotics	Tissue extracts which stimulated microbial growth	Mihich (1972)
Probiotics	Organisms and substances which contribute to intestinal microbial balance	Parker (1974)
Probiotics	A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance	Fuller (1989)
Direct-fed microbial products	Products that are purported to contain live (viable) microorganisms (bacteria and/or yeast)	FDA (1995)
Probiotics	Live microorganisms which when administered in adequate amounts confer a health benefit on the host	FAO/WHO (2001)
Probiotics	Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host	Hill et al. (2014)

Table 1.2. Taxonomic classification and physiological characteristics of direct-fed microorganisms approved for use in dog and cat foods.

Taxonomic Classification[1]		Physiological Characteristics		
Phyla and Genus	Species	Gram +/-	Spore-Forming	Oxygen Tolerance
Firmicutes				
Bacillus	*amyloliquefaciens, coagulans, lentus, licheniformis, pumilus, subtilis*	+	yes	microaerophile and facultative anaerobe
Enterococcus	*cremoris, diacetylactis, faecium, intermedius, lactis, thermophilus*	+	no	facultative anaerobe
Lactobacillus	*acidophilus, animalis, brevis, bulgaricus, casei, cellobiosus, curvatus, delbrueckii, fermentum, helveticus, lactis, planatarum, reuteri*	+	no	microaerophile and facultative anaerobe
Leuconstoc	*mesenteroides*	+	no	facultative anaerobe
Pediococcus	*acidilactici, cervisiae, pentosaceus*	+	no	facultative anaerobe
Bacteroidetes				
Bacteriodes	*amylophilus, capillosus, ruminocola, suis*	-	no	obligate anaerobe
Actinobacteria				
Bifidobacterium	*adolescentis, animalis, bifidum, infantis, longum, thermophilum*	+	no	obligate anaerobe
Propionibacterium				
Propionibacterium	*freudenreichii, shermanii*	+	no	obligate anaerobe

[1]NCBI Taxonomy Browser (https://ncbi.nlm.nih.gov)

Table 1.3. Summary of log reduction in microorganism viability under various extrusion processing conditions

Microorganism	Food Material	Process Conditions	Viable Cell Loss	Reference
Bacillus cereus	commercial pet food diet	NR	1.08 log	Biourge et al. (1998)
Bacillus stearothermophilus	animal feed mash	Extruder: single screw RT: 3 – 11 s IBM: 24.5 - 34.5% Die Temp.: 110 °C	1 log	Okelo et al. (2008)
Clostridium sporogenes	mechanically deboned turkey and white corn flour	Extruder: twin screw RT: 3.4 min IBM: 32% Die Temp.: 93.3 °C	2 log	Li et al. (1993)
Clostridium sporogenes	mechanically deboned turkey and white corn flour	Extruder: twin screw RT: 3.4 min IBM: 32% Die Temp.: 115.6 °C	4-5 log	Li et al. (1993)
Enterococcus faecium	dry dog food ration (corn flour, poultry by-product meal, corn gluten meal, rice meal, vitamins, and minerals)	Extruder: single screw RT: 71 s – 105 s IBM: 21.68% Die Temp.: 120 - 140 °C	6 log	Zhou (2016)
Enterococcus faecium	balanced carbohydrate-protein meal (chicken meal, rice, potassium chloride, ptoassium sorbate)	Extruder: single screw RT: NR IBM: 28.1% Die Temp.: 81.1 °C	5 log	Bianchini et al. (2012)
Enterococcus faecium	balanced carbohydrate-protein meal (chicken meal, rice, potassium	Extruder: single screw	1.4 - 5.81 log	Bianchini et al. (2014)

Microorganism	Food Material	Process Conditions	Viable Cell Loss	Reference
	chloride, ptoassium sorbate)	RT: 48 - 62.5 s IBM: 27.4 - 27.8% Temp 55.5 - 75 °C		
Enterococcus faecium	balanced carbohydrate-protein meal (chicken meal, rice, potassium chloride, ptoassium sorbate)	Extruder: single screw RT: 48 - 62.5 s IBM: 26.8 - 27.3% Temp: 80.3 - 100.5 °C	2.3 to >5.87 log	Bianchini et al. (2014)
Salmonella	oat flour	Extruder: single screw RT: 18 – 46 s IBM: 14 – 26% Die Temp.: 83 - 103 °C	5 log	Verma et al. (2018)
Salmonella typhiumurium	animal feed mash	Extruder: single screw RT: 7 s IBM: 28.5% Die Temp.: 83 - 103 °C	8 log	Okelo et al. (2008)
Salmonella enterica	balanced carbohydrate-protein meal (chicken meal, rice, potassium chloride, ptoassium sorbate)	Extruder: single screw RT 48 - 62.5 s IBM 27.3-27.6% Temp 55.5 - 68 °C	4 - 6.5 log	Bianchini et al. (2014)
Salmonella enterica	balanced carbohydrate-protein meal (chicken meal, rice, potassium chloride, ptoassium sorbate)	Extruder: single screw RT: 48 - 62.5 s IBM: 25.6 - 26.8% Die Temp.: 77 - 101 °C	>6.86 log	Bianchini et al. (2014)

Microorganism	Food Material	Process Conditions	Viable Cell Loss	Reference
Streptococcus thermophilus	whey protein isolate	Extruder: twin screw RT: 25 s IBM: 4 - 5% Die Temp.: 143 °C	4.2 log	Quéguiner et al. (2007)
Streptococcus thermophilus	whey protein isolate	Extruder: twin screw RT: 35 – 40 s IBM: 4 – 5% Die Temp.: 133 °C	4.9 log	Quéguiner et al. (2007)
Bacillus cereus	commercial pet food diet	Coated on exterior of kibble after expansion-extrusion and drying; stored in commercial packaging at room temperature in a dry well-ventilated warehouse for 12 months	0.1 - 0.4 log	Biourge et al. (1998)

Abbreviations: NR = not reported; RT = extruder residence time; IBM = in-barrel moisture content; Die Temp. = maximum temperature measured at the die.

Table 1.4. Summary of reported gastrointestinal health effects of Bacillus probiotics in dogs

			Significant Effects[2]				
Probiotic Strain	Dose (CFU/day)[1] and Length of Treatment	Animal Description	Fecal Quality Characteristics	Apparent Nutrient Digestibility	Fecal Fermentation End Products	Fecal Microbiome	Reference
Bacillus CIP 5832 (Paciflor)	7.5 x 10^8 CFU/day for 14 days	5 healthy adult German Pointer and German Shepherd dogs (age 5-10 y; BW 24 ± 3 kg)	Not evaluated	No differences observed	Not evaluated	Not evaluated	Biourge et al. (1998)
Bacillus subtilis (C-3102)	~1.8 x 10^8 CFU/day for 30 days	12 healthy Beagle puppies (age 7-8 mo; BW 9.0 ± 1.2 kg)	• firmer feces • dryer feces (avg. 36.5% DM in CON vs. 39.1% DM in PRO)	No differences observed	• lower fecal ammonia content (avg. 0.56% in CON vs. 0.45% in PRO)	Not evaluated	Felix et al. (2010)
Bacillus subtilis and *Bacillus licheniformis*	Range (based on animal BW and single	36 client-owned dogs of various breeds	• shorter time from start of treatment to last abnormal stool (avg.	Not evaluated	Not evaluated	Not evaluated	Herstad et al. (2010)

Probiotic Strain	Dose (CFU/day)[1] and Length of Treatment	Animal Description	Significant Effects[2]: Fecal Quality Characteristics	Apparent Nutrient Digestibility	Fecal Fermentation End Products	Fecal Microbiome	Reference
(additional strains included: *Lactobacillus farciminis, Pediococcus acidilactici,* and *Lactobacillus acidophilus* MA 64/4E; ZooLac Propaste)	or double dose) 2.9 x 10^9 - 1.7 x 10^{10} CFU/day for 8 days	suffering from acute canine gastroenteritis (age 4.1 ± 3.3 y; BW 9.0 ± 1.2 kg)	2.2 d in CON vs. 1.3 d in PRO) • shorter time from start of treatment to last symptom reported (avg. 2.2 d in CON vs. 1.4 d in PRO)				
Bacillus amyloliquefaciens CECT 5940	1.0 x 10^8 CFU/day for 39 days	16 healthy adult Beagles (age 2-7 y; BW 14.0 ± 0.89 kg; 8 males, 8 females)	No differences observed	No differences observed	No significant differences observed	• decrease in Clostridia (5.64 CFU/g feces in CON vs. 2.94 CFU/g feces in PRO)	González-Ortiz et al. (2013)

Probiotic Strain	Dose (CFU/day)[1] and Length of Treatment	Animal Description	Significant Effects[2]: Fecal Quality Characteristics	Significant Effects[2]: Apparent Nutrient Digestibility	Significant Effects[2]: Fecal Fermentation End Products	Significant Effects[2]: Fecal Microbiome	Reference
Bacillus subtilis C-3102 (Calsporin®)	6.5 x 10^7 CFU/100 g diet (food intake not reported) for 28 days	40 client-owned dogs of various breeds with chronic diarrhea (23 males, 17 females; age 1 - 13 y)	• improved fecal odor • reduced flatulence incidence • improvement in severity degrees of diarrhea	Not evaluated	Not evaluated	Not evaluated	Paap et al. (2016)

Bacillus subtilis C-3102 (Calsporin®)	~2.7 x 10^8 CFU/day for 28 days	16 healthy adult Beagles (age 4-8 y; BW 17.0 ± 0.64 kg; 10 spayed females, 6 intact males)	• firmer feces • higher fecal DM content (range 26.4-30.5% DM in CON vs. range 29.7-30.9% DM in PRO)	• higher ATTD of fat (avg. 86.6% in CON vs. 88.4% in PRO) • higher ATTD of NFE (avg. 85.3% in CON vs. 87.2% in PRO) • trend towards higher ATTD of DM (avg. 77.0% in CON vs. 79.1% in PRO) • trend towards higher ATTD of OM (avg. 82.1% in CON vs. 83.7% in PRO)	• decline in fecal NH_3 (range 126-140 µmol/g DM in CON vs. range 109-117 µmol/g DM in PRO) • decline in fecal pH (range 6.23 - 6.64 in CON vs. range 5.94-6.50 in PRO) • increase in total fecal SCFA content (avg. 670 µmol/g DM in CON vs. 787 µmol/g DM in PRO) • trend towards lower total fecal BCFA (avg. 26.9 µmol/g DM in CON vs. 20.9 µmol/g DM in PRO)	Not evaluated	Schauf et al. (2019)

Probiotic Strain	Dose (CFU/day)[1] and Length of Treatment	Animal Description	Significant Effects[2]: Fecal Quality Characteristics	Significant Effects[2]: Apparent Nutrient Digestibility	Significant Effects[2]: Fecal Fermentation End Products	Significant Effects[2]: Fecal Microbiome	Reference
Bacillus subtilis and *Bacillus licheniformis*	~1.5 x 10^7 CFU/day for 25 days	16 adult healthy Beagles (age 4 y; BW 10.3 ± 1.1 kg; 8 in-tact males, 8 in-tact females)	• firmer feces • less fetid fresh feces	No differences observed	• reduced total biogenic amines (avg. 303.4 mg/kg feces in CON vs. 209.9 mg/kg feces in PRO) • reduced putrescine (avg. 71.2 mg/kg in CON vs. 47.5 mg/kg in PRO) • reduced spermidine (avg. 27.8 mg/kg feces in CON vs. 19.9 mg/kg feces in PRO) • reduced cadaverine (avg. 129.8 mg/kg feces in CON vs. 88.6 mg/kg feces in PRO)	Not evaluated	Bastos et al. (2020)

Probiotic Strain	Dose (CFU/day)[1] and Length of Treatment	Animal Description	Significant Effects[2]: Fecal Quality Characteristics	Apparent Nutrient Digestibility	Fecal Fermentation End Products	Fecal Microbiome	Reference
					• reduced phenols in fresh feces (avg. 37.2% of chromatograph peak area in CON vs. 19% in PRO) • reduced quinoline in fresh feces (avg. 14.6% of chromatograph peak area in CON vs. 3.1% in PRO) • trend towards increased fecal isobutyrate (avg. 0.79 µmol/g in CON vs. 0.91 µmol/g in PRO)		

Probiotic Strain	Dose (CFU/day)[1] and Length of Treatment	Animal Description	Significant Effects[2]: Fecal Quality Characteristics	Apparent Nutrient Digestibility	Fecal Fermentation End Products	Fecal Microbiome	Reference
Bacillus subtilis C-3102 (Calsporin®)	~1 x 10^6 CFU/day for 33 days	16 healthy adult Beagles (age 1 y; BW 8.9 ± 1.1 kg; 8 males, 8 females)	• firmer stools • higher fecal DM content (avg. 30.3% DM in CON vs. 33.9% DM in PRO) • Reduced fecal odor	No differences observed	• Increased propionate • Reduced ammonia	• Increased bacterial diversity • Greater abundance of *Bacteroides*, *Faecalibacterium*, and *Allobaculum*	de Lima et al. (2020)

Abbreviations: CFU = colony-forming units; BW = body weight; ATTD = apparent total tract digestibility; DM = dry matter; OM = organic matter; SCFA = short-chain fatty acids; BCFA = branched-chain fatty acids.

[1] Doses preceded by a tilde (~) were approximated based on the supplement concentration and daily food intake of the animals.

[2] Significant effects represent statistical differences ($P<0.05$) and trends ($0.05\leq P<0.10$) between control groups (CON) versus probiotic supplemented groups (PRO) reported in each experiment.

Chapter 2 - Optimization of Extrusion Processing and Drying Conditions for the Survival of *Bacillus coagulans* GBI-30, 6086 in Commercial Pet Food Applications

Abstract

In companion animal nutrition, probiotics (direct-fed microbials) are considered functional ingredients that benefit the gastrointestinal and immune health of the host. *Bacillus coagulans* GBI-30, 6086 is a spore-forming bacterial strain that has been reported to survive environmental stresses, heat processing, and extreme-pH conditions. Extrusion cooking is the most widely used method to produce commercial dog and cat foods, however the thermal and mechanical forces exerted during extrusion and drying present a challenge for guaranteeing the viability of live microorganisms after processing. Two experiments were conducted to determine the reduction in viability of the organism at graded flour inoculation doses (0, 6.2, 6.7, and 7.3 $\log_{10}$ colony forming units per gram (CFU/g)) through extrusion cooking with varying levels of extruder water inputs (10, 12, and 20 kg/h), extruder screw speeds (400, 500, and 600 rpm), and dryer settings (49°C for 10 min; 107°C for 16 min; and 66°C for 46 min). Enumeration of bacterial colony forming units was performed on pre- and post-processing samples. Extrusion data were analyzed using a general linear model using the GLIMMIX procedure, and dryer data were analyzed as a completely randomized design with one-way analysis of variance (SAS v. 9.4, SAS Institute, Inc., Cary, NC) with significance accepted at a level of 95% confidence ($\alpha = 0.05$). The results indicate that the low SME extrusion conditions (in-barrel moisture of 35%, extruder screw speed of 400 rpm, and specific mechanical energy of 129 kJ/kg) resulted in the greatest retention ($P<0.05$), with a mean $\log_{10}$ reduction of viable spores of 0.44, 2.15, and 2.67 for the low, moderate, and severe extrusion

conditions, respectively. Viability of the spores through three dryer conditions were observed to be similar across all treatments. This study also demonstrated that the greatest losses of viability occurred during extrusion rather than drying, and that in-barrel moisture and extruder screw speed are two operational parameters may be modified for the optimization of *Bacillus coagulans* survival in extruded foods.

Introduction

In companion animal nutrition, probiotics (direct-fed microbials) are live microorganisms that benefit the gastrointestinal and immune health of the host (FAO/WHO, 2001). Probiotics can be administered to pets in capsulated form but are increasingly featured as functional supplements in premium foods, snacks, and treats (Packaged Facts, 2019). Several of the health-promoting activities associated with probiotics depend on the presence of an adequate number of viable cells being delivered into the animal's intestinal tract, making selection of suitable strains and mode of delivery to the pet key factors to consider for successful incorporation into a food product (Champagne et al., 2005; Markowiak and Ślizewska, 2018; Terpou et al., 2019; Yirga, 2015).

