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Characterization and Evaluation of the Probiotic Properties of the Sporeforming Bacteria, *Bacillus coagulans* Unique IS-2

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Characterization and Evaluation of the Probiotic Properties of the Sporeforming Bacteria, *Bacillus coagulans* Unique IS-2

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Characterization and Evaluation of the Probiotic Properties of the Sporeforming Bacteria, *Bacillus coagulans* Unique IS-2

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Probiotics are live organisms, that when consumed in adequate amounts, confer a health benefit to the host. To achieve probiotic status, each potential strain's functional properties and their safety to the consumer must be comprehensively evaluated. Probiotic effects have been observed to be strain specific, therefore each new strain of interest must be characterized according to their phenotypic and genetic characteristics. There is a list of characteristics that potential probiotic strains should have to be considered as a probiotic. Potential probiotic strains should be evaluated for their acid and bile salt resistance, antimicrobial activity and adherence and colonization to intestinal cells in the lower gastrointestinal tract. Additionally, those that may be used as additives to food products should be evaluated for their survival during processing and storage of those products.

The most common, and most researched, probiotic strains belong to the *Lactobacillus* and *Bifidobacterium* genera. However, there have been many reports of low viability of these strains in the delivery product by the end of the shelf life. This can cause problems as there is a desired level of probiotics that needs to be consumed to promote health benefits on the host. This problem has led the probiotic industry to research the probiotic properties of sporeforming organisms. Spores are dormant structures that are more

resistant to heat, cold, acidity and desiccation. This inherent protection could help improve the survival of the organism in the product and through transit of the gastrointestinal tract.

Bacillus coagulans is a Gram positive, lactic acid producing, facultative anaerobe and sporeforming bacteria strain that is drawing the attention of the probiotic industry. While research is still limited on this organism, there have been studies showing improvement of irritable bowel syndrome, *Clostridium difficile* induced colitis, rheumatoid arthritis and major depressive disorders associated with irritable bowel syndrome. Additionally, reports have shown the ability of *Bacillus* spores to reach and germinate in the gut, which is an important characteristic of potential probiotic strains. The purpose of this research is to evaluate the probiotic properties of the commercial *Bacillus coagulans* Unique IS-2, for its potential inclusion in food products.

To my cat, Pebbles, who entered my life the day before I started graduate school and was there every morning to wake me up when I didn't want to get out of bed and who was always right on the other side of the door to greet me when I came home. All my love.

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CHAPTER 1: LITERATURE REVIEW

1.1. Probiotics

Throughout the past century, food market trends have been driven by consumers desire for healthier foods. This had led to the exponential increase in new products on the shelf boasting health benefits to the consumer. The concept of health benefiting foods has been coined functional foods. These are foods consumed as part of the everyday diet that provides beneficial effects on human health, such as reducing cholesterol or high blood pressure (Charalampopoulos, Wang, Pandiella, & Webb, 2002). Foods can be found under the umbrella term, known as functional foods, through the addition of synthesized food ingredients, naturally occurring bioactive substances, or added bioactive substances (Grajek, Olejnik, & Sip, 2005). Probiotics are among the most commonly added substances to produce functional foods. This has led to a significant increase in research on probiotic organisms and their effects on the human body. Currently probiotics are being sold as dietary supplements, as inclusions into food products and as skin creams. While dietary supplements and skin creams are popular uses of probiotics, this report focuses on probiotics as inclusions into food products.

The idea of probiotics originated from over 100 years ago when Döderlein and Metchnikoff claimed lactic acid producing bacteria would produce health benefits when consumed (De Vecchi & Drago, 2006). This discovery motivated scientists to conduct further research on the health promoting properties of microorganisms after consumption. This led to an industry that has flourished for probiotics through increasing market share every year. In 2017, the probiotic market was over $1.8 billion USD and the food and beverage sector, as the largest sector, was greater than $125 million USD (Ahuja & Deb,

2018). By 2024, the food and beverage sector is expected to increase by 6.5% (Ahuja & Deb, 2018).

Currently, the most widely used definition of probiotics is "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). While there is no official intake level for foods, it is generally accepted that probiotics should be consumed at a level between 10^7-10^9 CFU/g of product to ensure the health benefits (Ashraf & Shah, 2011; Konuray & Erginkaya, 2018). Dietary probiotic health benefits are achieved by the arrival of the probiotic to the gut, where they positively impact the natural gut microbiota by increasing the growth of good bacteria and hindering the growth of undesirable or pathogenic bacteria (Sanders, 2003). Thus, potential probiotic strains must be evaluated for specific characteristics before their use in functional foods. Orally consumed probiotics therefore must survive transit through the digestive tract until they reach the colon, which is the target organ. For a strain to be considered a probiotic, it should be proven safe for human consumption. must survive reaching the target organ by having acid and bile tolerance, and be able to grow and colonize in the gut to promote health benefits (Gotcheva et al., 2002).

1.2. Microorganisms Used as Probiotics

The most common microorganisms used as probiotics are Lactic Acid Bacteria (LAB), with the majority belonging to the *Lactobacillus* or *Bifidobacterium* genera. Less commonly used probiotics belong to the genera of Lactococcus, Pediococcus, Leuconostoc, and Streptococcus. More recently, there are also non-lactic acid bacteria

being used as probiotics from the genera *Bacillus, Saccharomyces, Clostridium* and even from the Gram negative *Escherichia coli* (Foligné, Daniel, & Pot, 2013).

Traditionally, LAB strains are Gram positive rods, anaerobe or facultative, catalase negative, non-motile and non sporeforming (Song, Ibrahim, & Hayek, 2012). These strains also produce health benefiting substances such as lactic acid, hydrogen peroxide, antimicrobials and/or other bacteriocins in order to be competitive and interact with the host microbiota (Holzapfel, Haberer, Geisen, & Schillinger, 2001). For functional food products, *Lactobacillus* and *Bifidobacterium* are included in refrigerated products such as yogurt, milk, and cheeses. Refrigerated products are commonly used to deliver the probiotic cultures due to the limited viability of these strains to survive in other products and environments. Indeed, reports have shown that by the end of the shelf life, there is a significantly decreased amount of viable probiotic cells left in a product compared to the concentration expected to be present (Jayamanne & Adams, 2006; Shah, 2000).

The live and active nature of probiotic bacteria have led to issues of viability throughout the shelf life of the product and passage through the digestive tract in high enough numbers to reach the gut of the consumer. While probiotic strains are selected based on their acid and bile tolerance, one of the solutions to increase the viability of cells is a microencapsulation process. In an effort to extend the stability and shelf life of these probiotics, encapsulation or freeze-drying processes have been used to protect the bacteria during manufacturing, storage, and survival through the digestive tract until arrival in the gut (Weinbreck, Bodnár, & Marco, 2010). The encapsulation process,

which is the most efficient method, uses food grade biopolymers to coat the probiotic cells and has been proved to increase the viability of probiotics in some dairy products, such as yogurt, ice cream and cheese (Annan, Borza, & Hansen, 2008; Kailasapathy, 2002; Martín, Lara-Villoslada, Ruiz, & Morales, 2015; Weinbreck et al., 2010). Microencapsulated *Lactobacillus acidophilus* and *Bifidobacterium* spp survived better in yogurt than free cells (Godward, 2000). Additionally, microencapsulated *Lactobacilli*, *Bifidobacterium bifidum,* and *Bifidobacterium infantis* were observed to improve survival by at least 40% in the freezing of ice milk for frozen desserts (Godward, 2000; Kailasapathy, 2002). However, another study observed no significant difference between the survival of microencapsulated *Lactobacillus acidophilus, Bifidobacterium* spp and the free cells in ice cream during storage for 6 months at -20˚C (Godward, 2000). While there has been improved viability, there is still much to be researched on new technologies to improve the overall process (Martín et al., 2015). Therefore, having a probiotic that already possesses the ability to survive processing conditions and transit through the body, could help producers avoid an extra processing step and reduce their production costs.

1.3. Sporeforming Bacteria Used as Probiotics

Spores are found universally in soils or water samples across multiple temperature environments, have been associated with insects and animals and these spores associated with insects or animals have been shown to germinate, grow and then sporulate inside the host animal (Cutting, Simon M., Ricca, 2014). Due to their presence in the environment, it was generally determined that spores can be found in the gastrointestinal tract of insects

and animals due to the consumption of water, soil, air or food that contains the spores. However, recent work has led to the theory that sporeforming bacteria may be able to grow and interact within the gastrointestinal tract (Fakhry, Sorrentini, Ricca, De Felice, & Baccigalupi, 2008).

Spores are formed as a survival response to harmful environments that vegetative cells would not survive in. Sporeforming bacteria have the ability to sense their environment and when unfavorable conditions are sensed, they can switch their metabolism over to the formation of spores (Errington, 2003). The model organism for spore formation is *Bacillus subtilis*, as it was the first reported genome of a spore forming organisms and research has shown that the spore formation process is similar among all spore formers. Sporulation begins with an asymmetric cell division where the smaller portion will become the spore. The larger portion, called the mother cell, then engulfs the smaller part and creates the outer protective layers of the spore (Figure 1).

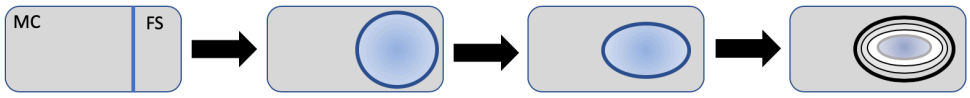

Figure 1. Spore formation process starts with the asymmetric division between the mother cell (MS) and the forespore (FS). The MS then engulfs the forespore which is when the formation of the spore layers can begin. Adapted from Henriques & Moran, Jr. (2007).

The main components of the spore (from the inside) are the core (condensed chromosome), cortex layer (peptidoglycan), undercoat region and the protein rich inner and the outer coat layers (Figure 2) (Cutting, 2011; Henriques & Moran, Jr., 2007).

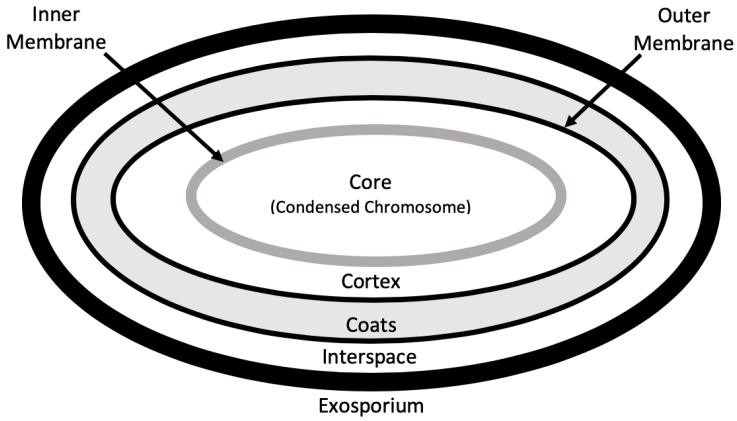

Figure 2. Diagram of a *Bacillus* Spore. Sizes of the spore layers are not drawn to scale. Adapted from Setlow (2014).

The spore is still a live cell, just in a dormant state, with almost no detectable metabolic activity, and increased protection against extreme temperatures, UV radiation, oxidation, and low water environments (Nicholson et al., 2003). Even in the low metabolic state, the spore is constantly monitoring their environment for favorable growth conditions and will respond by germinating when favorable growth conditions are detected. While the process of germination is not completely understood, a germination factor, such as a specific nutrient, may be needed to penetrate the spore coat in order to activate germination receptors, which begins the process of increasing metabolic activity to shed the spore coat and become vegetative cells again (Moir, 2006). These germination factors vary between species and strains however, they are commonly amino acids, sugars or purine nucleosides. For example, L-valine and L-alanine are germination triggers for *Bacillus subtilis* (Atluri, Ragkousi, Cortezzo, & Setlow, 2006).

With the viability issues of traditional probiotic strains, there has been an increased interest in the probiotic properties of *Bacillus* strains. *Bacillus* strains have created an interest in the probiotic community due to their inherent ability to form spores, their ability to produce antimicrobials, their normal prevalence in food products, and their greater chance of survival through the gastrointestinal tract (Sorokulova, 2013). When in their spore form, they are more resistant to extremes such as heat, cold, acidity, and dryness, and can survive as a spore for a long time. The bacteria will stay as a spore until it reaches an environment that is suitable for growth with adequate moisture, pH, temperature and nutrients. Once the bacteria reaches a suitable environment the spore will take in water and begin the germination process back to a more active vegetative state (Setlow, 2014).

Bacillus species' ability to form spores is a desirable trait to the probiotic industry as the bacteria would have its own inherent protection. The spore is still a live culture and when included in a product as a spore, the bacteria would be able to withstand harsh processing environments and remain stable in the product as a spore throughout the shelf life when held under the proper conditions. This could potentially extend the shelf life of products as the probiotic level would stay at the desired content for a longer time compared to traditional probiotics.