Extrusion is the most widely used cooking technology to produce pet foods today, accounting for an estimated 37% of total U.S. pet food retail sales (PFI, 2020). In comparison to other methods of cooking such as canning, baking, or freeze-drying, extrusion presents a unique set of challenges to survival of living microorganisms in that it is a high-temperature short-time process that utilizes both specific thermal energy (STE) and mechanical energy (SME) to cook and expand the material followed by convective oven drying to render the final product shelf-stable (Riaz, 2000). One of the main goals of food processing is to improve microbial safety, and many studies have accordingly evaluated the use of extrusion as a kill-step (Anderson et al., 2017; Bianchini et al., 2012; Verma and Subbiah, 2020) that complies with current food safety

regulations (FDA, 2011). This is counterproductive to the intentional inclusion of probiotic microorganisms, as the efficacy of probiotics in extruded pet foods products has been scrutinized due to deficiencies identified in viability counts compared to manufacturer label claims (Weese and Arroyo, 2003).

The microbiological lethality of extrusion is frequently attributed to the disruptive effects of thermal energy (Smelt and Brul, 2014). 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). Okelo et al. (2008) proposed that thermophilic spore-forming Bacillus stearothermophilus could serve as a suitable organism for validating sterilization of feeds through extrusion. By this reasoning, the survival of spore-forming probiotic microorganisms would also be expected to endure extrusion processing. However, Biourge et al. (1998) evaluated the survival of Bacillus CIP 5832 and reported a loss of spore viability >99% after extrusion, suggesting that there are mechanisms of destruction during extrusion which are not yet fully understood.

Less is known about the effects of mechanical energy (shear forces exerted on the material by the screw) on microorganism survival. Bulut et al. (1999) demonstrated a 5.3-log destruction of vegetative Microbcaterium lacticum cells by increasing the shear forces in a twin-screw extrusion system by varying the extruder water inputs. To our knowledge, the effect of mechanical energy on the destruction or retention of microbial spores has not been reported. *Bacillus coagulans* GBI-30, 6086 is a sporulated gram-positive bacteria that has been reported to demonstrate thermostability and resistance to acidic pH, which are valuable characteristics for a probiotic candidate in heat-treated foods (Jao et al., 2011; Konuray and Erginkaya, 2018). While

extrusion has been validated as an effective process control for pathogen destruction, the possibility that certain beneficial microorganisms may survive the cooking process, as evidenced by successful incorporation into several human food products, is promising. We hypothesized that reducing specific mechanical energy could be used to improve spore retention. Therefore, the goals of this research were to evaluate the survival capacity of *Bacillus coagulans* GBI-30, 6086 in a grain-free high-protein pet food produced under a range of inoculation levels, extrusion conditions, and drying conditions representative of commercial pet food production.

Materials and Methods

Although *Bacillus coagulans* GBI-30, 6086 is a non-pathogenic microorganism that is generally regarded as safe (GRAS) for human consumption, BSL-1 procedures were followed and approved by the Institutional Biosafety Committee (IBC) under protocol #1187 at Kansas State University for this study.

Raw Materials

A grain-free high-protein pet food formulation was developed to be nutritionally adequate for healthy adult dogs (AAFCO, 2020). The raw materials comprising the dry base ration were purchased from and blended by a commercial mill (Fairview Mills, Bern, KS). Ration particle sizes was reduced using a hammer mill to pass a 2 mm screen prior to inoculation and extrusion. The ingredient and proximate composition of the base ration are described in Table 2.1. *Bacillus coagulans* GBI-30, 6086 was obtained from the ingredient manufacturer (Kerry, Inc., Beloit, WI) in powdered form at a concentration of 1.5×10^{10} colony forming units (CFU)/g.

Dry Inoculation of Base Rations

Target quantities of the organism (Table 2.2) were blended into a dry base ration in a series of 1:5 ratio dilutions for 5 min in a paddle mixer (Hobart N50-60 5 qt Planetary Mixer, Troy, OH)

to produce a 9-kg sub-batch. The inoculated rations were incorporated into 227-kg batches and blended in a commercial double-ribbon mixer (Wenger Manufacturing, Sabetha, KS) for 5 min. Inoculation of the base ration was carried out at the Bioprocessing Industrial and Value Added Products (BIVAP) facility at Kansas State University. Enumeration of *Bacillus coagulans* was conducted at the Pet Food Microbiology and Toxicology Laboratory at Kansas State University.

Experiment 1: Extruder Conditions

The extrusion experiment was conducted as a 3 x 4 factorial arrangement of treatments with three levels of extruder SME conditions (low, moderate, and severe SME) and four levels of ration inoculation dose (control, low, moderate, and high CFU/g) were evaluated to determine the effect of starting dose and SME on survival of the microorganism through extrusion. The experimental rations were passed through a gravimetric feed hopper into a differential diameter cylinder preconditioner (Wenger Manufacturing Inc., Sabetha, KS) at a calibrated feed rate of 10 kg/h. The preconditioner configurations were set to a constant cylinder speed (400 rpm) and water flow (6.8 ± 0.2 kg/h), with no steam addition. The temperature of the preconditioner downspout (27.8°C ± 0.4) was measured using a digital probe attached at the exit of the preconditioner and the temperature reading was recorded from the control panel output. The preconditioned material was fed into a pilot-scale single-screw extruder (X-20, Wenger Manufacturing Inc., Sabetha, KS). The extruder screw configuration and barrel temperature profile are shown in Figure 1. Two circular dies (4.5 mm diameter inserts) were used to produce a standard size kibble for small to medium size dogs. The knife configuration included 6 hard blades set to a constant speed (mean 968 rpm). The extruder shaft speed, operational torque, steam flow, water flow, knife speed, and extruder zone temperatures were recorded from the control panel outputs. Power consumption (total kW) was measured by a three-phase digital power logger (Fluke 1738, Everette, WA)

attached to the electrical outlet supplying energy to the extruder. Different combinations of extruder shaft speed (400, 500, and 600 rpm) and extruder water flow (10, 12, and 20 kg/h) were used to generate three levels of SME conditions. The extruded kibbles were transported pneumatically from the extruder exit into a 3-pass horizontal wire belt conveyor dryer (Wenger Manufacturing Inc., Model 4800, Sabetha, KS). The product was dried at 107°C for 16 min of retention time (8 min each for the first and second passes, respectively), followed by 8 minutes for a third pass in the ambient cooler.

The run sequence was designed to allow the equipment to reach steady state for a minimum of 45 min using the uninoculated ration (CON), and to reduce the risk of cross contamination between production runs the treatments were produce in order of increasing dose (PLO, PMD, PHI). A minimum of 30 minutes between each treatment changeover was allowed to ensure transfer of the previous treatment through the system. Sample collection points included the raw base rations, off the extruder, and off the dryer at 15 minute intervals throughout the duration of each treatment. Samples were collected into sterile Whirl-Pak bags and stored frozen (-20°C) upon collection pending viability analysis. Three independent replications were conducted on separate days, and enumeration of bacterial colony forming units was performed on samples collected from the raw base ration, after extrusion, and after drying.

Experiment 2: Dryer Conditions

To investigate the effect of drying time and temperature of *Bacillus coagulans* survival, the high-dose ration (PHI) from Experiment 1 was processed using a moderate extruder profile (500 rpm screw speed and 12 kg/h extruder water flow) and transported pneumatically from the extruder exit into a three-pass horizontal wire belt dryer (Wenger Manufacturing Inc., Model 4800, Sabetha, KS). The dryer study was conducted as a completely randomized design with three time-

temperature combination treatments: high temperature-short time (HTst; 149°C for 10 min), moderate temperature-moderate time (MTmt; 107°C for 16 min), and low temperature-long time (LTlt; 66°C for 46 min). Three independent replications were conducted on separate days, and enumeration of bacterial colony forming units was performed on the raw base ration and after drying. Samples were collected into sterile Whirl-Pak bags and stored frozen (-20°C) upon collection pending viability analysis.

Product Measurements and Process Calculations

The wet mass flow rate was measured by collecting material flowing out of the extruder into a bucket for 60 s. Product bulk density in the raw material, off the extruder, and off of the dryer, was measured using a 1 L cup.

Specific mechanical energy (SME) was calculated according to the following equation:

$$SME\ (\frac{kJ}{kg}) = \frac{\left(\frac{\tau - \tau_0}{100}\right) \times P_{rated} \times (\frac{N}{N_{rated}})}{\dot{m}}$$

where τ is the % torque, or motor load, τ_o is the no-load torque (34%), N is the screw speed in rpm, N_r is the rated screw speed (508 rpm), P_r is the rated motor power (37.3 kW), and m is the total mass flow in kg/s.

In-barrel moisture (IBM) was calculate as described below:

$$IBM, \% = \frac{m_f * X_f + m_{ps} + m_{pw} + m_{es} + m_{ew}}{m_f + m_{ps} + m_{pw} + m_{es} + m_{ew}}\ x\ 100\%$$

where m_f is the dry feed rate, X_f is moisture content of the feed material, m_{ps} is the steam injection rate in the preconditioner (kg/h), m_{pw} is water injection rate in the preconditioner (kg/h),

m_{es} is water injection rate in the extruder, m_{es} is the steam injection rate in the extruder (kg/h), and m_{ew} is the rate of water injected in the extruder.

Barrel residence time of the feed material during the low, moderate, and severe SME conditions was determined during extrusion runs at steady state to verify the transit time of the material through the extruder barrel. Powdered red color dye was injected at the feeder inlet at t-0, and emerging samples were collected at 5-s intervals for 3 minutes. Samples were frozen at -20°C pending analysis of color intensity using a colorimeter (Chroma Meter CR-400, Konica Minolta, Ramsey, NJ). The resulting color concentration was quantified using the CIELAB color space model and to fit a distribution curve as described by Fichtali et al. 1995 and Iwe et al. 2001) and the mean residence time t in minutes was derived.

Bacteriological Enumeration

The viable colonies of *Bacillus coagulans* were determined using the microbiological enumeration assay described in USP Monograph FCC 10, First Supplement for *Bacillus coagulans* GBI-30, 6086 with modifications made to accommodate analysis of 50-g ration and kibble samples (Appendix I). The raw counts were expressed as colony forming units per gram of dry matter (CFU/g DM) and $\log_{10}$ transformed for statistical analysis.

Statistical Analysis

Processing data were analyzed using a general linear model from the (GLIMMIX procedure, SAS v. 9.4, SAS Institute, Inc., Cary, NC), with Fisher's least-significant difference post-hoc test. Results were considered significant at a level of 95% confidence ($\alpha = 0.05$). Day was designated as the blocking factor and a 30 min batch defined the experimental unit. Microbial data are expressed as the mean values and standard deviations after log transformation. Non-parametric Spearman rank-order correlation coefficients (r_s) were computed to explore the strength

and direction of the relationship between in-barrel moisture content (%), extruder shaft speed (rpm), die exit temperature (°C), barrel residence time (s), and extruder water flow (kg/h) with *Bacillus coagulans* reduction. Dryer data were analyzed as a completely randomized design using one-way analysis of variance (SAS v. 9.4, SAS Institute, Inc., Cary, NC) with three time-temperature treatments and Fisher's LSD test for mean comparisons.

Results and Discussion

Experiment 1: Extrusion Conditions

The processing measurements and calculations summarizing data from three replicates are given in Table 2.3. Although the processing was carried out in a pilot-scale extruder, the experimental conditions were selected to represent a range of settings that would be practical for the commercial production of pet food. Pet foods are typically produced under moderate moisture (<35% IBM) conditions in order to ensure adequate hydration of the material, gelatinization of starch, and acceptable kibble structure and quality (Varsha and Mohan, 2016; Pacheco et al., 2018). Operating at levels below 22% IBM is reported to increase SME in single-screw extrusion systems (Baller et al., 2018), whereas operating at greater than 36% tends to reduce flow viscosity and lower conversion of specific mechanical energy into heat (Chen et al., 2010).

Since moisture and extruder shaft speed are direct process inputs that can be controlled, the experimental extrusion conditions were aimed at generating a range from high level of mechanical stress (lowest moisture input/highest screw speed) to low mechanical stress (highest moisture input/lowest screw speed). The water injected into the preconditioner and extruder was at room temperature, and no steam was added at either stage to minimize the introduction of thermal energy into the system. During production, IBM decreased linearly (35.5%, 29.1%, and 27.8%) and extruder shaft speed increased linearly (401, 501, and 602 rpm) for the low, moderate, and severe

extrusion profiles, respectively. We were successful in creating low mechanical stress conditions for the low treatment (122 kJ/kg), however the moderate and high mechanical stress profiles generated a similar amount of SME (219 and 195 kJ/kg, respectively). We expect this was due to the level of extruder water inputs, which were not separated by the same magnitude for the three treatments. This was because in order to process at the highest screw speed of 600 rpm, a certain minimum water input was required to allow the material to flow continuously and prevent surging. Since we did not achieve a distinct separation of mechanical energies for the high and moderate extruder profiles, the following discussion will bear this in mind.

The enumeration results for *Bacillus coagulans* in the raw base rations, off the extruder, and off of the dryer are reported in Table 2.4. The two-way analysis of variance revealed that extruder SME ($P<0.0001$), but not ration inoculation dose ($P=0.9409$) nor the interaction ($P=0.6715$), influenced the survival of *Bacillus coagulans.* Overall mean $\log_{10}$ reduction was lower for the low SME extruder condition (0.44 ± 0.37 $\log_{10}$ CFU/g DM), compared to the moderate SME (2.15 ± 0.28 log CFU/g DM) and severe SME (2.67 ± 0.20 $\log_{10}$ CFU/g DM) conditions **(**Figure 2**)**.

There are several factors that influence the microbiological lethality of an extrusion system. For pet food extrusion, these include direct process inputs as well as intermediate variables such as specific mechanical energy (Bianchini et al., 2012). Likimani and Sofos (1990) proposed that the damage of spores during extrusion was primarily related to the die temperature and barrel residence time for a corn-soybean meal ration processed at 18% IBM. However, microorganisms have been reported to have high variability in their thermal resistance, even within the same species (O'Bryan et al., 2006). To explore the association of the process variables in our study, non-parametric Spearman rank order test was utilized to generate correlations to the log reduction of

Bacillus coagulans as a function of select process variables (Table 2.5). The results of this analysis provided *Bacillus coagulans* that extruder screw speed has a strong, positive monotonic association with $\log_{10}$ reduction of *Bacillus coagulans* (Figure 3), whereas extruder water inflow has a strong, negative monotonic association with $\log_{10}$ reduction of *Bacillus coagulans* (Figure 4).

Several reports documented an increased thermal resistance of pathogenic microorganisms in low-moisture environments (Anderson et al., 2017; Finn et al., 2013; Liu et al., 2018). However, in high-moisture dairy products *Bacillus coagulans* has also been observed to persist (Wang et al., 2009). Only when mild heat was applied in combination with an extended holding time (100 – 150 s) was a significant reduction in spore viability observed. Moisture has been shown to provide a protective effect on nutritional properties of foods, as well as prevent the formation of Maillard reaction products (Van Der Burgt et al., 1996; Van Rooijen et al., 2013). These effects are partly attributed to the changes in rheological properties of the melt, leading to a reduction in viscosity of the material as well as allowing water to absorb some of the heat generated by the friction of the screw (Chang et al., 1998). In principle, these events may also spare some of the impacts of thermal energy acting on the probiotic, thereby improving survival.

Experiment 2: Dryer Conditions

In this study, we aimed to investigate whether the dryer conditions might affect retention of *Bacillus coagulans* spores, because this step involves heat which may serve as an additional hurdle for probiotic microorganisms to overcome in order to remain viable in a finished product. For this experiment, the PHI ration was processed under moderate extrusion conditions, followed by drying under three time-temperature combinations (Table 2.6). The enumeration of *Bacillus coagulans* revealed similar retention for the three experimental treatments, with mean $\log_{10}$ CFU/g

reductions of 3.07, 2.91, and 2.58 for the LTlt, MTmt, and HTst conditions, respectively (Table 2.7). Greater variability in survival was observed for LTlt treatment relative to the MTmt and HTst treatment, however.

Extruded pet foods are dried to prolong shelf-life, optimize product density, and prepare the kibbles for subsequent enrobing with fat and/or flavor before packaging. Drying is a complex operation involving the transfer of heat and mass resulting in the removal of water from the semi-solid pet food kibbles. Most extruded pet food kibbles exit the extruder containing 20 – 30% moisture and a water activity that would promote the growth of spoilage microorganisms. The final moisture content when exiting the dryer is typically 10% or less, depending on the formula composition (i.e., whether or not the product contains humectants) and desired product texture (i.e., dry and crunchy, or soft-moist). Without proper drying, extruded petfood can begin to develop microbial and fungal growth within a matter of hours to days. The dryer settings can be modified, however the time and temperature settings are commonly optimized to reduce organoleptic changes and result in a final product with a target bulk density and moisture content.

Conveyor ovens such as the type used in this study rely on convection heating to dry wet kibble (Poireir, 2003). High-moisture pet food kibbles leaving the extruder are transported pneumatically to the dryer system inlet and distributed onto a perforated wire mesh bed. During transport to the dryer, the kibble may cool by several degrees and may lose a small fraction of moisture as steam flashes off of the hot kibble. Once in the dryer, hot air fueled by natural gas is forced through the mesh bed with the assistance of fans to circulate the air, allowing air to come into contact with the product. During the first phase of drying (a “constant drying rate” period), heat transfer from the air to the surface moisture increases the vapor pressure of the free (unbound) water in the product and the moisture evaporates and is carried away in the humidified air by an

exhaust fan. The amount of evaporation required to dry the product is dependent on the starting and final moisture content, and is independent of the rate of water removal (Prabhat K. Nema, Barjinder Pal Kaur, 2018). Once the kibble reaches a critical moisture content at which the surface moisture has been removed, the moisture located near the center of the pieces to migrate towards the surface. During this phase, the rate of moisture evaporation is reduced (a "falling rate period"), thereby increasing the temperature of the kibble (Poireir, 2003).

Drying is an essential process in the production of shelf-stable extruded pet foods, yet the dryer conditions are frequently overlooked in kill-step validation studies because the general strategy for preservation is preventing growth of spoilage microorganisms rather than destruction of pathogens (Amit et al., 2017). Microbial inactivation by drying is thought to occur mainly by thermal stress that result in DNA damage, cell wall deformation, and denaturation of microbial protein (Tripathi and Giri, 2014; Iaconelli et al., 2015). However, drying also introduces osmotic stress on microorganisms, which results when the cellular water concentration falls below a certain critical level. This can result in loss of cell membrane integrity and function. Gram-positive bacteria tend to be more resistant to osmotic stress compared to Gram-negative bacteria due to the differences in the structural composition and stabilization of their cell layers (Mille et al., 2005). In addition, drying may have different effects on different microorganisms (Terpou et al., 2019). For example, *Salmonella* and *Enterococcus faecium* have been reported to exhibit increased thermal resistance under low moisture conditions and reduced water activity (Liu et al., 2018). Because *Bacillus coagulans* is in a sporulated state, its survival capacity through drying is greater than that of vegetative cells (Janštová and Lukášová, 2001; Murrell and Scott, 1966). *Bacillus coagulans* has been reported to have D-values (the time required to achieve one log reduction in viable cell concentration) of 7.05 min at 95 °C, 2.56 min at 100 °C, 1.18 min at 105 °C, and 0.20

min at 110 °C in thermally processed tomato juice with a pH of 4.0, and 4.56 min at 100 °C, 1.20 min at 105 °C, 0.27 min at 110 °C, 0.07 min at 115 °C when at a pH of 4.3 (Peng et al., 2012). This work demonstrated a reduction in thermal resistance of *Bacillus coagulans* at a lower pH. No D-values have been established for *Bacillus coagulans* in complex semi-solid matrices such as pet food kibble. However, considering that the pH of the kibble (6.3-6.7) is higher relative to tomato juice (4.0-4.3), it is plausible that the D-values would increase at a given temperature.

In the present study, the kibbles exiting the extruder contained an average of 23% moisture content, which would require the removal of 15.3 kg of water for every 100 kg wet product dried to a final 10% moisture content. Because the kibbles contained a similar moisture level on entering the dryer, and retained similar physical characteristics (kibble size and bulk density), and chemical matrix (ingredient and nutrient composition), the amount of free (unbound water) removed from the material would also be similar. Because of this property, the cumulative thermal stress imposed on the *Bacillus coagulans* spores by evaporation would have been similar for the three treatments and explain these results. However, the amount of time spent in each drying phase (i.e., constant rate versus falling rate) may explain the variability in survival observed. For the LTlt treatment, the dryer air (66 °C) was below the boiling point of water. This suggests that the moisture transfer from the kibble to the air would have occurred at a slower rate, and would depend heavily on the ability of the air to contact the kibble equally throughout the dryer bed. For kibbles that were not uniformly distributed (i.e., either due to uneven spreading or location within the dryer) may have experienced less thermal stress compared to kibbles in a more exposed location. This may explain why more variability was seen in the LTlt relative to the other dryer conditions.

Conclusion

The results of the current study confirm that the extrusion process had a greater impact on *Bacillus coagulans* survival compared to the drying process. The greatest retention (< 1 $\log_{10}$ CFU/g DM reduction) was observed with an in-barrel moisture level of 35%, extruder shaft speed of 400 rpm, and calculated SME input of 129 kJ/kg. However, these conditions resulted in an extrudate with high bulk density and moisture content, presenting a challenge for maintaining a product of acceptable quality. Formulation levels of *Bacillus coagulans* for application in extrusion systems with lower in-barrel moisture, higher screw speeds, or higher specific mechanical energy inputs are recommended to account for a margin of at least 3-$\log_{10}$ CFU above the target number of viable cells in the finished product.

This study also demonstrated that extrusion processing parameters can be modified to influence the retention of *Bacillus coagulans* spores. However, the survival of spore-forming strains through extrusion should not be generalized to all microorganisms, vegetative cells in particular, since individual strains may respond differently to the same processing conditions. Since a relationship between specific mechanical energy and probiotic survival exists, and effects of thermal energy are well-documented, future researchers should consider the ratio of specific thermal:mechanical energy on probiotic survival through commercial pet food extrusion.

Author Contributions to the Chapter

HLA: experiment conduction, data and sample collection, sample analysis, statistical analysis, data interpretation, and manuscript preparation.

CGA: experiment design, data interpretation, and manuscript revision.

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Chapter 2 Figures

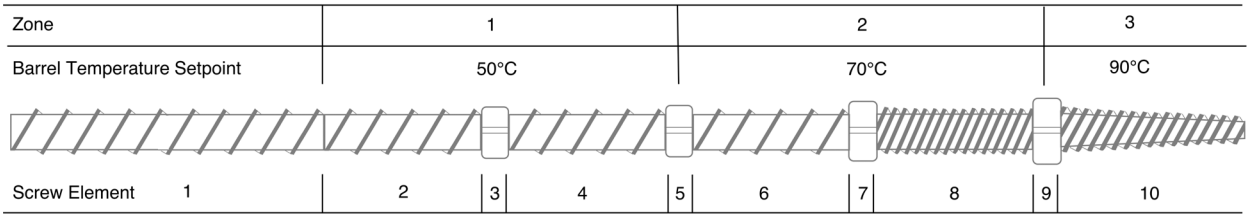

Figure 2.1 Schematic of extruder screw profile from feed end (left) to discharge end (right). Screw Element 1 = inlet screw, single flight; 2 = full pitch, single flight, uncut; 3 = steam lock, small diameter; 4 = full pitch, single flight, uncut; 5 = steam lock, small diameter; 6 = full pitch, single flight, uncut; 7 = steam lock, medium diameter; 8 = half-pitch, double flight, uncut; 9 = steam lock, large diameter; 10 = half-pitch, double flight, cone. Head Element 1 = inlet smooth; 2 = center spiral; 3 = center spiral; 4 = center spiral; 5 = center spiral; 6 = cone spiral; Total screw length: 825.5 mm.

Chapter 2 Tables

Table 2.1. Dry pet food base ration ingredient composition and proximate analysis on an as-is basis

Formulation	Amount
Ingredients	
Chicken Meal, %	38.30
Peas, Dehydrated, %	22.12
Sweet Potatoes, Flaked, %	22.12
Tapioca Flour, %	5.53
Pea Protein, %	5.53
Beet Pulp, %	3.32
Potassium Chloride, %	0.55
Salt, %	0.55
Dicalcium Phosphate, %	0.55
Titanium Dioxide, %	0.44
DL-Methionine, %	0.28
Choline Chloride, %	0.22
Fish Oil, %	0.17
Vitamin Premix[1], %	0.17
Trace Mineral Premix[2], %	0.11
Bacillus coagulans (15B CFU/g)	*
Analyzed Nutrient Composition	
Moisture, %	6.78
Crude Protein, %	34.10
Crude Fat, %	6.93
Crude Fiber, %	3.72
Ash, %	7.69
Nitrogen-Free Extract (NFE)[3], %	40.78

[1] 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, Folic Acid.

[2] 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, Ethylenediamine Dihydroiodide.

* Each experimental ration contained differing levels of *Bacillus coagulans* as reported in Table 2.

Table 2.2. Inoculation of four pet food base rations with *Bacillus coagulans* before processing.

	Inoculation Treatment[1]			
Bacillus coagulans Application	CON	PLO	PMD	PHI
Application Method	None	Base Ration	Base Ration	Base Ration
Amount in Formula[2], %	0.00	0.003	0.03	0.30
Log CFU/g in Base Ration	0.00	6.23	6.69	7.33
Total CFU/g in Base Ration	0.00	1.71×10^6	4.89×10^6	2.16×10^7

[1] Treatments: CON = control; PLO = probiotic low dose; PMD = probiotic moderate dose; PHI = probiotic high dose.
[2] *Bacillus coagulans* was added in a powdered form with 15 billion colony-forming units (CFU)/g (Kerry Inc., Beloit, WI, USA).

Table 2.3. Experiment 1 processing parameter means for three experimental extrusion specific mechanical energy (SME) conditions (N=36).

Process Parameter	Extrusion Treatment[1]			SEM[2]	*P*-value[3]
	Low SME	Moderate SME	Severe SME		
Raw Material					
Feed Moisture (%)	9.25	8.93	8.97		
Bulk Density (g/L)	566.02	582.21	579.19		
Feeder Screw Speed (rpm)	7.00	7.00	7.00		
Preconditioner					
Cylinder Speed (rpm)	400	400	400		
Steam Flow (kg/h)	0	0	0		
Water Flow (kg/h)	6.83	6.85	6.96		
Discharge Temp. (°C)	28.17	27.99	28.08	0.102	0.4635
Feed Moisture (%)	13.33^{b}	14.91^{ab}	16.44^{a}	0.819	0.0025
Extruder					
Screw Speed (rpm)	401.83	500.79	602.17		
Steam Flow (kg/h)	0	0	0		
Water Flow (kg/h)	19.58	11.68	9.97		
Knife Speed (rpm)	966.71	969.41	966.79		
Zone 1 Temp. (°C)	49.75	49.73	49.92		
Zone 2 Temp. (°C)	70.42	69.2	70.67		
Zone 3 Temp. (°C)	89.17	89.88	89.54		
Motor Load (%)	41.42^{c}	44.69^{a}	43.38^{b}	0.454	<0.0001
Power (kW)	6.28^{b}	9.43^{a}	9.81^{a}	0.387	<0.0001
Feed Moisture (%)	31.35^{a}	24.13^{b}	20.96^{c}	1.014	<0.0001
In-Barrel Moisture (%)	35.47^{a}	29.13^{b}	27.78^{c}	0.17	<0.0001
Bulk Density (g/L)	500.98^{a}	359.01^{b}	344.90^{b}	8.195	<0.0001
Die Exit Temp. (°C)	107.71^{b}	134.34^{a}	138.04^{a}	3.562	<0.0001
Wet Flow Rate (kg/h)	83.18^{a}	73.71^{b}	70.81^{b}	1.39	<0.0001
SME (kJ/kg)	122.12^{b}	219.30^{a}	195.12^{a}	8.728	<0.0001
Barrel Residence Time (s)	91.8	93.3	87.3		
Dryer					
Drying Temp. (°C)	107.22	107.22	107.22		
Drying Time (min)	16	16	16		
Cooling Time (min)	8	8	8		
Bulk Density (g/L)	465.75^{a}	337.13^{b}	334.08^{b}	10.076	<0.0001
Feed Moisture (%)	13.56^{a}	5.89^{b}	5.01^{b}	0.769	<0.0001

[1] Treatments: Low SME = 20 kg/h extruder water flow with 400 rpm screw speed; Moderate SME = 12 kg/h extruder water flow with 500 rpm screw speed; Severe SME = 10 kg/h extruder water flow with 600 rpm screw speed.
[2] SEM: standard error of the mean
[3] P-values represent Type III fixed effects of extrusion profile.

Table 2.4. Enumeration results for *Bacillus coagulans* survivors expressed as mean and standard deviation (SD) for log10 CFU/g on a dry matter (DM) basis (N=27).

		Sampling Point					
		Base Ration		Off-Extruder		Off-Dryer	
Inoculation Treatment[1]	Extrusion Treatment[2]	Mean	SD	Mean	SD	Mean	SD
PLO	Low SME	6.16	± 0.12	6.24	± 0.10	5.38	± 0.68
	Moderate SME	6.27	± 0.33	4.02	± 0.59	4.43	± 0.61
	Severe SME	6.06	± 0.31	3.39	± 0.25	3.61	± 0.48
PMD	Low SME	6.63	± 0.12	6.35	± 0.18	6.58	± 0.10
	Moderate SME	6.60	± 0.04	4.87	± 0.61	4.21	± 0.43
	Severe SME	6.78	± 0.14	3.64	± 0.23	3.95	± 0.26
PHI	Low SME	7.05	± 0.27	6.61	± 0.06	6.56	± 0.09
	Moderate SME	7.02	± 0.57	4.23	± 0.98	4.79	± 0.76
	Severe SME	7.26	± 0.67	4.09	± 0.46	4.52	± 1.19

[1] Treatments: PLO = probiotic low dose; PMD = probiotic moderate dose; PHI = probiotic high dose.

[2] Treatments: Low SME = 20 kg/h extruder water flow with 400 rpm screw speed; Moderate SME = 12 kg/h extruder water flow with 500 rpm screw speed; High SME = 10 kg/h extruder water flow with 600 rpm screw speed.

Table 2.5. Spearman correlation coefficients (rs) for select extrusion processing parameters and log10 reduction of *Bacillus coagulans* (n=27).

Process Parameter	r_s	*P*-value
In-Barrel Moisture, %	-0.65669	0.0002
Die Exit Temperature, °C	0.5528	0.0004
Specific Mechanical Energy (SME), kJ/kg	0.51291	0.0062
Barrel Residence Time, s	-0.20383	0.3078

[1]Spearman correlation coefficients with magnitude |0.00-0.19| are very weak; |0.20-0.39| are weak; |0.40-0.59| are moderate; |0.60-0.79| are strong; and |0.80-1.00| are very strong indicators of a monotonic association.

Table 2.6. Experiment 2 processing parameter means for three experimental dryer condition (N=9).

	Dryer Treatment[1]				
Process Parameter	LTlt	MTmt	HTst	SEM[2]	*P*-value[3]
Raw Material					
Feed Moisture (%)	9.07	9.10	9.09		
Bulk Density (g/L)	583.00	580.33	573.00		
Feeder Screw Speed (rpm)	7.00	7.00	7.00		
Preconditioner					
Cylinder Speed (rpm)	400.00	400.00	400.00		
Steam Flow (kg/h)	0.00	0.00	0.00		
Water Flow (kg/h)	6.63	7.07	6.75		
Discharge Temp. (°C)	28.00	28.00	28.00		
Feed Moisture (%)	16.21	14.97	17.52	0.878	0.2028
Extruder					
Screw Speed (rpm)	501.33	501.33	501.50		
Steam Flow (kg/h)	0.00	0.00	0.00		
Water Flow (kg/h)	11.42	10.78	11.60		
Knife Speed (rpm)	968.00	968.00	967.67		
Zone 1 Temp. (°C)	50.33	52.56	50.33		
Zone 2 Temp. (°C)	70.50	69.28	70.33		
Zone 3 Temp. (°C)	90.17	88.11	90.50		
Motor Load (%)	45.33	46.22	44.50	0.686	0.2815
Power (kW)	11.17	10.03	9.44	0.742	0.3707
Feed Moisture (%)	23.15	23.28	23.80	0.922	0.8744
In-Barrel Moisture (%)	28.83	28.68	29.11	0.278	0.5778
Bulk Density (g/L)	348.00	355.00	362.58	15.923	0.8164
Die Exit Temp. (°C)	133.67	133.06	133.17	1.573	0.9584
Wet Flow Rate (kg/h)	76.40	73.15	74.00	1.489	0.3441
Dryer					
Drying Temp. (°C)	65.56	107.22	148.89		
Drying Time (min)	46.00	16.00	10.00		
Cooling Time (min)	8.00	8.00	8.00		
Feed Moisture (%)	7.27[a]	5.90[a]	4.04[b]	0.432	0.0054
Bulk Density (g/L)	336.83	324.17	323.00	15.899	0.7991

[1] Treatments: LTlt = low temperature-long time (66°C for 46 min); MTmt = Moderate temperature-moderate time (107°C for 16 min); HTst = high temperature-short time (149°C for 10 min).
[2] SEM: standard error of the mean
[3] P-values represent Type III fixed effects of dryer treatment.