Sporeforming probiotics would therefore be consumed in the spore form, which would help protect the bacteria during transit through the gastrointestinal tract prior to reaching the gut. The spore would be resistant to the low pH of the stomach and bile salts of the

small intestine (Elshaghabee, Rokana, Gulhane, Sharma, & Panwar, 2017; Hyronimus, Le Marrec, Sassi, & Deschamps, 2000; Spinosa et al., 2000). Thus, the spore would have high survivability on its route to the gut where it would find a suitable environment and begin the germination process (Cutting, 2011; Fakhry et al., 2008). A molecular method observed *Bacillus* spores germinated in the jejunum of mice (Casula & Cutting, 2002). Additionally, *Bacillus clausii* spores have been observed to survive and persist in the human gut, with feces containing the spores for up to 12 days after consumption (Ghelardi et al., 2015).

1.3.1. Lactobacillus sporogenes

Lactobacillus sporogenes was discovered by Horowitz-Wlassowa and Nowotelnow in 1933 (De Vecchi & Drago, 2006). Due to the environment in which it was found and its properties, it closely resembled the *Lactobacillus* genus and was thus named Lactobacillus sporogenes. As science progressed and identification of microorganisms were improved upon, it was determined this microorganism was misclassified. Bergey's Manual of Determinative Bacteriology 8th edition, stated that any spore bearing rods that produce lactic acid, are catalase positive, and are facultative anaerobes should be classified as *Bacillus* (De Vecchi & Drago, 2006). Therefore, this species, due to its spore forming capabilities, could not belong to the *Lactobacillus* genus and was then reclassified as Bacillus sporogenes. More recently, it was discovered that B. sporogenes had substantial similarity with *B. coagulans* and was reclassified as such.

1.3.2. *Bacillus coagulans* Used as Probiotics

Bacillus coagulans was first described in 1915 as a spoilage organism in canned milk and was determined to be tolerant to acid and thermal conditions (De Clerck et al., 2004). It is Gram positive, rod-shaped (singularly or in short chains), motile and can grow between 30-55˚C, with an optimal pH growth between 5.5 – 6.5. *Bacillus coagulans* has the ability to form spores and is a facultative anaerobe (De Vecchi & Drago, 2006). It is also one strain that has drawn the attention of the probiotic industry due to its sporeforming abilities and potential probiotic properties.

Bacillus coagulans strains have been showed to have heterogeneous physiological characteristics, leading to different names for similar strains, that sometimes requires DNA analysis to discern how closely related they are (De Vecchi & Drago, 2006). However, *B. coagulans* is the scientifically correct and accepted name though many people are still misclassifying the organism as Lactobacillus sporogenes. One reason is that *Lactobacillus* probiotics are natural residents of the gastrointestinal tract and have a history of safe use, while *Bacillus* strains have not been as closely studied for safety. Therefore, using the *Lactobacillus* name may help the probiotic industry reap the benefit from the known safety of *Lactobacillus* strains to avoid questions around the lesser studied safety of *Bacillus coagulans,* even though that is the scientifically correctly identified name (De Vecchi & Drago, 2006; Drago & De Vecchi, 2009).

Bacillus coagulans has been evaluated for its probiotic properties in *in vitro* and *in vivo* models. Studies have been used to observe its anti-inflammatory and immune-

modulating effects. *Bacillus coagulans* reduced inflammation by inhibited ROS formation and reducing PMN cell migration in *in vitro* experiments (Jensen, Benson, Carter, & Endres, 2010). Additionally, *B. coagulans* was used to treat *Clostridium difficile* in mice and it was found to improve stool consistency by improving the gut mucosa barrier (Fitzpatrick, Small, Greene, Karpa, & Keller, 2011). Some strains of *Bacillus coagulans* have been granted Generally Recognized as Safe (GRAS) status by the U.S. Food and Drug Administration for its application in dairy products (Konuray & Erginkaya, 2018). The protein rich spore coat helps protect *B. coagulans* from harsh conditions, such as heat, cold, or dryness during processing conditions and the harsh acid and bile conditions found in the upper digestive system during digestion (Hyronimus et al., 2000). Then upon entry into the colon environment, conditions are much less harsh, allowing for the spore to germinate and vegetative cells start to reproduce themselves while initiating, which can interact with the gut microbiota (Song et al., 2012).

1.4. Probiotic Properties

As mentioned previously, the definition of probiotics is "live microorganisms that when consumed in adequate amounts, confer a health benefit to the host" (Hill et al., 2014). This definition is very broad to encompass the multiple strains of microorganisms used as probiotics. Due to the broad definition, groups and industry have agreed upon a list of properties that organisms should possess to be effective as a probiotic. In a joint meeting, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) outlined that probiotic effects have been found to be strain specific, therefore each individual strain must be identified with the genus and species

and be tested for these effects to justify the claim of the health benefit associated with them (Araya et al., 2002). In addition to the genus and species, the probiotic organism must be completely characterized by studying toxin production, virulence factors, antibiotic resistance and production and adherence or colonization of the gut (Anadón, Rosa Martínez-Larrañaga, & Aranzazu Martínez, 2006; Gotcheva et al., 2002).

Health benefits arise by the arrival of the probiotic to the gut, where they positively impact the natural gut microbiota by increasing the growth of good bacteria and hindering the growth of undesirable or pathogenic bacteria (Sanders, 2003). Therefore, potential probiotic strains must be evaluated for specific health benefits through *in vitro* testing and then corroborated with *in vivo* animals and human studies (Araya et al., 2002). Most probiotics are consumed orally therefore they must survive transit through the digestive tract until they reach the colon. To confer a health benefit, probiotics should be considered safe for human consumption and should be tested for their acid and bile tolerance, adherence to intestinal cells, and potential antimicrobial activity to reduce pathogenic bacteria (Araya et al., 2002; Drago & De Vecchi, 2009; Gotcheva et al., 2002).

Probiotics strains that possess the above qualities *in vitro* should then be tested for their safe consumption through *in vivo* testing in animal and human studies to determine if they are able to produce a health benefit on the host (Araya et al., 2002). Additionally, probiotic strains should be able to survive processing and remain stable during the shelf life of the product.