Table 2.7. Enumeration of *Bacillus coagulans* after extruding and drying under low temperature-long time (LTlt), moderate temperature-moderate time (MTmt), and high temperature-short time (HTst) dryer conditions (n=9).

	Dryer Treatment[1]				
Count Parameter	LTlt	MTmt	HTst	SEM[2]	*P*-Value[3]
Total CFU/g DM, Base Ration	6.62×10^7	1.69×10^7	6.05×10^6		
Total CFU/g DM, Off-Dryer	3.05×10^4	2.29×10^4	1.45×10^4		
Log_{10} CFU/g DM Reduction	3.07	2.91	2.60	0.4161	0.7322

[1] Treatments: LTlt = low temperature-long time (66°C for 46 min); MTmt = Moderate temperature-moderate time (107°C for 16 min); HTst = high temperature-short time (149°C for 10 min).
[2] SEM: standard error of the mean
[3] P-values represent Type III fixed effects of dryer treatment.

Chapter 3 - Evaluation of Graded Levels of *Bacillus coagulans* GBI-30, 6086 on Apparent Nutrient Digestibility, Stool Quality, and Intestinal Health Indicators in Healthy Adult Dogs

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 *Bacillus coagulans* on stool quality, nutrient digestibility, and intestinal health markers in healthy adult dogs. Extruded diets containing graded levels of probiotic 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 ten individually housed adult beagle dogs (7 castrated males, 3 spayed females) of similar age (5.75 ± 0.23 years) and body weight (12.3 ± 1.5 kg). The study was designed as 5 x 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 probiotic (PEX), and low, moderate, and high probiotic 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 to

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 probiotic treatment (PEX) or the coated probiotic treatments (PCL, PCM, and PCH) compared to CON. These results suggest that *Bacillus 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.

Introduction

Functional pet foods, such as those containing direct-fed microbials ("probiotics") are considered a key growth driver in the $36.9 billion market of dog and cat foods in the United States (Di Cerbo et al., 2017; APPA, 2020). Probiotics are defined as live bacteria, which when consumed at adequate levels provide a health benefit to the host (Hill et al., 2014). Foods containing probiotics are considered to be "functional" in that they offer enhanced health benefits beyond supplying essential nutrients when consumed on a regular basis (Hasler, 2002). In companion animal nutrition, probiotics 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 probiotics 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; Tzortizis et al., 2004; Yu et al., 2008; Bajagai et al., 2016). The most widely used probiotics 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 probiotic 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* probiotics 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)$\cdot d^{-1}$ resulted in a slight improvement to digestibility of dry matter, protein, lipid, and metabolizable energy, although the differences were not significant compared to a non-probiotic 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$\cdot d^{-1}$ and observed higher apparent digestibility of crude fat and nitrogen-free extract as well as a trend towards higher dry matter and organic matter digestibility compared to non-probiotic treated diets. Bastos et al. (2020) did not find improvements to nutrient digestibility in dogs in response to supplementation with *Bacillus subtilis* and *Bacillus licheniformis* at a dose of 7.47×10^6 CFU$\cdot d^{-1}$, however improvements in fecal scores and a reduction in fecal biogenic amines were observed. These investigations demonstrate variability in the effectiveness of *Bacillus* probiotics for improving nutrient digestibility and highlight the importance of identifying the minimal effective dosage of novel probiotic strains.

Of interest in this research area is *Bacillus coagulans* GBI-30, 6086, a novel probiotic 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 *Bacillus 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 *Bacillus 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 *Bacillus 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 3.1). Five experimental diets were developed to contain no probiotic (CON) or graded levels of *Bacillus 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 probiotic 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, 10^9 CFU

consumed per dog per day (Table 3.2). Food-grade TiO_2 (FDandC 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, *Bacillus 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 blended in a double-ribbon mixer for 5 min. The remaining four treatments were produced without probiotic 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 processing of all treatments and were recorded from the control panel output. Extruded kibbles were transported pneumatically from the extruder exit into a 3-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 *Bacillus coagulans* by blending the probiotic powder with the flavor digest in a paddle mixer for 5 min one week 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 *Bacillus coagulans* CFU was performed in triplicate at the Pet Food Microbiology and Toxicology Laboratory at Kansas State University following the procedures described in USP Monograph FCC 10, First Supplement for *Bacillus coagulans* GBI-30, 6086 with modifications made to accommodate analysis of 50 g kibble samples.

Animal Feeding

The feeding trial was conducted at the Kansas State University Large Animal Research Center where ten healthy adult Beagle dogs (3 spayed females, 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 m x 1.20 m) equipped with an acrylic-coated mesh floor to allow for 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 h light and 8 h dark for each 24 h cycle. Food allowance was controlled by pre-weighing portions for each animal and feeding twice daily (at 08:00 and 17:00 h) in equal portions at each meal. Orts were removed and weighed after 30 min of feeding. Initial food quantities on d 0 were determined by weighing the dogs and calculating the daily metabolizable energy requirement for inactive lab kennel dogs (95 x BWkg0.75) (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 x 5 replicated Latin square consisting of 5 periods with 16 d of acclimation to the diet followed by 5 d of total fecal collection for a total duration of 105 d. Random assignment of experimental treatments to each of the ten 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; 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). 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 total fecal collection (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 to 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:

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

Marker Method:

$$Nutrient\ Digestibility, \% = 1 - \frac{\%\ Nutrient\ in\ Feces\ x\ \%\ TiO_2\ in\ Food}{\%\ Nutrient\ in\ Food\ x\ \%\ TiO_2\ in\ Feces}\ x\ 100\%$$

Ammonia concentration in the fresh fecal samples was determined according to the colorimetric method described by Chaney and Marbach (1962). Fecal SCFA and BCFA content were determined by gas-liquid chromatography (Erwin et al., 1961) using a capillary column (15 m x 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. 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 absence of colony growth) and reached the intended concentrations in the inoculated diets (Table 3.2). 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 probiotics as a valuable component of their pet's diet. Part of the working definition of probiotics 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 *Bacillus 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; Endres et al., 2011). Studies with *Bacillus* spp. in dogs have investigated doses in the range of 6 to 9 $\log_{10}$ CFU·d^{-1} (Bastos et al., 2020; Schauf et al., 2019), 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 probiotics adds cost to products, identifying the minimum effective dose for a given strain is desirable.

The probiotic application methods we selected were aimed at representing commercially relevant practices used in the pet food industry. Adding probiotics 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 viability of probiotic 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 probiotic powder with the flavor digest for 5 min before coating was used to facilitate the uniform distribution of *Bacillus 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 probiotics into the base ration before extrusion is becoming increasingly common. For treatment PEX, a 0.03% (w/w) inclusion of the probiotic in the formula resulted in 4.58 x 10^6 CFU·g^{-1} of *Bacillus coagulans* in the base ration before extrusion, and a final count of 1.06 x 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 x 10^6 CFU·g^{-1}. To our knowledge, ours is the first study to evaluate both a heat-processed and direct coating application of probiotics to dogs.

Animals

All ten dogs remained healthy throughout the study as confirmed by veterinary staff. The mean BW of the dogs was 12.3 kg (range 10.8 – 13.8 kg) at d 0 and 13.5 kg (range 11.4 - 15.6 kg) at d 105. A paired t test indicated 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 metabolizable energy calculations (NRC, 2006), which may have overestimated their energy expenditure compared to 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 $g \cdot d^{-1}$ (P = 0.1364; Table 3.3). Because the probiotic dosage level was developed based on an expected 200 $g \cdot d^{-1}$ intake, this indicates at least 98.5% of the target dose of *Bacillus coagulans* CFU was consumed for each probiotic containing treatment. No differences in food intake were expected because the dietary treatments only differed with respect to the probiotic application method and dose.

Fecal Characteristics

Wet fecal output (range 113 – 126 g·d-1; P = 0.1356), fecal moisture content (range 69.7 - 70.25%; P = 0.6415), defecations per day (range 1.98 – 2.18; P = 0.3041), and fecal score (range 3.68 – 3.77; P = 0.5507) did not differ between treatments (Table 3.3). In humans, supplementation with *Bacillus coagulans* has demonstrated increased intestinal peristalsis and improved fecal scores in subjects with functional constipation (Minamida et al., 2015), as well as improve bowel movement frequency, shape, and color (Ara et al., 2002) when administered at a level 108 CFU·d-1. Animal studies with *Bacillus 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 that stool quality, moisture content, and defecation frequency would be maintained throughout the study. These results are supported by the findings of other probiotic strains fed to healthy adult dogs, with no observed changes compared to 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 ATTD of dry matter (range 80.3 – 81.5%; P = 0.3023), organic matter (range 84.8 – 86.1%; P = 0.1656), crude protein (range 83.1 – 84.1%; P = 0.3620), or gross energy (range 82.7 – 83.8%; P = 0.1938) between treatments (Table 3.4). However, ash digestibility varied widely across treatments (range 31.6 – 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 to 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 3.4). 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) were found, as well as a trend (increase of 1.96%; *P* = 0.0743) in crude protein digestibility for PCH compared to CON. However, Tukey's post-hoc test revealed that the protein digestibility means did not differ significantly.

Few studies have investigated the effects of probiotics 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 x 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 probiotic supplementation.

The mode of action of *Bacillus 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 *Bacillus 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 probiotics 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 *Bacillus coagulans* PSB-07 is known to depend on intrinsic and extrinsic factors (i.e., temperature, pH, carbon and nitrogen substrates), ranging from approximately 100 – 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 – 28,100 units/g of dietary protein (Behrman et al., 1969). There is also general agreement that probiotic effects *in vivo* are species and often strain specific (Rowland, 2010). Pasupathy et al. (2001) reported an increase in daily weight gain in growing puppies when supplemented with *Lactobacillus 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 $CFU \cdot d^{-1}$. However, Sun et al. (2019) reported no difference in apparent total tract digestibility when supplementing dogs with *Weissella cibaria* at levels up to 1.5×10^{11} $CFU \cdot d^{-1}$ compared to a non-probiotic 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 – 5.49; P = 0.4402), fecal ammonia concentration (range 94 – 107 $\mu mol \cdot g\ DM^{-1}$; P = 0.8414), total SCFA (range 171 – 197 $\mu mol \cdot g\ DM^{-1}$; P =

0.7924), and total BCFA (range 9 – 12 μmol·g DM^{-1}; P = 0.5766) were not different among treatments (Table 3.5). The relative proportions of acetate (range 52.2 – 54.0%; P = 0.6637), propionate (range 36.9 – 37.8%; P = 0.9212), butyrate (range 9.1 – 10.7%; P = 0.2327), isovalerate (range 44.7 – 52.2%; P = 0.1199), isobutyrate (range 32.3 – 36.5%; P = 0.2216), and valerate (range 14.8 – 23.0%; P = 0.1224) were also not different among treatments.

Many of the beneficial effects associated with probiotics 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 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 have 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 to *Lactobacillus* spp. that produce a

greater level of lactic acid. Among *Bacillus* strains, it has been reported that *Bacillus coagulans* tends to have improved lactic acid production efficiency compared to in *B. thermoamylovorans*, *B. licheniformis*, and *B. subtilis* in batch fermentation models (Poudel et al., 2015); however, most 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 to 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 probiotic (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 probiotics ("synbiotics") have demonstrated the ability to reduce fecal pH and ammonia and increase fecal SCFA (Swanson et al., 2002; Patra, 2017; Gagné et al., 2013; Strompfová et al., 2013; Markowiak and Ślizewska, 2018). Further investigation could be conducted in a low-oligosaccharide formula to determine if the probiotic fermentation activity of *Bacillus coagulans* may be detected.

Probiotic Application Methods

Overall, we did not find evidence to support that adding *Bacillus 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 to the non-probiotic control. Adding probiotics into a pre-blend before extrusion may offer manufacturing advantages such as simplified raw

material logistics, optimized mixing uniformity, and less need for specialized coating processes. However, it would seem counterproductive to intentionally subject a probiotic 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 et al., 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 months. Depending on the microorganisms of concern and intensity of heat treatment, the goal is to render pathogenic cells injured beyond repair while preserving viability of probiotics. 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, probiotic 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 to CON. These differences were not seen for PCL and PCM, however, which suggests that the minimum effective dose of *Bacillus 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 are 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 *Bacillus coagulans* at a higher dose before extrusion would not incite similar changes as coating the kibble with an equivalent number of CFU.

Conclusion

In summary, the current study provides evidence that supplementation with *Bacillus 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 probiotic to thermal treatment through extrusion and drying did not appear advantageous to the gastrointestinal health parameters measured in this study compared to a non-probiotic 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 probiotic 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 probiotic 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 *Bacillus 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.

Author Contributions to the Chapter

HLA: experiment conduction, data and sample collection, sample analysis, statistical analysis, data interpretation, and manuscript preparation.

CGA: experiment design, data interpretation, and manuscript revision.

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Chapter 3 Tables

Table 3.1. 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
Bacillus 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
Metabolizable Energy[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, 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, Ethylenediamine Dihydroiodide.
[4] Metabolizable Energy (ME) of diets was calculated using modified Atwater factors of 3.5, 3.5, and 8.5 kcal/g for

energy from crude protein, nitrogen-free extract, and crude fat, respectively (NRC, 2006).
*Each experimental diet contained differing levels of *Bacillus coagulans* applied as reported in Table 3.2.

Table 3.2. 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 x 10^6	0.00	0.00	0.00
Analyzed CFU·g^{-1} in Diet[4]	0.00	1.06 x 10^4	5.92 x 10^4	6.86 x 10^5	6.84 x 10^6
Dose (CFU·dog^{-1}·d^{-1})[5]	0.00	2.12 x 10^6	1.18 x 10^7	1.37 x 10^8	1.37 x 10^9

[1] CON = control; PEX = probiotic applied before extrusion; PCL = probiotic applied as coating at low dose; PLC = probiotic applied as coating at moderate dose; PCH = probiotic applied as coating at high dose.
[2] Formula inclusion as percent of batch weight with *Bacillus coagulans* added as a powder with 15 billion colony-forming units (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}

Table 3.3. 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 = probiotic applied before extrusion; PCL = probiotic applied as coating at low dose; PCM = probiotic applied as coating at moderate dose; PCH = probiotic applied as coating at high dose.
[2] *P*-value represents Type 3 Test of Fixed Effects for Diet

Table 3.4. Apparent total tract digestibility (ATTD) of dogs fed diets with differing levels of *Bacillus coagulans* estimated by total fecal collection (TFC) and titanium dioxide (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

^{abc} Means within a row with different superscripts differ ($P < 0.05$).
^{xy} Means within a row with different superscripts differ ($P < 0.10$).
[1] Treatments: CON = control; PEX = probiotic applied before extrusion; PCL = probiotic applied as coating at low dose; PCM = probiotic applied as coating at moderate dose; PCH = probiotic applied as coating at high dose.
[2] *P*-value represents Type 3 Test of Fixed Effects for Diet

Table 3.5. 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] Treatments: CON = control; PEX = probiotic applied before extrusion; PCL = probiotic applied as coating at low dose; PCM = probiotic applied as coating at moderate dose; PCH = probiotic applied as coating at high dose.
[2] Total short-chain fatty acids (SCFA) (acetate + propionate + butyrate); Individual SCFA are expressed as a percent of total SCFA.
[3] Total branched-chain fatty acids (BCFA) (isobutyrate + isovalerate + valerate); individual BCFA are expressed as a percent of total BCFA.
[4] *P*-value represents Type 3 Test of Fixed Effects for

Chapter 4 - Characterization of the Fecal Microbiome of Healthy Adult Beagle Dogs Supplemented with Graded Levels of *Bacillus coagulans* GBI-30, 6086

Abstract

Direct-fed microbials ("probiotics") are commonly added to companion animal diets with the goal of improving intestinal health, nutrient utilization, and immune health of pets. One of the proposed mechanisms by which probiotics exert their effects is by altering microbial composition in the colon through competition for nutrients, pathogen inhibition, and interaction with the host immune cells thereby influencing the functional capacity of the resident microbiota. This study aimed to evaluate the effect of *Bacillus coagulans* GBI-30, 6086 supplemented at graded doses on the fecal microbiome composition of healthy adult dogs. High-protein, grain-free extruded diets containing graded levels of probiotic applied before extrusion or as a topical coating were fed to ten individually housed adult beagle dogs (7 castrated males, 3 spayed females) of similar age (5.75 ± 0.23 years). The study was designed as 5 x 5 replicated Latin square with 16-d adaptation followed by 5-d total fecal collection for each period. The 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. Fresh fecal samples (n=50) were analyzed by 16S rRNA gene pyrosequencing. Community diversity was evaluated in R (v4.0.3, R Core Team, 2019). Relative abundance of select taxonomic levels were analyzed using the MIXED procedure in SAS (version 9.3, SAS Institute, Inc., Cary, NC) with treatment and period as fixed effects and dog as a random effect. Means were separated using a post hoc Tukey adjustment for multiple comparisons. Results were considered significant at $P<0.05$. Firmicutes comprised the greatest proportion of observational taxonomic units (mean

81.2% ± 5), followed by Actinobacteria (mean 9.9% ± 4.4), Bacteroidetes (mean 4.5% ± 1.7), Proteobacteria (mean 1.3% ± 0.7), and Fusobacteria (mean 1.1% ± 0.6). No evidence of a shift in predominant phyla, class, family, or genus taxonomic levels were found with the exception of the *Bacillus* genus, which was observed to have a greater relative abundance (P=0.0189) in the low probiotic coating (PCL) and high probiotic coating (PCH) treatment groups compared to the extruded probiotic (PEX) and non-probiotic control (CON) groups. Alpha-diversity indices (Richness, Chao1, ACE, Shannon, Simpson, Inverse Simpson, and Fisher) were similar for all treatments. Beta-diversity metrics (principal coordinate analysis and multi-dimensional scaling) did not provide evidence of clustering for UniFrac distances among treatment groups. Altogether, our data show that supplementation with *Bacillus coagulans* GBI-30, 6086 at a dose of up to 9 $\log_{10}$ CFU/day did not significantly alter the overall diversity of the fecal microbiome of healthy adult dogs over a 21-d period.