1.4.1. Acid and Bile Tolerance

Bora et. al. (2009) evaluated the physiochemical properties of *B. coagulans* and found that the pH of aqueous media affected the stability of the spores. Evaluation of the spores between pH 1.2 and 8.0, showed there was close to a 20% decrease in viability of the *B. coagulans* spores at the extreme acidic (pH 1.2) and alkaline (pH 8.0) conditions, with the optimum stability at pH 6.8 (Bora et al., 2009). Majeed et al. (2016) evaluated resistance to gastric acid of a different strain of *B. coagulans* and saw similar results in the acidic region. There was no significant difference found between pH 3.0 to pH 8.0 in spore count over 4 hours, conversely, the most acidic condition, pH 1.5, had a loss of 2.1 logs after 4 hours (Majeed, Nagabhushanam, Natarajan, Sivakumar, Eshuis-de Ruiter, et al., 2016). Both of these studies show some decreased viability of *B. coagulans* spores at low pH conditions, which may be similar to the stomach environment. However, *in vivo* and *in vitro* experiments have shown that *Bacillus* spores can survive passage through the upper gastrointestinal tract and germinate in the colon environment (Casula & Cutting, 2002; Maathuis, Keller, & Farmer, 2010). Casula and Cutting (2002) fed mice *Bacillus* spores with a genetically engineered gene, *ftsH-lacZ,* that is expressed in vegetative cells. After consumption of the spores, 2 logs of vegetative cells were detected in the jejunum and ileum of the small intestine. As only spores were fed, the detection of vegetative cells must have been a result of the spores germinating in the intestine of the mice (Casula & Cutting, 2002). Additionally, Ganeden observed around a 10% germination of their *B. coagulans* strain, GanedenBC^{30}, in a TIM-1 model of the stomach and small intestine, indicating germination in the gut is possible (Maathuis et al., 2010).

While *in vivo* methods would be the best for evaluating the survival and stability of probiotic bacteria in the human digestive tract, these methods are expensive and labor intensive. Therefore, *in vitro* methods are most commonly used to evaluate the acidic and bile salt resistance of probiotic and potentially probiotic strains, typically through adjusted broth media for 4 hours (Hyronimus et al., 2000; Lee, Park, Choi, & Cho, 2012; Maathuis et al., 2010). During these studies, *Bacillus* species have shown the ability to survive acidic conditions with pH as low as 2.0 to varying degrees, depending on the strain. While pH 2.0 showed greater loss of viability, some strains of *Bacillus* had greater than 49% viability remaining after 2 hours at pH 3.0 (Lee et al., 2012). However, Maathuis et al. (2010) observed a 70% survival of *B. coagulans* spores through a TIM-1 dynamic model of the stomach, where the stomach pH dropped from pH 6.5-2.0 over 2 hours. Additionally, for most strains of *Bacillus* evaluated, survival of the strains were even better in the presence of bile salts (Hyronimus et al., 2000; Lee et al., 2012; Maathuis et al., 2010). As these strains were tested as vegetative cells, it is expected that spores would have a similar or greater survival at acidic and bile conditions.

1.4.2. Germination in the Gut

After surviving the upper gastrointestinal tract, conditions in the lower gastrointestinal tract could be suitable for the spores to germinate and start producing more vegetative cells, which can interact with the gut microbiota upon reaching the colon (Song et al., 2012). In 2010, in an *in vitro* model of the stomach and small intestine, survival of the *B. coagulans* strain GanedenBC^{30} had 70% spore survival but germination of the spores was <10% (Maathuis et al., 2010). The survival through the stomach was promising, but the

gemination of the spores in the small intestinal was very low. In 2019, the germination of GanedenBC^{30} was studied again using a computer controlled *in vitro* model of the gastrointestinal tract, simulating an adult human (Keller, Verbruggen, Cash, Farmer, & Venema, 2019). The difference was that in this study the probiotic was introduced to the model with a calculated meal that would help provide germination triggers that would be present during normal consumption. During this study they found that >90% of spores had germinated in the *in vitro* model of the gastrointestinal tract in the presence of the meal (Keller et al., 2019).

Animals studies have shown that animals fed *Bacillus coagulans* had an increased population of LAB in the gut microbiota. Additionally, other animal studies showed *B. coagulans* helped remedy hypercholesterolemia, had antidiarrheal affects, and improved some symptoms of *Clostridium difficile* induced colitis (Aminlari et al., 2018; Bomko et al., 2017; Fitzpatrick et al., 2011; Majeed, Natarajan, et al., 2016; Wu et al., 2018) . However, after consumption of spores in rats, *B. coagulans* was determined to only be a transitory member of the intestine and passed quickly to be eliminated in the feces (Abhari et al., 2015).

Additionally, probiotic *B. coagulans* has been found safe for consumption and successful in human clinical studies as a treatment for irritable bowel syndrome, reduction of cholesterol, a therapy for rheumatoid arthritis, as an anti-diarrheal and secondary treatment to antibiotic therapy (Endres et al., 2009, 2011; Majeed, Natarajan, et al., 2016;

Majeed & Nagabhushanam, 2016; Mandel, Eichas, & Holmes, 2010). Additionally, it may increase immune response against some respiratory infections (Baron, 2009).

1.4.3. Thermal Resistance During Processing

Some *Lactobacillus* and *Bifidobacterium* strains have been evaluated in fruit juice supplements and it was observed that the pH, pasteurization time and temperature affected the viability of the probiotic bacteria (Sheehan, Ross, & Firzgerald, 2007). Acidic environments were for the most part tolerated by a majority of strains tested, but thermal processing had a greater effect on the inactivation of the probiotics. However, *B. coagulans* has not been studied as much. In one of the few studies available, the inactivation of *B. coagulans* specifically, was found to be affected by the composition of the food matrix, processing temperature and product pH (Palop, Raso, Pagán, Condón, & Sala, 1999). The D-value of *B. coagulans* in buffer was found to decrease to less than a third when the pH changes from pH 7.0 to pH 4.0. Additionally, the D-value decreased even more, about one sixth, with the same change in pH when included in tomato or asparagus food matrices (Palop et al., 1999). Therefore, these factors should always be evaluated when determining the product application of the *B. coagulans* probiotic strain.