Introduction

The canine gastrointestinal tract contains an immensely diverse microbial community that consists of bacteria, archaea, fungi, viruses, and prokaryotes (Handl et al., 2011). An estimated 10^{14} microbial cells comprise the resident microbiota, with the highest density of cells (10^{11}) harbored in the colon (Hooda et al., 2012; Hoffmann et al., 2016; Honneffer et al., 2017). With the aid of rapidly evolving high-throughput DNA sequencing technologies, characterization of the fecal microbiome of humans and animals has helped identify several important roles of the gut microbiota in nutrient and energy utilization, mitigation of pathogens, improvement of intestinal health, and modulation of the immune system (Suchodolski, 2011). Manipulation of the microbiota through dietary modification (e.g. macronutrients, fiber sources, or food type) or ingestion of direct-fed microbials ("probiotics") has gained popularity as a strategy for therapeutic intervention in certain diseases associated with microbial disturbances, such as after antibiotic use, inflammatory bowel disease, acute gastroenteritis, obesity, and recently, aging (Pilla and Suchodolski, 2020). Probiotics are formally defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001; Hill et al., 2014). In addition to clinical importance in therapy for disease, probiotics are also commonly administered to healthy dogs with the goal of maintaining intestinal health, optimizing nutrient utilization, and supporting immune system function. One of the purported ways probiotics confer these effects is by positively influencing the resident microbiota through competition for substrates, production of anti-microbial compounds, reduction of luminal pH, and interaction with the host immune cells (Ng et al., 2009). However, the microbiota-modulating action of a given microorganism is highly dependent on the strain and dose being administered, which necessitates the evaluation of novel strains to verify efficacy (Azad et al., 2018).

Bacillus coagulans GBI-30, 6086 is a spore-forming bacterial strain well-suited for incorporation into commercially prepared foods (Konuray and Erginkaya, 2018). Previous work in our lab has demonstrated retention of spore viability through extrusion of pet food kibble. Improvements to gross energy and organic matter digestibility were also observed in adult dogs when supplemented at a level of 10^9 colony forming units (CFU) of *Bacillus coagulans* GBI-30, 6086 per day (Acuff and Aldrich, 2020). Furthermore, *Bacillus coagulans* has shown potential to alter microbial composition and function in humans, swine, and rodent models (Ara et al., 2002; Nyangale et al., 2015; Abhari et al., 2016; Wu et al., 2018). We hypothesized that the consumption of an adequate number of *Bacillus coagulans* CFU would increase the predominance of saccharolytic and proteolytic bacterial taxa in the canine fecal microbiota, thereby supporting the digestion of energy-yielding residues for the animal. No previous studies have examined the changes to the canine fecal microbiota that occur following supplementation with this novel probiotic strain. Therefore the objective of this research was to characterize the fecal microbiome of healthy adult dogs fed a grain-free, high-protein extruded pet food supplemented with graded doses of *Bacillus coagulans* GBI-30, 6086.

Materials and Methods

Ethics Statement

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

Experimental Diets

A grain-free high-protein pet food base ration was formulated to be nutritionally adequate for healthy adult dogs (AAFCO, 2020; Table 4.1). Raw materials were purchased from and

blended by a commercial mill (Fairview Mills, Bern, KS) with particle size reduced via hammer mill to pass a 2 mm screen before inoculation and extrusion. Five experimental diets were developed to contain graded levels of *Bacillus coagulans* applied either internally to the base ration or as a topical coating to the exterior of the kibble (Table 4.2). The experimental levels of probiotic were selected to achieve a minimum of one $\log_{10}$ separation between treatments with 0, 10^6, 10^7, 10^8, 10^9 CFU consumed per dog per day. Enumeration of viable organisms was performed at the Pet Food Microbiology and Toxicology Laboratory at Kansas State University (Manhattan, KS) following the microbiological enumeration assay described in USP Monograph FCC 10, First Supplement for *Bacillus coagulans* GBI-30, 6086 with modifications made to accommodate analysis of 50 g of kibble samples. Proximate analysis of the diets was completed at a commercial laboratory (Midwest Laboratories, Omaha, NE) to validate nutritional composition and obtain an estimate of caloric density before initiating the animal feeding study.

Probiotic Application Methods

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 x 10^{10} colony forming units (CFU) $\cdot g^{-1}$. For one dietary treatment (PEX), a target quantity of the organism was blended into the dry 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 blended in a double-ribbon mixer for 5 min. Inoculation of the base ration was carried out at the Bioprocessing Industrial and Value Added Products (BIVAP) facility at Kansas State University.

The remaining experimental diets contained no probiotic in the base ration, and instead were inoculated with graded levels of probiotic applied as a topical coating on the exterior of dried kibbles. A dry inoculation of the flavor digest was utilized; wherein, the target dose of the organism

was blended with the flavor digest in a paddle mixer for 5 min. Dried kibbles were sprayed with liquified chicken fat to reach a level of 8% of the batch by weight in a rotating barrel mixer. After application of the chicken fat, inoculated digest was sifted onto the rotating kibbles over a 5 min period at a level of 1% of the batch by weight. A negative control diet was assembled by coating uninoculated kibbles with chicken fat and uninoculated digest only. The sequence of coating proceeded from control (CON), extruded (PEX), low (PCL), moderate (PCM), and high (PCH) probiotic doses with a cleanout procedure between treatments to minimize carry over between treatments. Topical coating of all diets was completed in the Pet Food Processing Laboratory at Kansas State University.

Diet Production

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 processing of all treatments and were recorded from the control panel output.

Extruded kibbles were transported pneumatically from the extruder exit into a 3-pass horizontal wire belt dryer (Wenger Manufacturing Inc., Model 4800, Sabetha, KS). The product was dried at 110 °C for 8 minutes and 12 minutes of retention time for the first and second conveyor passes, respectively, followed by 10 minutes for a third pass in the ambient cooler. The diets were coated with chicken fat and either uninoculated or inoculated flavor digest as previously described. Coated diets were packaged in multiwall bulk kraft paper bags with a polyethylene

interior liner and stored in an indoor temperature-controlled location throughout the duration of the study.

Animal Feeding

The feeding trial was conducted at the Kansas State University Large Animal Research Center where ten healthy adult Beagle dogs (3 spayed females, 7 castrated males) of similar age (5.75 ± 0.23 yr), body weight (12.3 ± 1.5 kg), and body condition score (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 m x 1.20 m) equipped with an acrylic-coated mesh floor to allow for separation of urine and feces. The animals were maintained in a temperature-controlled (23 °C) modular building with automatic light timers set to 16 h light and 8 h dark for each 24 h cycle. Food was provided twice daily (at 08:00 and 17:00 h) in equal portions at each meal, and orts were weighed and recorded daily. Initial food quantities on d 0 were determined by weighing the dogs and calculating the daily metabolizable energy requirement for inactive lab kennel dogs (95 x $BW_{kg}^{0.75}$) (NRC, 2006). Throughout the study, body weight was recorded weekly and caloric portioning was adjusted ± 5% for the subsequent week to maintain body weight. Body condition score was recorded on the first and final day of the experiment. Water was provided for ad libitum consumption. Health of the dogs was assessed daily by veterinary staff and enrichment activities (grooming, exercise, group play, and one-on-one interactions) were scheduled once per week during the first two weeks of each experimental period.

Sample Collection

The study was conducted as a 5 x 5 replicated Latin square consisting of 5 periods with 16 d of acclimation to the diet followed by 5 d of total fecal collection for a total duration of 105 d. Dogs were randomly assigned to the five experimental diets over the course of five periods,

allowing each dog to serve as its own control. During each 5 d collection period, a fresh sample (within 15 minutes of defecation) was collected using a sterile Whirl-pak bag, and 2 g aliquots were transferred with a spatula into plastic microcentrifuge tubes and stored at -80 °C for pending fecal DNA extraction.

Fecal DNA Extraction and Sequencing

The DNA was extracted from 200 mg of each stool sample (n=50) using a QIAamp Power Fecal Pro DNA Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions (Handbook 02/2020). DNA concentration was assessed on a qbit (Qubit 2.0, Invitrogen by Life Technologies, Carlsbad, CA). The 16S V3/V4 gene was amplified using the Illumina 16S Metagenomic Sequencing library prep protocol (Illumina, Inc., San Diego, CA) as specified by the manufacturer.

Data Analysis

Raw reads were trimmed for quality using FastQC v0.11.9 with a cutoff length for paired end reads at 465 base pairs. The remaining reads were analyzed using Mothur v1.44.1 (Schloss et al., 2009). The unique 16S reads and output by Mothur, were then aligned to reference sequences from the SILVA rRNA database (Quast et al., 2013) for closed-reference observational taxonomic unit (OTU) assignment. Near-identical sequences were merged using VSEARCH v2.15.1 (Rognes et al., 2016).

Alpha-diversity was evaluated using observed unique sequences, Chao1, ACE, Shannon, Simpson, Inverse Simpson and Fisher indices. Beta-diversities of normalized OTU were determined by a principal coordinate analysis (PCoA) in R (v4.0.3, R Core Team, 2019). To determine the taxonomic differences between treatment groups, a MIXED protocol was performed in SAS (version 9.3, SAS Institute, Inc., Cary, NC) with treatment and period as fixed effects and

dog as a random effect. Means were separated with a post hoc Tukey adjustment for multiple comparisons. Results were considered significant at $P<0.05$.

Results and Discussion

Dietary Treatments

The ability of probiotics to impart health benefits to animals has prompted a rapid increase in the development of probiotic-containing pet foods, treats, and supplements in the marketplace (APPA, 2020; Di Cerbo et al., 2017). A grain-free high protein pet food formula was selected as the dietary format in order to represent pet foods which are likely to contain probiotics as a functional component. For the probiotic application methods we selected, adding probiotics 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 viability of the organism (Biourge et al., 1998; González-Forte et al., 2014; Rodrigues et al., 2020). However, given the resilience of sporulating microorganisms and mounting evidence that heat-killed microorganisms may still impart health benefits to the host (Hasegawa et al., 1996; Jensen et al., 2017; Piqué et al., 2019), an extruded treatment (PEX) was included to investigate the effects of a heat-processed application to the topical coating. *Bacillus coagulans* was not detected in the control diet (indicated by absence of colony growth) and reached the intended concentrations in the inoculated diets (Table 4.2), with a minimum of 1-log change in CFU achieved between doses. For treatment PEX, a 0.03% (w/w) inclusion of the probiotic in the formula resulted in 4.58×10^6 CFU·g^{-1} of *Bacillus 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}.

Animals

All ten dogs remained healthy throughout the duration of the study. The dogs had a mean BW of 12.3 kg (range 10.8 – 13.8 kg) at the start of the study and 13.5 kg (range 11.4 - 15.6 kg) at d 105. Mean body condition score remained the same (6.3 ± 1.2). The dogs consumed a similar amount of food across all treatments with a mean consumption of 197 ± 4 $g \cdot day^{-1}$. Because the probiotic dosage level was developed based on an expected 200 $g \cdot day^{-1}$ intake, this confirmed that a minimum of 98.5% of the target probiotic dose was consumed for each treatment. Part of the working definition of probiotics is based on the presence of an adequate amount of viable cells necessary to impart a benefit to the host. However, the efficacious amount 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 *Bacillus coagulans* GBI-30, 6086 for humans is between 1 x 10^8 and 3 x 10^9 CFUs with a no-observed-adverse-effect-level of 1.36 x 10^{11} $CFU \cdot bw_{kg}^{-1} \cdot d^{-1}$ (Endres et al., 2009). Studies with *Bacillus* spp. in dogs have investigated doses in the range of 6 to 9 $\log_{10}$ $CFU \cdot d^{-1}$ (Bastos et al., 2020; Schauf et al., 2019), so our treatments were designed accordingly to deliver graded doses that spanned the range reported in the literature.

Fecal Microbiota Community Composition

One of the fundamental goals of characterizing the composition of the microbiome is to determine which microorganisms are present and at what levels. Relative abundance is widely accepted and applied to describe how common or rare bacterial taxa occur in a particular microbial ecosystem (Morton et al., 2019). With this approach, the proportions of microorganisms as a percent of total observational taxonomic units are used in place of absolute counts because this allows quantification of organisms independent of the initial microbial load in the original sample. The information gained from a relative abundance profile allows the comparison of different

animals, under different experimental conditions, or states of health and disease (Shreiner et al., 2015). An understanding of the microbial community may also help in identifying their functional roles in contributing to health-promoting benefits for the host (Langille, 2018).

The relative abundance of bacterial phyla for each dog and treatment is illustrated in Figure 1. Overall, nine bacterial phyla were identified, among which Firmicutes comprised the greatest proportion of OTU (mean 81.2% ± 5.0), followed by Actinobacteria (mean 9.9% ± 4.4), Bacteroidetes (mean 4.5% ± 1.7), Proteobacteria (mean 1.3% ± 0.6), and Fusobacteria (mean 1.1% ± 0.6), and less than 1% of Patescibacteria, Deferribacterota, and Campilobacterota, Chlorobacteria. At the class taxonomic level, fifteen bacterial classes were identified, among which Clostridia represented the greatest proportion of OTU (52.6% ± 5.1), followed by Bacilli (24.4% ± 4.7), Actinobacteria (6.0% ± 3.8), Bacteroidia (4.6% ± 1.7), and Coriobacteriia (3.8% ± 1.1). At the family taxonomic level, sixty bacterial families were identified among which *Lachnospiraceae* represented the greatest overall abundance (30.4% ± 5.8), followed by *Erysipelotrichaceae* (16.0% ± 4.1), *Peptostreptococcaceae*, (10.6% ± 3.4), *Erysipelatoclostridiaceae* (4.6% ± 1.9), *Bifidobacteriaceae* (3.2% ± 4.3), *Bacteroidaceae* (2.8% ± 1.2), *Coriobacteriaceae* (2.4% ± 0.8), *Ruminococcaceae* (2.2% ± 0.9), *Clostridiaceae* (2.2% ± 1.2), *Prevotellaceae* (1.6% ± 0.8), *Eggerthellaceae* (1.3% ± 0.8), *Actinomycetaceae* (1.2% ± 0.8), *Fusobacteriaceae* (1.1% ± 0.5). There were 10.4% of OTU that were unclassified at the family level, and all remaining families each comprised less than 1% total OTU. More than one hundred bacterial genera were identified, among which *Holdemanella* comprised the highest proportion of OTU (6.7% ± 2.8), followed by *Turicibacter* (5.2% ± 3.1), *Bacteroides* (2.7% ± 1.2), *Catenibacterium* (2.3% ± 1.6), *Collinsella* (2.1% ± 0.7), *Allobaculum* (1.3% ± 0.7), *Actinomyces* (1.2% ± 0.8), *Erysipelatoclostridium* (1.1% ± 0.6), *Blautia* (1.1% ± 0.4), *Fusobacterium* (1% ±

0.4). There were 54.4% of OTU with unclassified genera, and all remaining genera each comprised less than 1% of total OTU.