1.4.4. Survival/Stability During Shelf Life

While the most important property of a probiotic is that it can promote a health benefit on the host, it must also survive and remain stable in the delivery product until the host is able to ingest the probiotic. Thus, the probiotic must remain viable during the shelf life of the product so that the concentration of probiotic is equal to or higher than what is

required to obtain the health benefit imparted by the bacteria. Therefore, the composition of the food will play an important role in the viability of the probiotic during shelf life. Important product characteristics that may affect the viability would be pH, water activity, nutrients, oxygen and temperature of storage (Song et al., 2012). It is also important for the spore to not germinate during the shelf life so that it is in the protective spore coat upon consumption. Additionally, the addition of probiotics should not change the quality of the final product. Therefore, organoleptic properties should be monitored to ensure the added spores do not affect the product throughout the shelf life. Sensory of the probiotic developed product should be similar to commercial products without probiotics (Song et al., 2012).

1.5. Products that Contain *Bacillus coagulans*

The most widely used probiotics are from the *Lactobacillus* and *Bifidobacterium* genera and are mostly included in dairy products. However, they are not resistant to heat. Probiotics that are resistant to heat would be expected to potentially be included in a wide range of products (Konuray & Erginkaya, 2018). *Bacillus coagulans* had gained interest due to its heat resistant spore form. While this species has not been widely studied for viability, there are some products that contain *B. coagulans* as a probiotic. These products include yogurts, fermented beverages, fruit juices, sausages, pasta, muffins and cereal based function foods (Cutting, 2011; Fares et al., 2015; Jafari et al., 2017; Konuray & Erginkaya, 2018).

1.6. References

Abhari, K., Shekarforoush, S. S., Sajedianfard, J., Hosseinzadeh, S., & Nazifi, S. (2015). The effects of probiotic, prebiotic and synbiotic diets containing *Bacillus coagulans* and inulin on rat intestinal microbiota. *Iranian Journal of Veterinary Research*, *16*(3), 267–273.

Ahuja, K., & Deb, S. (2018). Global Market Insights. Retrieved February 20, 2019, from https://www.gminsights.com/industry-analysis/probiotics-market

Aminlari, L., Shekarforoush, S. S., Hosseinzadeh, S., Nazifi, S., & Sajedianfard, J. (2018). Effect of Probiotics *Bacillus coagulans* and *Lactobacillus plantarum* on Lipid Profile and Feces Bacteria of Rats Fed Cholesterol-Enriched Diet. https://doi.org/10.1007/s12602-018-9480-1

Anadón, A., Rosa Martínez-Larrañaga, M., & Aranzazu Martínez, M. (2006). Probiotics for animal nutrition in the European Union. Regulation and safety assessment. *Regulatory Toxicology and Pharmacology*, *45*(1), 91–95. https://doi.org/10.1016/j.yrtph.2006.02.004

Annan, N. T., Borza, A. D., & Hansen, L. T. (2008). Encapsulation in alginate-coated gelatin microspheres improves survival of the probiotic *Bifidobacterium adolescentis* 15703T during exposure to simulated gastro-intestinal conditions. *Food Research International*, *41*(2), 184–193. https://doi.org/10.1016/j.foodres.2007.11.001

Araya, M., Morelli, L., Reid, G., Sanders, M. E., Stanton, C., Pineiro, M., & Ben Embarek, P. (2002). Guidelines for the Evaluation of Probiotics in Food. *Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of*

Probiotics in Food, 1–11. https://doi.org/10.1111/j.1469-0691.2012.03873

Ashraf, R., & Shah, N. P. (2011). Selective and differential enumerations of *Lactobacillus delbrueckii* subsp. *bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus casei* and *Bifidobacterium* spp. in yoghurt - A review. *International Journal of Food Microbiology*, *149*(3), 94–208. https://doi.org/10.1016/j.ijfoodmicro.2011.07.008

Atluri, S., Ragkousi, K., Cortezzo, D. E., & Setlow, P. (2006). Cooperativity Between Different Nutrient Receptors in Germination of Spores of *Bacillus subtilis* and reduction of this cooperativity by alterations in the GerB Receptor. *Journal of Bacteriology*, *188*(1), 28–36. https://doi.org/10.1128/JB.188.1.28

Baron, M. (2009). A patented strain of *Bacillus coagulans* increased immune response to viral challenge. *Postgraduate Medicine*, *121*(2), 114–118. https://doi.org/10.3810/pgm.2009.03.1971

Bomko, T. V., Nosalskaya, T. N., Kabluchko, T. V., Lisnyak, Y. V., & Martynov, A. V. (2017). Immunotropic aspect of the *Bacillus coagulans* probiotic action. *Journal of Pharmacy and Pharmacology*, *69*(8), 1033–1040. https://doi.org/10.1111/jphp.12726

Bora, P. S., Puri, V., & Bansal, A. K. (2009). Physicochemical Properties and Excipient Compatibility studies of Probiotic *Bacillus coagulans* Spores. *Scientia Pharmaceutica*, *77*, 625–637. https://doi.org/10.3797/scipharm.0904-01

Casula, G., & Cutting, S. M. (2002). Probiotics: Spore Germination in the Gastrointestinal Tract. *Society*, *68*(5), 2344–2352. https://doi.org/10.1128/AEM.68.5.2344

Charalampopoulos, D., Wang, R., Pandiella, S. S., & Webb, C. (2002). Application of cereal and cereal components in functional foods: a review. *International Journal of Food Microbiology*, *79*(1–2), 131–141.

Cutting, Simon M., Ricca, E. (2014). Bacterial spore-formers: friends and foes. *FEMS Microbiology Letters*, *358*, 107–109. https://doi.org/10.1111/1574-6968.12572

Cutting, S. M. (2011). Bacillus probiotics. *Food Microbiology*, *28*, 214–220. https://doi.org/10.1016/j.fm.2010.03.007

De Clerck, E., Rodriguez-Diaz, M., Forsyth, G., Lebbe, L., Logan, N. A., & DeVos, P. (2004). Polyphasic Characterization of *Bacillus coagulans* Strains, Illustrating Heterogeneity within this Species, and Emended Description of the Species. *Systematic and Applied Microbiology*, *27*(1), 50–60. https://doi.org/10.1078/0723-2020-00250

De Vecchi, E., & Drago, L. (2006). *Lactobacillus sporogenes* or *Bacillus coagulans*: misidentification or mislabelling? *International Journal of Probiotics and Prebiotics*, *1*(1), 3–10.