Several researchers have attempted to define how a healthy microbiome compares to those affected by disease. However, high variation between individuals and confounding extrinsic factors (i.e. diet, environment, or medications) add complexity to assigning a baseline microbiome that encompasses all circumstances (Bäckhed et al., 2012). Much of our understanding of what constitutes a healthy microbiome comes from studying the microbial richness and diversity in healthy dogs. This has been the focus of several recent and extensive reviews, and the general consensus is that a healthy microbiome is characterized by a high level of species diversity and richness, and sustaining these attributes is beneficial to the animal's health (Deng and Swanson, 2015; Blake and Suchodolski, 2016; Barko et al., 2018; Huang et al., 2020; Pilla and Suchodolski, 2020). Disruption of the resident microbiota can lead to a state of dysbiosis characterized by poor microbial diversity, a reduction of health-promoting microorganisms, and increase in the relative abundance of pathogenic species (Suchodolski, 2016; AlShawaqfeh et al., 2017; Brüssow, 2020).

To our knowledge, the present study is the first to characterize the fecal microbiome of healthy adult dogs fed a high-protein grain-free extruded diet. Our results are in agreement those reported in the literature describing the five predominant phyla Firmicutes, Actinobacteria, Bacteroidetes, Fusobacteria, and Proteobacteria comprising 99% of the canine microbiota. Although the relative proportion of each differ considerably depending on the animal's diets which may elicit different effects depending on the diet format, nutrient composition (i.e., crude protein (CP) and crude fat (CF) content), as well as fiber and starch sources. The diet matrix and the extent to which it is digested and absorbed in the small intestine determines the load of protein, lipid, and starch residues that become substrates for fermentation in the colon, influencing the proliferation

of bacterial groups best suited to utilizing these substrates (Hooda et al., 2012; Wernimont et al., 2020). For instance, certain bacterial families, such as *Bifidobacteriaceae* and *Clostridiaceae* have been linked to protein and energy digestion in dogs (Zentek et al., 2003). Bermingham et al., (2017) also reported finding shifts in 27 bacterial families and 53 genera of healthy adult dogs fed a raw diet (76% CP, 18% CF, and 0.6% fiber as fed) compared to a commercial extruded food (with 30% CP, 27% CF, and 2% fiber as fed) over a 63 d study. Most notably, the relative abundances of *Bacteriodes*, *Prevotella*, *Peptostreptococcus* and *Faecalibacterium* was lower and *Fusobacterium*, *Lactobacillus* and *Clostridium* were all more abundant for the raw-fed group. Another interesting finding from their work was that *Clostridiaceae, Erysipelotrichaceae and Bacteroidaceae* were found to be highly correlated to fat and protein digestibility. In a similar experiment, Sandri et al. (2017) fed dogs raw diet (with 15% CP, 10% CF, and <1% fiber as fed) or a commercial extruded diet (with 24% CP, 9%, and 2% fiber as fed), and found a higher abundance of Proteobacteria and Fusobacteria in dogs fed the raw food. Kim et al. (2017) also evaluated the changes in fecal microbiota of dogs fed a raw diet (with 90% meat and 10% vegetables as fed) compared to commercial extruded diets (with 18-21% CP, and 8–10% CF, and 3-5% fiber as fed) and found that the number of OTU in the raw-fed group was significantly higher than in the commercial diet-fed group. Likewise, Herstad et al., (2017) reported an increase in *Clostridiaceae, Dorea, Coriobacteriales, Coriobacteriaceae,* and *Slackia* bacterial taxa in the feces of dogs fed a high minced beef diet (with 46% CP, 33% CF, and <1% fiber as fed) compared to a commercial extruded food (with 27% CP, 16% CF, and 1% fiber as fed). In a study evaluating several extruded prescription diets, Mori et al. (2019) observed a decrease in Actinobacteria and Firmicutes and an increase in Fusobacteria in dogs fed a weight loss diet (with 30% CP, 9.5% CF, 28% fiber) compared to a non-allergenic diet (with 18% CP, 16.5% CF, and 6% fiber). In more

recent research by Sandri et al. (2019), the abundances of *Erysipelotrichaceae incertae sedis*, *Eubacterium*, *Anaerobacter* and *Sarcina* was observed to increase in the feces of dogs fed raw diets (with 10-12% CP, 4% CF, and 1% fiber as fed) in comparison to an extruded control diet (with 24% CP, 15% CF, and 2% fiber as fed). In this study, the genera *Prevotella*, *Alloprevotella*, *Suttarella* were also lower in dogs fed a raw diet containing chickpeas, whereas *Lactobacillus* was lower in a raw diet containing peas. The addition of potato fiber in extruded diets (with 26% CP, 15% CF, and 11% fiber as fed) was also found to increase the Firmicutes phyla and *Faecalibacterium* genus in healthy dogs (Panasevich et al., 2015). Further investigation of fiber sources has also demonstrated microbiota modulation potential. Duysburgh et al. (2020) developed an *in vitro* system to emulate the canine microbial environment with the ability to test for shifts in bacterial taxa resulting from dietary changes such as introduction of fermentable fibers. Most notably, in a comparison of both *in vitro* and *in vivo* tests, Firmicutes increased in both models significantly when 3% (w:w) fructooligosaccharides from chicory root was evaluated. This supports the broad and influential role diet plays in shifting the composition of resident microbiota.

A key objective of our study was to determine if supplementation with *Bacillus coagulans* GBI-30, 6086 would induce changes in the relative abundance of bacteria at various taxonomic levels. Supplementation with probiotics is increasingly used as a strategy to modulate the microbiome, and *Bacillus spp.* are considered good candidates for inclusion in food products because of their enhanced survivability through food processing stresses and the gastrointestinal tract (Konuray and Erginkaya, 2018). The mechanisms by which probiotics can inhibit pathogen growth include lowering the luminal pH by producing organic acids (i.e. lactic acid and acetic acids), producing antibacterial compounds (i.e. hydrogen peroxide and bacteriocins); increasing extracellular secretion of mucin; interacting with immune cells; as well as by the competing for

nutrients within the intestinal lumen (Corr et al., 2009; Tizard and Jones, 2018). Probiotics within the *Lactobacillus spp*. and *Bifidobacterium spp*. families have been shown to have positive effects on microbial shifts in humans (Jones and Versalovic, 2009; Lee et al., 2003). In a study evaluating the fecal microbiota of healthy dogs supplemented with probiotic mixture containing *Streptococcus*, *Bifidobacterium*, and *Lactobacillus* strains, Rossi et al. (2020) observed a significant decrease in the abundance of *Clostridium perfringens* and a significant increase in the abundance of beneficial *Bifidobacterium* and *Lactobacillus* organisms. *Bacillus spp*. are known to produce several biologically active metabolites (i.e. bacteriolytic enzymes, bacteriocins, and antibiotics) with antibacterial and antifungal activity (Ozawa et al., 1979; Nagal et al., 1996; Urdaci and Pinchuk, 2004). Recently, *Bacillus coagulans LBSC* supplementation demonstrated positive modulation in a static human gastrointestinal *in vitro* model, most notably an increase in phyla such as Actinobacteria and Firmicutes, and a decrease of Bacteroidetes, Proteobacteria, Streptophyta and Verrucomicrobia (Maity et al., 2020). Supplementation of a high-protein diet (containing 60% casein) with *Bacillus coagulans* was shown to increase intestinal *Bifidobacteria* and decrease *Clostridia perfringens* in rats (Ara et al., 2002). Schauf et al. (2019) evaluated the effect of *Bacillus subtilis* supplemented with a low energy extruded diet (with 20% CP, 11% CF and 28% fiber) and observed an improvement to fat and starch digestibility, an increase in fecal short chain fatty acids, and a decrease in fecal ammonia of healthy dogs over a 28 d period. While the researchers did not analyze fecal samples for microbial DNA in this study, these findings suggest that there were changes occurring in the fermentation activities of the microbiota. Moreover, *Bacillus spp.* have also been shown to reduce pathogen colonization, improve feed conversion, and enhance weight gain in poultry, pigs, ruminants, and aquatic species (Mingmongkolchai and Panbangred, 2018; Al-Shawi et al., 2020). Kaewtapee et al. (2017) also

reported an increase in the abundance of *Roseburia spp*. when supplemented with *B. subtilis* and *B. licheniformis* in both high-protein and low-protein diets in pigs. Taken together, the results suggest that the taxonomic shifts observed may depend on the *Bacillus* species being utilized as well as the animal they are provided to.

In the present study, we did not detect a shift in any taxa with the exception of the *Bacillus* genus, which was observed to have a greater relative abundance (P=0.0189) in the PCL and PCH treatment groups compared to PEX and CON groups. Nyangale et al. (2014) similarly reported an increase in *Bacillus spp*. in an *in vitro* system using fecal inoculum from elderly humans supplemented with *Bacillus coagulans* compared to a placebo. Other studies have also corroborated that the species of supplementation may be detected, however the changes in the resident microbiota may not be prominent enough to produce microbial population shifts. Other reports in the literature have found similar results on supplementation with probiotics. Garcia-Mazcorro et al. (2011) investigated a synbiotic (containing 5 x 10^9 CFU of a mixture of seven probiotic strains, and a blend of fructooligosaccharides and arabinogalactans) that administered to healthy adult dogs daily for 21 d and did not observe a shift in predominant taxa except those which corresponded to the probiotic strains administered. Kelley et al. (2012) demonstrated that a *Bifidobacterium animalis* probiotic supplement effectively improved stool quality in kenneled German Shepherd dogs, however the only changes detected in the fecal microbiota detected was in the *Bifidobacterum* genus.

Available studies in dogs evaluating the effect of different *Bacillus spp.* probiotic on the fecal microbiota have shown a reduction in fecal counts of pathogenic *Clostridia spp.* (Biourge et al., 1998; González-Ortiz et al., 2013). Schauf et al. (2019) fed healthy adult dogs a commercial extruded diet (20.9% protein, 11% fat, and 2.8% crude fiber) comprised primarily of wheat and

meat and bone meal and reported a reduction in fecal pH, an increase in fecal short chain fatty acids, and a decrease in fecal branched chain fatty acids in dogs supplemented with *B. subtilis* at a level of 2.5 x 10^8 CFU consumed per dog per day. Based on the available evidence, it could be said that the dietary composition and format have a more profound effect on system level shifts in the microbiome compared to the introduction of a probiotic alone. In our study, all dogs were fed the same extruded diet, differing only in the probiotic application method and dosage added. Another possible reason for the lack of taxonomic shifts observed may be due to the use of healthy mature dogs in our study. Microbiota shifts in mature animals have been reported to be minor in comparison to young or old populations (Guard et al., 2017; Masuoka et al., 2017), which may be in part due to the long-term stability of established microbiomes as is reported in humans (Lozupone et al., 2012; Faith et al., 2013). The equilibrium of the microbiome appears to occur in puppy and kittens shortly after weaning, which coincides with the transition to a solid food diet (Burton et al., 2016). Once at homeostasis, the resilience of a microbial ecosystem that allows its initial functional or taxonomic composition to be restored following disturbances such as after diet changes, antibiotic treatment, increase in pathogen load from the environment or endogenous population overgrowth, or during periods of stress or disease (Sommer et al., 2017). Microbiomes with higher resiliency may explain why some animals are more prone to gut-related disorders (Fassarella et al., 2020). Researchers in this area have also acknowledged the notion of a tipping point, which is a critical aspect that determines when an abrupt shift in microbiota composition or functionality will change (Lahti et al., 2014). It is possible that the introduction of a transient probiotic elicits effects that are below the critical point necessary to stimulate a significant shift in the colon, due to the small relative abundance of ingested bacteria compared to the resident community (Derrien and van Hylckama Vlieg, 2015).

Alpha Diversity

In addition to studying relative abundance profiles, other methods of analysis are widely applied to describe microbiological data that originate in the field of community ecology (Gilbert and Lynch, 2019). Alpha diversity refers to the diversity within a sample and represents variation for each dog's microbiome. There are a number of computed indices that have been proposed to help describe the number of species in a sample ("richness") and how dominant or rare each species is relative to the others ("diversity") (Wagner et al., 2018). Among these, the number of OTU represents the number of clusters of 16S rDNA sequences that meet a 97% or higher threshold of identity. The similarity of the sequences is treated as a unique taxonomic unit of a bacterial species, and the number of sequences in each cluster represent the count of OTU in each sample and provide an estimate of species richness in each sample. OTU are quantifiable data that can be used to compute various alpha diversity indices. The Shannon Diversity Index accounts for both richness and diversity by measuring both the number of species and the inequality between species abundances. A large value indicates the presence of many species with evenly distributed abundances (Shannon and Weaver, 1949). The Simpson Index in contrast places more weight on the species diversity rather than richness, with a value between 0 and 1 indicating the probability that two randomly chosen OTU are from the same species. A smaller Simpson index value indicates a higher diversity estimation (Simpson, 1949). The Inverse Simpson Index is a closely related estimator that represents the probability that the richness of a community with uniform evenness would have the same level of diversity as a randomly chosen sample. Samples with a higher Inverse Simpson Index value are regarded as having higher diversity (Hill, 1973). Other indices we chose to include are the abundance-based coverage estimator (ACE) index which estimates the species richness after correction; a higher ACE value indicates a higher richness

(Chao and Lee, 1992). Fisher's Index is another richness estimator that incorporates the relationship between the number of species present and the number of individuals in a random sample from the population (Fisher et al., 1943). The last parameter presented in our data is the Chao1 Index, which estimates diversity using the most abundant taxa's. It is a nonparametric method for estimating the number of species in a community (Chao, 1984). The greater the Chao1 Index, the higher the expected species richness of the microbiota.

In the present study, alpha-diversity indices (Richness, Chao1, ACE, Shannon, Simpson, Inverse Simpson, and Fisher) were similar for all treatments (Table 4.4), suggesting that supplementation with the probiotic did not influence the species richness or diversity within the samples. Interestingly, the majority of studies comparing the fecal microbiome of healthy adult humans supplemented with probiotics have also shown no changes to alpha diversity, richness, or evenness when compared to a placebo. Schmidt et al. (2018) found no significant differences in alpha diversity measures between groups fed a bones and raw food (BARF) diet compared to a commercial canned or extruded foods, despite the BARF group consuming a higher amount of protein and fat. Similarly, Pilla et al. (2019) found that a synbiotic *Enterococcus faecium* did not alter alpha-diversity in dogs with enteropathy. On the other hand, Algya et al. (2018) observed that alpha-diversity of fecal microbial communities were lower in dogs fed grain-free roasted, refrigerated, and raw foods compared to extruded diets. Collectively, these results suggest that even though shifts in predominant bacterial taxa may be detected, these shifts do not necessarily correspond to changes to the richness or diversity metrics of the animal's microbiome.

Beta Diversity

Beta-diversity is another ecologically rooted approach used to study microbial community compositional differences among multiple sample groups (Whittaker, 1960). Among commonly

used beta-diversity metrics, principal coordinates analysis (PCoA) is a helpful ordination technique that allows the samples relationships to be plotted on a 2-dimensional scatterplot, with the axes representing fractions of variability. Each point on the scatterplot represents a single sample, and the distance between points represents how compositionally different the samples are from each other (Koleff et al., 2003). The distance between sample points can be computed by calculating the fraction of branch lengths of a combined phylogenetic tree that are not shared between two communities without regard to abundance of the microorganisms, providing a qualitative measure known as unweighted UniFrac (Lozupone and Knight, 2005; Lozupone et al. 2007). Consequently, PCoA plots can be interpreted as samples with high similarity appearing as clusters, and samples with high dissimilarity appearing randomly dispersed (Goodrich et al. 2014). Since PCoA can lead to linear bias in the plot configurations, we also chose to use non-metric multi-dimensional scaling (MDS), an algorithm that ranks the pair-wise distances between samples and maps them onto a two-dimensional plot that provides similar clustering visualization without requiring the dissimilarities between samples to be linear in nature (Ramette, 2007; Mbareche et al., 2020).