Drago, L., & De Vecchi, E. (2009). Should *Lactobacillus sporogenes* and *Bacillus coagulans* Have a future? *Journal of Chemotherapy*, *21*(4), 371–377. https://doi.org/10.1179/joc.2009.21.4.371

Elshaghabee, F. M. F., Rokana, N., Gulhane, R. D., Sharma, C., & Panwar, H. (2017). *Bacillus* as potential probiotics: Status, concerns, and future perspectives. *Frontiers in Microbiology*, *8*(AUG), 1–15. https://doi.org/10.3389/fmicb.2017.01490

Endres, J. R., Clewell, A., Jade, K. A., Farber, T., Hauswirth, J., & Schauss, A. G. (2009). Safety assessment of a proprietary preparation of a novel Probiotic, *Bacillus*

coagulans, as a food ingredient. *Food and Chemical Toxicology*, *47*(6), 1231–1238. https://doi.org/10.1016/j.fct.2009.02.018

Endres, J. R., Qureshi, I., Farber, T., Hauswirth, J., Hirka, G., Pasics, I., & Schauss, A. G. (2011). One-year chronic oral toxicity with combined reproduction toxicity study of a novel probiotic, *Bacillus coagulans*, as a food ingredient. *Food and Chemical Toxicology*, *49*(5), 1174–1182. https://doi.org/10.1016/j.fct.2011.02.012

Errington, J. (2003). Regulation of endospore formation in *Bacillus subtilis*. *Nature Reviews Microbiology*, *1*(2), 117–126. https://doi.org/10.1038/nrmicro750

Fakhry, S., Sorrentini, I., Ricca, E., De Felice, M., & Baccigalupi, L. (2008). Characterization of spore forming Bacilli isolated from the human gastrointestinal tract. *Journal of Applied Microbiology*, *105*(6), 2178–2186. https://doi.org/10.1111/j.1365-2672.2008.03934.x

Fares, C., Menga, V., Martina, A., Pellegrini, N., Scazzina, F., & Torriani, S. (2015). Nutritional profile and cooking quality of a new functional pasta naturally enriched in phenolic acids, added with β-glucan and *Bacillus coagulans* GBI-30, 6086. *Journal of Cereal Science*, *65*, 260–266. https://doi.org/10.1016/j.jcs.2015.07.017

Fitzpatrick, L. R., Small, J. S., Greene, W. H., Karpa, K. D., & Keller, D. (2011). *Bacillus Coagulans* GBI-30 (BC30) improves indices of *Clostridium difficile*-Induced colitis in mice. *Gut Pathogens*, *3*(1), 16. https://doi.org/10.1186/1757-4749-3-16

Foligné, B., Daniel, C., & Pot, B. (2013). Probiotics from research to market: The possibilities, risks and challenges. *Current Opinion in Microbiology*, *16*(3), 284–292. https://doi.org/10.1016/j.mib.2013.06.008

Ghelardi, E., Celandroni, F., Salvetti, S., Gueye, S. A., Lupetti, A., & Senesi, S. (2015). Survival and persistence of *Bacillus clausii* in the human gastrointestinal tract following oral administration as spore-based probiotic formulation. *Journal of Applied Microbiology*, *119*(2), 552–559. https://doi.org/10.1111/jam.12848

Godward, G. N. (2000). Studies on enhancing the viability and survival of probiotic bactiera in dairy foods through strain selection and microencapsulation. University of Western Sydney.

Gotcheva, V., Hristozova, E., Hristozova, T., Guo, M., Roshkova, Z., & Angelov, A. (2002). Assessment of Potential Probiotic Properties of Lactic Acid Bacteria and Yeast Strains. *Food Biotechnology*, *16*(3), 211–225. https://doi.org/10.1081/FBT-120016668

Grajek, W., Olejnik, A., & Sip, A. (2005). Probiotics, prebiotics and antioxidants as functional foods. *Acta Biochimica Polonica*, *52*(3), 665–671. https://doi.org/10.1093/brain/awt108

Henriques, A. O., & Moran, Jr., C. P. (2007). Structure, Assembly, and Function of the Spore Surface Layers. *Annual Review of Microbiology*, *61*(1), 555–588. https://doi.org/10.1146/annurev.micro.61.080706.093224

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C., & Sanders, M. E. (2014). The International Scientific Association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *National Review Gastroenterol Hepatol*, *11*(11), 506–514. https://doi.org/10.1038/nrgastro.2014.66

Holzapfel, W. H., Haberer, P., Geisen, R., & Schillinger, U. (2001). Taxonomy and

important features of probiotic microorganisms in physiologic properties of lactic acid bacteria. *American Journal of Clinical Nutrition*, *73*(2), 365S. https://doi.org/10.1093/ajcn/73.2.365s

Hyronimus, B., Le Marrec, C., Sassi, A. H., & Deschamps, A. (2000). Acid and bile tolerance of spore-forming lactic acid bacteria. *International Journal of Food Microbiology*, *61*, 193–197.

Jafari, M., Mortazavian, A. M., Hosseini, H., Safaei, F., Mousavi Khaneghah, A., & Sant'Ana, A. S. (2017). Probiotic *Bacillus*: Fate during sausage processing and storage and influence of different culturing conditions on recovery of their spores. *Food Research International*, *95*, 46–51. https://doi.org/10.1016/j.foodres.2017.03.001

Jayamanne, V. S., & Adams, M. R. (2006). Determination of survival, identity and stress resistance of probiotic bifidobacteria in bio-yoghurts. *Letters in Applied Microbiology*, *42*(3), 189–194. https://doi.org/10.1111/j.1472-765X.2006.01843.x

Jensen, G. S., Benson, K. F., Carter, S. G., & Endres, J. R. (2010). GanedenBC30 cell wall and metabolites: Anti-Inflammatory and Immune Modulating Effects *in Vitro*. *BMC Immunology*, *11*.

Kailasapathy, K. (2002). Microencapsulation of probiotic bacteria: technology and potential applications. *Current Issues Intestinal Microbiology*, *3*, 39–48.

Keller, D., Verbruggen, S., Cash, H., Farmer, S., & Venema, K. (2019). Spores of *Bacillus coagulans* GBI-30, 6086 show high germination, survival and enzyme activity in a dynamic, computer-controlled *in vitro* model of the gastrointestinal tract. *Beneficial Microbes*, *10*(1), 77–87. https://doi.org/10.3920/BM2018.0037

Konuray, G., & Erginkaya, Z. (2018). Potential Use of *Bacillus coagulans* in the Food Industry. *Foods*, *7*(6), 92. https://doi.org/10.3390/foods7060092

Lee, J., Park, I., Choi, Y., & Cho, J. (2012). *Bacillus* strains as feed additives: *In vitro* evaluation of its potential probiotic properties, 577–585.