The results of PCoA (Figure 2A) and MDS (Figure 2B) plotting in our study did not provide evidence of clustering for the unweighted UniFrac distances among our five treatment groups. Most studies assessing beta-diversity in the fecal microbiome of dogs have focused on dietary interventions such as food format, novel ingredients, ingredient composition. For instance, Algya et al. (2018) found that there was a tendency for clustering of UniFrac distances, but a clear difference was not detected between dogs fed raw, grain-free, and extruded diets. In contrast, Schmidt et al. (2018) did find significant clustering through PCoA plots in the evaluation of dogs fed raw diets compared to dogs fed a commercial extruded food. Jarett et al. (2019) reported no

differences in PCoA using Bray-Curtis dissimilarity in dogs fed 0 or 24% cricket meal over a 29 d period. Kim et al. (2017) used principal component analysis (PCA) to assess beta diversity and found evidence of clustering along principle component 2 but not principle component 1 using Fast UniFrac distance metrics for dogs fed either commercial extruded diets or a raw (bones, raw meat, and vegetables). They noted that the natural group was also characterized by higher richness and diversity compared to the commercial diet fed group. Only a limited number of studies have investigated the effect of probiotic supplementation on beta-diversity in dogs, and they have reported mixed results. Rossi et al. (2020) did not observe changes to beta-diversity in dogs supplemented with a multi-strain probiotic (containing *Streptococcus thermophlus, Bifidobacterium lactis,* and several *Lactobacillus* species) over a 60 d supplementation period while consuming a commercial extruded food as the base diet. Similarly, Pilla et al. (2019) found no changes when supplementing a synbiotic (containing Enterococcus faecium, fructooligosaccharides, and gum Arabic) in dogs with food-responsive chronic enteropathy over a 6 week period compared to a placebo control group. Interestingly, Jha et al. (2020) reported a significant shift in beta-diversity for PCoA4 but not PCoA1-3 plots for healthy dogs fed a synbiotic multi-strain probiotic (that included *L. reuteri, P. acidilactici, E. faecium, L. acidophilus, B. animalis , L. fermentum,* and *L. rhamnosus* with inulin) over a 28 d period. This had a small effect on the overall gastrointestinal microbiome composition. Xu et al. (2019) reported that both young (<8 months old) and elderly (60-156 months old) dogs were clearly distinguished from a control group after 60 d of supplementation with a multi-strain probiotic (containing *L. casei, L. plantarum, and B. animalis*); however these differences were not observed in dogs of early adulthood age (9 – 24 months). This further supports the idea that the extent to which probiotics may be able to impart a benefit could in part be related to the age of the pet.

Conclusion

This study investigated the effects *Bacillus coagulans* GBI-30, 6086 added to grain-free high-protein extruded diets at graded levels has on the fecal microbiome of healthy adult dogs. No evidence of significant alternations in the resident bacterial taxa or changes in alpha and beta diversity indices over the 21 d supplementation periods were observed. However, a transient increase of the *Bacillus* genus administered was noted. Our results suggest that probiotic supplementation in healthy adult dogs allows for the maintenance of the compositional, diversity, and richness indices in the gut microbiota, and the maintenance of a stable microbiome may be viewed as a benefit of probiotic consumption. The findings of this study may help to guide consumers, veterinarians, and researchers on the supplementation of probiotics in healthy dogs. Furthermore, while the focus of this work was on detecting shifts in predominant taxa and indicators of bacterial richness and diversity, the benefits of probiotics for healthy individuals are not limited to modulation of the microbiota. The microbiota-modulating effect of *Bacillus coagulans* may be enhanced in populations where dysbiosis is common, such as puppies during weaning, in dogs with underlying gastrointestinal disorders, after treatment with antibiotics, or in aging pets. More research regarding the use of probiotics in commercial pet foods is necessary to develop a stronger body of evidence to support their utility in both health and unhealthy population groups.

Author Contributions to the Chapter

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HLA and CGA designed the study, performed the experiment, alpha diversity statistical analysis, and wrote the manuscript.

MPH and PPH provided technical support with DNA extraction and quantification.

TD, AL, TNG, and RMP performed PCR, 16S rRNA sequencing, library preparation, bioinformatics analysis including constructing principal coordinate analysis (PCoA) plots.

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Chapter 4 Figures

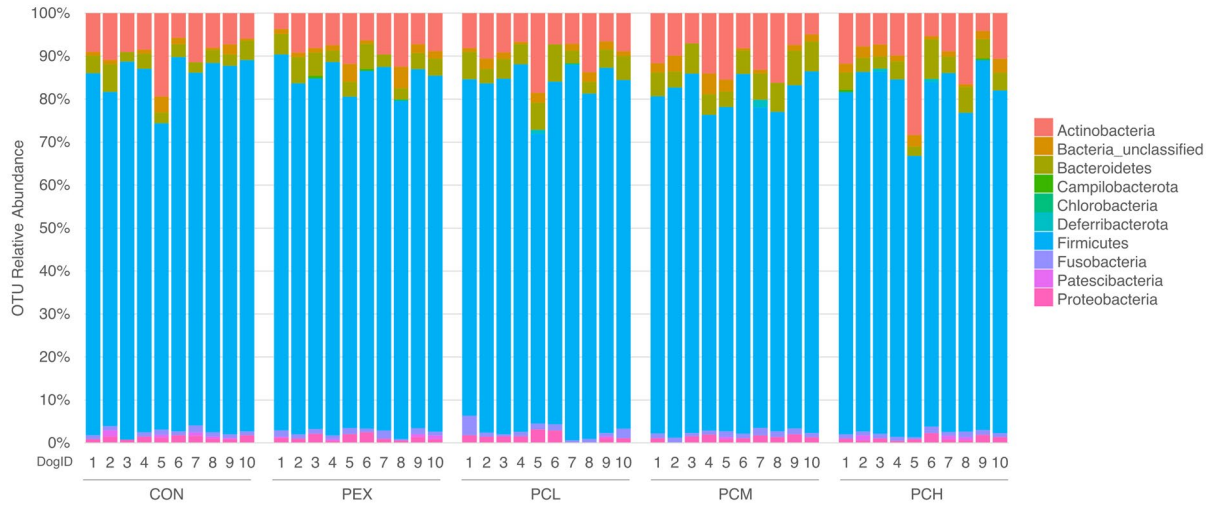

Figure 4.1. Relative abundance of bacterial phyla in fecal samples from health adult dogs (n=10) fed supplemented with graded levels of *Bacillus coagulans* GBI-30, 6086. Treatments: CON = control; PEX = probiotic applied before extrusion; PCL = probiotic applied as coating at low dose; PLC = probiotic applied as coating at moderate dose; PCH = probiotic applied as coating at high dose.

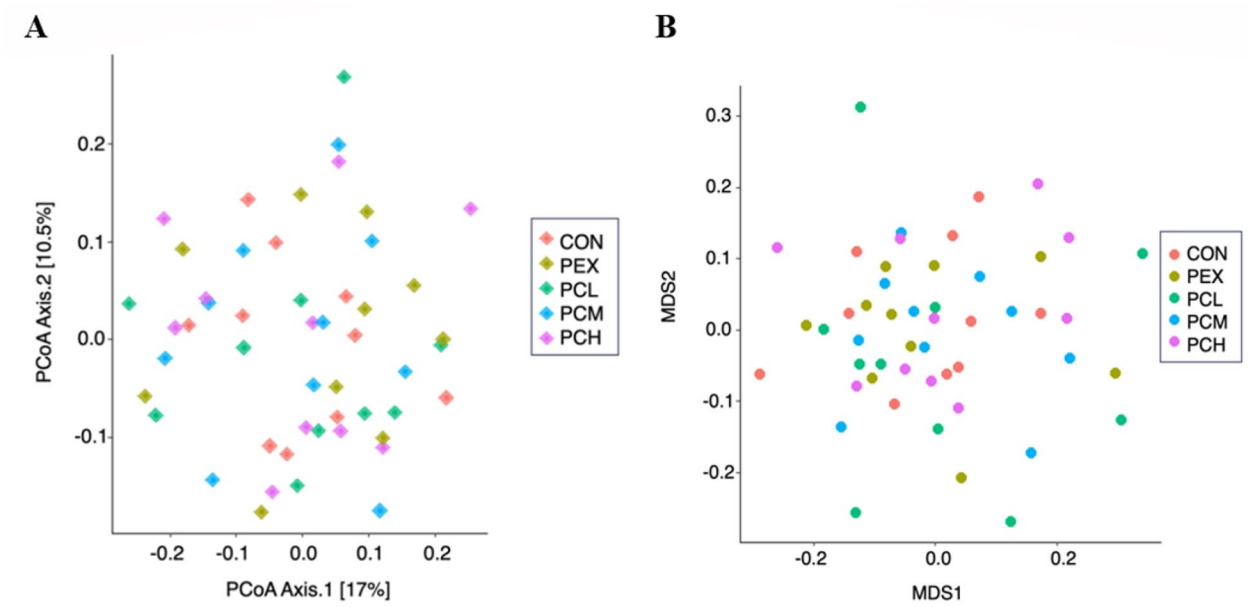

Figure 4.2. Principal coordinate analysis (PCoA) explaining 17% and 10.5% of the variability in OTU (A) and non-metric multi-dimensional scaling (MDS) (B) plots of Bray-Curtis UniFrac distances for samples of the five probiotic treatment groups. Treatments: CON = control; PEX = probiotic applied before extrusion; PCL = probiotic applied as coating at low dose; PLC = probiotic applied as coating at moderate dose; PCH = probiotic applied as coating at high dose.

Chapter 4 Tables

Table 4.1. Ingredient composition and proximate analysis (as-is basis) of a grain-free pet food formula produced to evaluate the effect of *Bacillus coagulans* on the fecal microbiome of healthy adult dogs.

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
Bacillus coagulans (15B CFU·g^{-1})[4]	*
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
Metabolizable Energy[5], 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, 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, Ethylenediamine Dihydroiodide.
[4] *Bacillus coagulans* in powdered form with 15 billion colony-forming units (CFU)·g^{-1} (Kerry, Inc., Beloit, WI, USA)
[5] Metabolizable Energy (ME) of diets was calculated using modified Atwater factors of 3.5, 3.5, and 8.5 kcal/g for energy from crude protein, nitrogen-free extract, and crude fat, respectively (NRC, 2006).
*Each experimental diet contained differing levels of *Bacillus coagulans* applied as reported in Table 2.

Table 4.2. 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 x 10^6	0.00	0.00	0.00
Analyzed CFU·g^{-1} in Diet[4]	0.00	1.06 x 10^4	5.92 x 10^4	6.86 x 10^5	6.84 x 10^6
Dose (CFU·dog^{-1}·d^{-1})[5]	0.00	2.12 x 10^6	1.18 x 10^7	1.37 x 10^8	1.37 x 10^9

[1] CON = control; PEX = probiotic applied before extrusion; PCL = probiotic applied as coating at low dose; PLC = probiotic applied as coating at moderate dose; PCH = probiotic applied as coating at high dose.
[2] Formula inclusion as percent of batch weight with *Bacillus coagulans* added as a powder with 15 billion colony-forming units (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}

Table 4.3. Predominant bacterial phyla, class, family, and genus (expressed as percentage of sequences) in feces of dogs fed graded doses of *Bacillus coagulans* GBI-30, 6086.

				Treatment[1]						
Phylum	Class	Family	Genus	CON	PEX	PCL	PCM	PCH	SEM[2]	*P*-Value[3]
Actinobacteria				10.60	10.55	9.63	9.35	9.33	1.474	0.9441
	Actinobacteria			6.33	6.97	5.76	5.52	5.24	1.269	0.8787
		Actinomycetaceae	Actinomyces	1.28	1.12	1.36	1.02	1.05	0.272	0.8832
		Bifidobacteriaceae		3.29	4.19	3.09	2.94	2.55	1.396	0.9418
			Bifidobacterium	0.49	0.70	0.75	0.74	0.68	0.119	0.5277
		Corynebacteriaceae	Corynebacterium	1.00	0.61	0.68	0.68	0.66	0.184	0.5891
		Microbacteriaceae		0.38	0.51	0.28	0.24	0.50	0.101	0.2319
			Leucobacter	0.33	0.51	0.28	0.24	0.45	0.097	0.2617
		Propionibacteriaceae		0.11	0.29	0.06	0.19	0.25	0.099	0.4540
	Coriobacteriia			4.09	3.59	3.86	3.79	4.04	0.374	0.8766
		Coriobacteriaceae		2.33	2.24	2.39	2.49	2.28	0.250	0.9592
			Collinsella	1.89	2.03	2.06	2.26	2.06	0.234	0.8586
		Eggerthellaceae		1.39	1.19	1.03	1.19	1.65	0.276	0.5760
			Slackia	0.73	0.76	0.55	0.80	0.99	0.103	0.0678
Bacteroidetes				4.25	4.88	3.79	5.41	4.44	0.496	0.2063
	Bacteroidia			4.25	4.88	3.79	5.41	4.44	0.496	0.2063
		Bacteroidaceae	Bacteroides	2.41	3.27	2.24	3.03	2.79	0.343	0.2077
		Prevotellaceae		1.67	1.44	1.29	2.08	1.46	0.209	0.0934
			Alloprevotella	0.75	0.54	0.65	0.84	0.65	0.109	0.3572
			Prevotella	0.43	0.62	0.43	0.58	0.59	0.081	0.2707
			Prevotellaceae GA6A1 Group	0.38	0.28	0.21	0.49	0.23	0.090	0.1552

				Treatment[1]						
Phylum	Class	Family	Genus	CON	PEX	PCL	PCM	PCH	SEM[2]	*P*-Value[3]
Firmicutes				81.43	80.21	81.57	80.83	81.81	1.630	0.9582
	Bacilli			23.12	23.92	25.42	23.12	26.17	1.469	0.4807
		Bacillaceae		0.71	0.44	0.70	0.41	1.08	0.178	0.0769
			Bacillus	0.13[ab]	0.00[b]	0.34[a]	0.04[b]	0.32[a]	0.087	0.0189
			Oceanobacillus	0.53	0.40	0.30	0.33	0.59	0.129	0.4224
		Enterococcaceae	Enterococcus	0.07	0.09	0.13	0.15	0.22	0.099	0.8405
		Erysipelatoclostridiaceae		4.22	4.78	4.25	4.51	5.21	0.616	0.7725
			Candidatus Stoquefichus	0.22	0.24	0.24	0.23	0.18	0.094	0.9896
			Catenibacterium	2.26	2.19	2.30	2.27	2.32	0.545	0.9999
			Erysipelatoclostridium	0.89	1.34	0.93	0.96	1.43	0.200	0.1851
			Erysipelotrichaceae UCG-003	0.77	0.89	0.59	0.82	1.07	0.225	0.6612
		Erysipelotrichaceae		14.97	15.76	17.41	15.32	16.55	1.356	0.7157
			Allobaculum	1.62	1.09	1.53	1.04	1.10	0.237	0.2666
			Faecalitalea	0.98	1.04	0.75	0.91	0.83	0.126	0.5343
			Holdemanella	5.87	7.03	6.66	6.36	7.56	0.876	0.7093
			Turicibacter	5.13	5.01	6.32	4.89	4.55	1.035	0.7908
		Gemellaceae	Gemella	0.17	0.29	0.06	0.10	0.03	0.068	0.0708
		Lactobacillaceae		0.77	0.72	0.89	0.80	0.66	0.191	0.9295
			Lactobacillus	0.77	0.72	0.80	0.75	0.66	0.189	0.9869
		Leuconostocaceae		0.46	0.31	0.19	0.25	0.32	0.108	0.5069
			Leuconostoc	0.24	0.14	0.10	0.15	0.08	0.075	0.6193
			Weissella	0.22	0.17	0.09	0.10	0.24	0.068	0.4003

				Treatment[1]						
Phylum	Class	Family	Genus	CON	PEX	PCL	PCM	PCH	SEM[2]	*P*-Value[3]
		Staphylococcaceae		0.24	0.10	0.08	0.24	0.20	0.083	0.4745
			Staphylococcus	0.24	0.06	0.08	0.24	0.17	0.079	0.3446
		Streptococcaceae		0.63	0.58	0.55	0.68	0.93	0.182	0.5950
			Streptococcus	0.63	0.58	0.55	0.63	0.89	0.176	0.6592
	Clostridia			55.13	53.15	50.97	52.37	51.59	1.659	0.4454
		Anaerovoracaceae		0.39	0.65	0.58	0.36	0.46	0.115	0.3229
			Anaerovoracaceae	0.22	0.37	0.37	0.31	0.42	0.107	0.7151
		Butyricicoccaceae		0.67	0.79	0.68	0.73	0.81	0.094	0.7704
			Butyricicoccus	0.67	0.79	0.62	0.69	0.72	0.098	0.8072
		Clostridia UCG-014		0.48	0.46	0.26	0.45	0.45	0.089	0.3963
		Clostridiaceae		2.59	2.00	2.10	1.77	2.49	0.377	0.5116
			Clostridium Sensu Stricto 1	0.65	0.91	0.73	0.75	0.96	0.133	0.4348
		Eubacteriaceae	Eubacterium	0.39	0.37	0.24	0.18	0.42	0.115	0.5101
		Lachnospiraceae		31.74	30.18	27.95	32.53	29.50	1.865	0.4457
			Anaerocolumna	0.19	0.03	0.07	0.11	0.21	0.070	0.3417
			Blautia	0.93	1.12	0.98	1.22	1.12	0.123	0.4746
			Howardella	0.50	0.52	0.31	0.58	0.55	0.102	0.3795
			Lachnospira	0.34	0.28	0.22	0.29	0.25	0.091	0.9204
			Roseburia	0.14	0.21	0.08	0.21	0.12	0.084	0.7479
			Tyzzerella	0.63	0.81	0.75	0.83	0.82	0.106	0.6643
		Peptostreptococcaceae		0.12	0.10	0.11	0.09	0.11	0.010	0.2448
			Peptostreptococcus	0.09	0.23	0.10	0.05	0.08	0.075	0.4890
			Romboutsia	0.49	0.62	0.46	0.55	0.59	0.067	0.3994