Maathuis, A. J. H., Keller, D., & Farmer, S. (2010). Survival and metabolic activity of the GanedenBC30 strain of *Bacillus coagulans* in a dynamic *in vitro* model of the stomach and small intestine. *Beneficial Microbes*, *1*(1), 31–36. https://doi.org/10.3920/BM2009.0009

Majeed, M., & Nagabhushanam, K. (2016). A Double-Blind, Placebo-Controlled, Parallel Study Evaluating the Safety of *Bacillus coagulans* MTCC 5856 in Healthy Individuals. *Journal of Clinical Toxicology*, *06*(01), 1–9. https://doi.org/10.4172/2161-0495.1000283

Majeed, M., Nagabhushanam, K., Natarajan, S., Sivakumar, A., Ali, F., Pande, A., Majeed, S., & Karri, S. K. (2016). *Bacillus coagulans* MTCC 5856 supplementation in the management of diarrhea predominant Irritable Bowel Syndrome: a double blind randomized placebo controlled pilot clinical study. *Nutrition Journal*, *15*(1), 21. https://doi.org/10.1186/s12937-016-0140-6

Majeed, M., Nagabhushanam, K., Natarajan, S., Sivakumar, A., Eshuis-de Ruiter, T., Booij-Veurink, J., de Vries, Y. P., & Ali, F. (2016). Evaluation of genetic and phenotypic consistency of *Bacillus coagulans* MTCC 5856: a commercial probiotic strain. *World Journal of Microbiology and Biotechnology*, *32*(4), 1–12. https://doi.org/10.1007/s11274-016-2027-2

Majeed, M., Natarajan, S., Sivakumar, A., Ali, F., Pande, A., Majeed, S., & Karri, S. K.

(2016). Evaluation of Anti-Diarrhoeal Activity of *Bacillus coagulans* MTCC 5856 and its Effect on Gastrointestinal Motility in Wistat Rats. *International Journal of Pharma and Bio Sciences*, *7*(1), 311–316.

Mandel, D. R., Eichas, K., & Holmes, J. (2010). *Bacillus coagulans*: a viable adjunct therapy for relieving symptoms of rheumatoid arthritis according to a randomized, controlled trial. *BMC Complementary and Alternative Medicine*, *10*, 1–7.

Martín, M. J., Lara-Villoslada, F., Ruiz, M. A., & Morales, M. E. (2015). Microencapsulation of bacteria: A review of different technologies and their impact on the probiotic effects. *Innovative Food Science and Emerging Technologies*, *27*, 15–25. https://doi.org/10.1016/j.ifset.2014.09.010

Moir, A. (2006). How do spores germinate? *Journal of Applied Microbiology*, *101*(3), 526–530. https://doi.org/10.1111/j.1365-2672.2006.02885.x

Nicholson, W. L., Munakata, N., Horneck, G., Setlow, P., Melosh, H. J., & Setlow, P. (2003). Resistance of *Bacillus* Endospores to Extreme Terrestrial and Extraterrestrial Environments. *Microbiology and Molecular Biology Reviews*, *64*(3), 548–572. https://doi.org/10.1128/mmbr.64.3.548-572.2000

Palop, A., Raso, J., Pagán, R., Condón, S., & Sala, F. J. (1999). Influence of pH on heat resistance of spores of *Bacillus coagulans* in buffer and homogenized foods. *International Journal of Food Microbiology*, *46*(3), 243–249. https://doi.org/10.1016/S0168-1605(98)00199-8

Sanders, M. E. (2003). Probiotics: Considerations for human health. *Nutrition Reviews*, *61*(3), 91–99.

Setlow, P. (2014). Germination of spores of *Bacillus* species: What we know and do not

know. *Journal of Bacteriology*, *196*(7), 1297–1305. https://doi.org/10.1128/JB.01455-13

Shah, N. P. (2000). Probiotic Bacteria: Selective Enumeration and Survival in Dairy Foods. *Journal of Dairy Science*, *83*(4), 894–907. https://doi.org/10.3168/jds.S0022-0302(00)74953-8

Sheehan, V. M., Ross, P., & Firzgerald, G. F. (2007). Assessing the acid tolerance and the technological robustness of probiotic cultures for fortification in fruit juices. *Innovative Food Science and Emerging Technologies*, *8*, 279–284.

Song, D., Ibrahim, S., & Hayek, S. (2012). Recent Application of Probiotics in Food and Agricultural Science. *Intech Open*, *2*, 3–36. https://doi.org/10.5772/32009

Sorokulova, I. (2013). Modern Status and Perspectives of *Bacillus* Bacteria as Probiotics. *Journal of Probiotics & Health*, *1*(4), 1–5. https://doi.org/10.4172/2329-8901.1000e106

Spinosa, M. R., Braccini, T., Ricca, E., De Felice, M., Morelli, L., Pozzi, G., & Oggioni, M. R. (2000). On the fate of ingested *Bacillus* spores. *Research in Microbiology*, *151*(5), 361–368. https://doi.org/10.1016/S0923-2508(00)00159-5

Weinbreck, F., Bodnár, I., & Marco, M. L. (2010). Can encapsulation lengthen the shelf-life of probiotic bacteria in dry products? *International Journal of Food Microbiology*, *136*(3), 364–367. https://doi.org/10.1016/j.ijfoodmicro.2009.11.004

Wu, T., Zhang, Y., Lv, Y., Li, P., Yi, D., Wang, L., Zhao, D., Chen, H., Gong, J., & Hou, Y. (2018). Beneficial impact and molecular mechanism of *Bacillus coagulans* on piglets' intestine. *International Journal of Molecular Sciences*, *19*(7), 1–17. https://doi.org/10.3390/ijms19072084

CHAPTER 2: CHARACTERIZATION OF *BACILLUS COAGULANS* UNIQUE IS-2

2.1. Introduction

Bacillus coagulans is a Gram-positive rod, facultative, lactic acid producing and sporeforming bacteria. Due to its sporeforming abilities, *B. coagulans* has two different paths for its lifecycle. The vegetative one is the traditional life cycle of replicating, growing and proliferating in an environment rich with nutrients and capable of supporting life. When nutrients start to deplete or the environment changes to undesirable conditions, the cell can detect these and switch over to the life cycle that results in the production of endospores (Nicholson et al., 2003). The endospore is a protein rich protective layer that surrounds a dehydrated core that contains the chromosome (Fakhry et al., 2008). The spore is in a dormant state that can be transported by wind, water, or animal vectors and can survive for years. During this dormant spore state, the cell is continually monitoring the environment for desirable conditions. When conditions are favorable, the spore can begin to germinate and switch once again to the vegetative life cycle.