				Treatment[1]						
Phylum	Class	Family	Genus	CON	PEX	PCL	PCM	PCH	SEM[2]	*P*-Value[3]
			Terrisporobacter	0.15	0.28	0.11	0.14	0.26	0.125	0.8272
		Peptostreptococcales-Tissierellales		0.40	0.56	0.36	0.20	0.45	0.156	0.5657
		Ruminococcaceae		2.05	2.42	2.08	2.44	2.20	0.308	0.8375
			Faecalibacterium	0.55	0.62	0.55	0.55	0.68	0.085	0.7422
			Fournierella	0.53	0.62	0.53	0.58	0.62	0.070	0.7976
	Negativicutes			0.66	1.01	0.65	1.23	0.93	0.253	0.4497
		Acidaminococcaceae	Phascolarctobacterium	0.17	0.16	0.10	0.13	0.09	0.072	0.8884
		Selenomonadaceae		0.49	0.85	0.55	1.10	0.85	0.231	0.3407
			Megamonas	0.49	0.81	0.55	0.99	0.77	0.197	0.3717
Fusobacteria				0.88	1.27	1.16	1.17	1.23	0.201	0.6817
	Fusobacteriia			0.88	1.27	1.16	1.17	1.23	0.201	0.6817
		Fusobacteriaceae		0.88	1.23	1.07	1.17	1.15	0.169	0.6253
			Fusobacterium	0.84	1.17	0.71	1.09	1.04	0.122	0.0680
Proteobacteria				1.09	1.31	1.11	1.51	1.46	0.222	0.5539
	Gammaproteobacteria			0.97	1.28	1.11	1.51	1.46	0.208	0.3122
		Enterobacteriaceae		0.29	0.52	0.34	0.45	0.54	0.111	0.4371
			Escherichia-Shigella	0.25	0.52	0.34	0.36	0.54	0.097	0.1823
		Succinivibrionaceae		0.38	0.49	0.38	0.57	0.54	0.099	0.5338
			Anaerobiospirillum	0.34	0.43	0.32	0.40	0.40	0.091	0.9101
		Sutterellaceae		0.20	0.27	0.34	0.39	0.28	0.090	0.6131
			Sutterella	0.20	0.23	0.25	0.34	0.28	0.082	0.7739

[abc] Means within a row with different superscripts differ (P < 0.05).

[1] Treatments: CON = control; PEX = probiotic applied before extrusion; PCL = probiotic applied as coating at low dose; PCM = probiotic applied as coating at moderate dose; PCH = probiotic applied as coating at high dose.

[2] SEM = standard error of the mean
[3] *P*-value represents Type 3 Test of Fixed Effects for Diet

Table 4.4. Alpha-diversity indices Richness, Chao1, ACE, Shannon, Simpson, Inverse Simpson, and Fisher (LS means; 95% confidence interval) of the microbiome of healthy adult dogs (n=10) supplemented with graded levels of *Bacillus coagulans* GBI-30, 6086.

	Treatment[1]					
Indices	CON	PEX	PCL	PCM	PCH	*P*-value[2]
Richness	210.3; 168.5-252.2	185.3; 143.5-227.2	243.4; 201.6-285.3	200.0; 158.2-241.9	201.5; 159.7-243.4	0.3720
Chao1	823.3; 605.4-1041.2	730.3; 512.4-948.2	987.8; 769.9-1205.7	837.0; 619.1-1054.9	708.4; 490.5-926.3	0.3933
ACE	867.1; 648-1086.1	694.2; 475.1-913.2	979.3; 760.3-1198.4	786.4; 567.3-1005.4	738.9; 519.9-958.0	0.3780
Shannon	2.3; 2.2-2.4	2.4; 2.3-2.5	2.4; 2.3-2.5	2.5; 2.4-2.6	2.4; 2.3-2.5	0.3930
Simpson	0.8; 0.8-0.9	0.9; 0.8-0.9	0.9; 0.8-0.9	0.9; 0.9-0.9	0.9; 0.8-0.9	0.3153
Inverse Simpson	6.5; 5.8-7.3	6.9; 6.2-7.7	7.2; 6.4-7.9	7.6; 6.8-8.3	6.7; 5.9-7.4	0.3314
Fisher	27.7; 22.4-33.1	24.0; 18.6-29.3	32.0; 26.6-37.3	26.6; 21.3-32.0	26.3; 21-31.7	0.3158

[1] Treatments: CON = control; PEX = probiotic applied before extrusion; PCL = probiotic applied as coating at low dose; PCM = probiotic applied as coating at moderate dose; PCH = probiotic applied as coating at high dose.
[2] *P*-value represents Type 3 Test of Fixed Effects for Diet

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Appendix A - Enumeration Assay for *Bacillus coagulans* GBI-30, 6086 in Pet Food Kibble

1. **Prepare the Peptone Diluent:**

   a. Prepare a solution of 0.1% peptone in water (weight/volume) and adjust to a pH of 7.0 with a solution of lactic acid.

      4000 mL Batch:

      Combine:

4 g	Bacto Peptone
4000 mL	Distilled Water

      Adjust to pH 7.0

      Divide into 12 x 250 mL Bottles and ~ 9mL test tubes

      ▲**Notes:**

      - You may use BD Bacto™ Peptone (www.bd.com), or equivalent peptone suitable for microbiological analysis.
      - The solution is made by adding 1 g peptone to 1 L of water. No other ingredients.

   b. Using an autoclave, steam sterilize the solution at 121° for no longer than 15 minutes, then allow to cool in the unopened autoclave.

   c. Dispense the peptone solution into sterile containers as needed for preparing samples.

      ▲**Note:** If the peptone solution is made ahead in bulk, it may be stored at room temperature or refrigerated at 4°C. If refrigerated, allow to come to room temperature before use.

2. Prepare The Trace Mineral Solution:

a. Prepare a solution containing the following mineral concentrations in deionized water:

Table 1. Preparation of Trace Mineral Solution

Mineral	Concentration
Sodium chloride	10 mg/mL
Iron (II) sulfate, heptahydrate	18 mg/mL
Manganese (II) sulfate, monohydrate	16 mg/mL
Zinc sulfate, heptahydrate	1.6 mg/mL
Copper (II) sulfate, pentahydrate	1.6 mg/mL
Cobalt (II) sulfate, heptahydrate	1.6 mg/mL

▲Notes:

- The solution may be made ahead of time and refrigerated for up to 2 months.

- The fresh solution will be slightly pink in color. In the case of hydrated salts, users may substitute other hydration forms so long as the mineral salt concentration is maintained in the final solution.

- Freshly made, this solution will be pink in color. You may see some brown sediment after storage, that's okay. Use a stir bar to mix it. A pink to brown hue is okay to use.

3. Prepare the Glucose Yeast Extract BC Agar Medium

a. Prepare a solution containing the following reagents:

Table 2. Preparation of Glucose Yeast Extract BC Agar Medium

Reagent	Quantity	Quantity
Yeast extract powder	5.0 g	4.0 g
Peptone	5.0 g	4.0 g
D-Glucose	5.0 g	4.0 g
Potassium phosphate dibasic (K2HPO4)	0.5 g	0.4 g
Potassium phosphate monobasic (KH2PO4)	0.5 g	0.4 g
Magnesium sulfate	0.3 g	0.24 g
Trace mineral solution	1.0 mL	0.8 mL
Water	1000.0 mL	800.0 mL

b. Adjust the pH of the mixture to 6.3 with a solution of lactic acid.

c. Transfer the mixture to a large conical flask, add 15.0 g (12.0 g for 800 mL batch) of bacteriological agar to the flask, cover the flask with aluminum foil, and bring to boiling on a hot plate with stirring.

d. Allow the mixture to boil until the agar has completely dissolved, then sterilize in an autoclave at 121° for no longer than 15 min.

e. Once the autoclave can safely be opened, remove the flask and incubate in a water bath at 50° until needed for plating.

▲Note:

- The media solutions can be made ahead of time and stored at 4° C (heat gently to melt the agar prior to use).

- This media is not commercially available, and must be made specifically for this enumeration assay.

4. Sample Preparation

a. If the water activity (a_w) of the kibble sample is > 0.7, refrigerated storage at 4°C is recommended until enumeration is performed.

b. Transfer 50 g of kibble sample into a sterile stomacher bag.

c. Add 250 mL of previously sterilized Peptone diluent to the bag and mix at about 150–200 rpm for 5 min in a stomacher.

▲ Note: In some cases a smaller or larger sample size is required for accurate enumeration:

- For samples with serving size of 1–50 g, add 199–150 mL of peptone water to get a total volume of 200 mL or 299–250 mL of peptone water to get a total volume of 300 mL. (The dilutions for these preparations would be from 1 g/300 mL to 50 g/300 mL.)

- **50g of pet food kibble : 250 mL peptone water** is recommended to reduced variability in the data.

- For samples with serving size larger than 50 g, take a half serving size or use a representative sample of around 50 g or less, and add peptone water to get a total volume of 200–300 mL. In these cases, the term"serving size" refers to the sample size representing the declared enumeration value for the product.

- Kibble can be a challenge because the texture is usually very hard, which prevents uniform mixing and increasing the chances of the stomacher bags tearing. If the kibble is very hard, soak the kibble for 10-20 min in the mineral water to allow it to become soft. (May not require a full 10 – 20 minutes, use your judgement as the texture softens).

▲ **Note:** Take note of the soaking duration of each sample and keep consistent for all samples.

d. Check the pH of the sample suspension. If the pH is below 7.0, adjust it to a pH of 8.5 ± 0.2 with 5 N sodium hydroxide solution (not: pH of 9 is too high, would need to use lactic acid to bring it back down). If the pH is above 8.7, adjust it to a pH of 8.5 ± 0.2 with 5 N lactic acid solution.

▲ **Notes:**

- In most cases, pet food kibble samples will have a pH below 7, and will need to be adjusted using the 5 N sodium hydroxide solution. Without bringing the pH up to 8.5, a sample that's too acidic will be a double impact of damage during the heat activation step.

- Take care to avoid cross contamination of samples through the pH probe. You will need to shake the sample, and pour small amounts into separate cups to check the pH instead of inserting the probe into the stomacher bags.

- A 300 mL sample will allow for plenty of sample to use for the pH adjustment.

- Larger samples also help to avoid enumeration issues from non-uniform mixing within the kibble formula.

e. Homogenize the pH adjusted sample in a stomacher.

f. Transfer 20–30 mL of the homogenized suspension to a sterile 50-mL conical centrifuge tube with a cap.

g. Incubate the tube in a water bath held at 75°C for exactly 30 minutes.

▲**Notes:**

- Start the timer immediately once the stomacher bag is added to the water bath. Don't wait for the temperature to come to 75°C. As long as the temperature remains above 70°C, that will be alright. The first 5 minutes are often between 71°C - 73°C, that's okay and won't impact the results.

- The sample is heat activated at 75°C for 30min, so ensure that temperature does not significantly decrease when adding several samples simultaneously to the water bath.

- Continuous-pumping water baths prevent a large decrease in temperature.

h. At 30 minutes exactly, immediately cool the samples to below 45°C and homogenize again. This can be done by placing the bags back in the stomacher and allowing to cool, about 10 seconds per sample before pour plating.

i. Transfer 1.0 mL of the cooled sample suspension into a sterile test tube containing 9 mL of Peptone diluent, previously prepared.

j. Mix thoroughly by vortexing. This suspension represents a 0.5×10^{-3} dilution of the sample.

k. Repeat dilution in a succession of test tubes until the final dilution is expected to contain about 30 colony-forming units (CFU)/mL. The final three dilutions will be used in the analysis.

▲**Note:** Take care to plate the sample preparation dilutions within 10–20 min of preparation.

5. Analysis

a. For each Sample preparation tube to be plated, prepare Petri plates as follows:

- Aseptically transfer 1.0 mL of the Sample preparation separately into three appropriately labeled sterile 15-mm × 100-mm Petri plates.

- Then pour 15–20 mL of the molten BC agar medium into each plate. (1:10 dilution)

- Place the lid on each plate after adding the molten BC agar medium.

- Then gently swirl the plates to mix the Sample preparation and the BC agar medium.

▲**Note:** Be careful to avoid spillage onto the lid of the dish when swirling the plates.

b. Repeat this procedure for the additional two dilutions of the Sample preparation.

▲**Notes:**

- Each sample will require 9 petri plates, plus the two blank plates in the following step.

- If working with several samples, you will need to split them into groups of no more than 10 samples per assay to manage the space and timing for pour plating.

c. Prepare one blank plate that contains only BC agar medium and a second blank plate in which 1.0 mL of Peptone diluent has been mixed with BC agar medium.

d. Allow the plates to sit at room temperature until the BC agar medium solidifies.

e. Then invert the plates and incubate them at 40°C ± 2°C for 48 h.

f. After 48 h of incubation, count the colonies on the prepared plates, including both blank plates.

▲Notes:

- Plates containing between 30 and 300 colonies are considered ideal for counting.

- Count only colonies that appear as follows:
    - Surface colonies should be 1–5 mm in diameter (these will be larger than those within the medium)
    - white to cream in color
    - convex
    - with entire margins and smooth surfaces.
- Colonies *inside* the BC agar medium should:
    - Be 0.5–1 mm in diameter
    - Appear as cream-colored pinpoints within the medium.

g. Calculate the average number of colonies per plate, then multiply the average number of colonies counted by the reciprocal of the dilution factor to obtain the CFU/g of the sample. Report results in CFU/g.

▲Notes: For samples larger than 1 g, consider the sample size as one unit. Calculate the average number of colonies per plate, and then multiply the average number of colonies counted by the reciprocal of the dilution factor to obtain CFU/sample. To get CFU/g of the sample, divide CFU/ sample by actual gram weight sample size.

For example, if the sample size is 50 g and the final volume is 300 mL (50-g sample plus 250 mL of peptone water), the first dilution factor would be 1/300 (consider the whole sample size as 1 unit). If 5 more additional 1-in-10 dilutions were made, the total dilution factor would be:

$$1/300 \times 1/10 \times 1/10 \times 1/10 \times 1/10 \times 1/10 = 1/300 \times 1/10^{-5}$$

If the average colonies counted per plate of the last dilution is 200, the CFU/sample size would be:

$$200 \times 300 \times 10^5 = 6 \times 10^9 \text{ CFU/50 g and } 6/50 \times 10^9 = 1.2 \times 10^8 \text{ CFU/g}$$

The presence of any colonies not conforming to this description should be noted. Both blank plates should be entirely free of any type of colonies.

▲**Notes:**

- In the case of blank plates that contain colonies, the entire procedure must be repeated, potentially including the preparation of the Diluent and the BC agar medium, depending on which plate(s) contain colonies.\

- Bacillus subtilis is frequently detected from pet food products and will grow much faster than BC30 overtaking the plates as opposed to generating single colonies.

Acceptance Criteria:

- Even within the same lab, 10 – 20% variance is acceptable.

- 100% of the colonies observed meet the description provided in the Analysis section. The product contains no less than 100% of the declared colony count, in CFU/g.

Additional Notes:

- The name BC30 refers to the spores being able to germinate at 30°C.

- Low counts may not just be due to poor survivability, but due to the enumeration method used. In BC30's history, the pH adjustment was added to make sure the samples were more alkaline, and they saw 100% recovery after what was thought to be a poor survival issue. 8.3 – 8.7 is ideal for this strain.

- The probiotic material used in this study was 15 Billion CFU/g concentration.