Bacillus coagulans' sporeforming ability provides this species with inherent protection against heat, cold, dryness, and acidic conditions (Nicholson et al., 2003). This spore protection is a main reason why *B. coagulans* has gained interest in the probiotic industry. Some strains of *B. coagulans* have already achieved GRAS status and have been shown to survive mild heat treatments (Orrù et al., 2014). A probiotic is defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). This is a very broad definition that describes the various microorganisms used as probiotics. However, a WHO/FAO working group has

defined more specific guidelines that lists the characteristics a microorganism should have in order to be considered as a probiotic. One of these is that all probiotics must be identified with genus, species and strain, as probiotic characteristics have been found to be strain specific (Araya et al., 2002). While it has been widely thought that health benefits are strain specific, there has been evidence that there are certain sub-species, species or genus specific health benefits (Sanders et al., 2018). For example, species from the genus *Bifidobacterium* are known for their production of short chain fatty acids, which have been shown to have beneficial effects on the gastrointestinal tract (Sanders et al., 2018). Therefore, there cannot be complete assumptions of probiotic ability among all strains of a genus or even species, and each individual strain should still be characterized and evaluated for its probiotic properties. It is also recommended that identity of the strain be evaluated phenotypically and genetically (Araya et al., 2002).

Bacillus coagulans has been documented as a diverse species of bacteria with varying abilities among different strains (De Clerck et al., 2004). Although the morphology may be similar, biochemical properties may differ among different strains. Therefore, when evaluating a strain for probiotic properties it is also important to characterize them with regard to their morphology and biochemical processes. It can also be important to sequence the DNA of the strain under evaluation to fully characterize the organism genetically.

When evaluating the phenotype of a strain, in addition to its morphology and chemical properties, another important property to characterize for potential probiotic strains is

their ability to survive processes usually applied in food manufacturing. Both stability and loss of viability have been observed when evaluating products added with probiotics during processing and shelf life (Ashraf & Shah, 2011; Bora et al., 2009). Therefore, it's important to evaluate the stability and survivability of a strain during food processing when considering it as a potential probiotic. One of the important processing steps used by the food industry is the use of heat. Thus, the thermal resistance of probiotic strains is an important factor to be considered when evaluating their potential applications in the food industry.

Bacterial thermal resistance is dependent upon the species, heating matrix, and intrinsic and extrinsic factors in the environment such as temperature and pH (Desai & Varadaraj, 2010; Haberbeck, Alberto da Silva Riehl, de Cássia Martins Salomão, & Falcão de Aragão, 2012). Most traditional probiotic strains, such as *Lactobacillus* and *Bifidobacterium,* are chosen for their acid tolerance so they can survive the acidic conditions of the stomach, but they are generally not heat resistant (Gotcheva et al., 2002). This requires them to be added after any thermal processing step that is part of the manufacturing of the product, otherwise they would die during that process and never reach the gut of the consumer. Therefore, the food industry has an interest in finding probiotic strains that are heat resistant and could survive during thermal treatments usually applied during processing. This would allow the addition of probiotic strains to take place at the beginning of the process, avoiding any potential post processing contamination.

The heat resistance of microorganisms is usually evaluated by determining the thermal death time required for one decimal reduction of the population, or D-value, of the organism at a specific temperature. The D-Value is the time (usually in minutes) required to reduce the bacterial population by 90%, or 1 log, at a specific temperature. The higher the D-value, the more resistant to heat the microorganism will be. This information can be useful in determining if it can survive thermal processing or how much must be added to the product formulation to compensate for any loss during processing and ensure the final product has the desired bacterial concentration. Additionally, thermal resistance data is not always collected at high temperatures, rather a Z-value is used to estimate how resistant a microorganism would be over a range of temperatures (Palop et al., 1999). Therefore, if the D-values at high temperatures would be experimentally determined, it would provide more accurate information associated to the thermal resistance of organisms of interest. The purpose of this research is to characterize the *Bacillus coagulans* strain Unique IS-2 using biochemical tests and to determine its thermal resistance by establishing its D-values at different temperatures and in different food matrices.

2.2. Materials and Methods

2.2.1. Preparation of Frozen Stocks

A lyophilized powder containing spores of *Bacillus coagulans* Unique IS-2 was obtained from Nebraska Cultures, LLC, which is now UAS Laboratories, LLC (Wausau, WI). The spores were grown aerobically in Tryptic Soy Broth (TSB) at 55˚C for 24 hours. Overnight cultures were transferred twice to fresh TSB, to ensure healthy growth. The

final culture was mixed with 15% (v/v) glycerol and dispensed into 1 ml aliquots and were stored into sterile 1.5 ml centrifuge tubes for storage at -80˚C. Working stocks for biochemical tests were prepared by streaking frozen stocks onto Tryptic Soy Agar (TSA) and incubating at 55˚C for 24 hours. Cultures were transferred to fresh TSA plates twice to ensure healthy growth after frozen storage.

2.2.2. Catalase Test

One drop of 3% hydrogen peroxide was placed on a glass slide. A sterile loop was used to transfer one colony of *B. coagulans* into the hydrogen peroxide drop. A positive reaction resulted in immediate bubbling.

2.2.3. Oxidase Test

Filter paper was placed inside a sterile petri plate and soaked with oxidase reagent solution. Immediately after adding the oxidase reagent, a sterile loop was used to transfer one colony of *B. coagulans* onto the filter paper. A positive reaction resulted in a color change of pink to dark purple within 10-30 seconds.

2.2.4. Indole Test

5 ml sterile 1% tryptone broth was dispensed into sterile test tubes. A sterile loop was used to inoculate one colony of *B. coagulans* into the tryptone broth. The inoculated media was incubated at 35˚C for 24 hours. After 24 hours, 5 drops of Kovac's Reagent was added to the tryptone broth tubes. A positive reaction was a dark red color formation on the surface layer of the broth.

2.2.5. Gelatin Hydrolysis

A sterile loop was used to stab inoculate a heavy load of multiple *B. coagulans* colonies into nutrient gelatin media tubes (Becton, Dickinson and company, Sparks, MD). Inoculated media tubes and control tubes were incubated at the optimal growth temperature of 55˚C for up to 14 days, checking every 24 hours for liquification. Every 24 hours, control and inoculated tubes were placed in an ice bath until control tube became solid (media liquefies over 28˚C). Inoculated tubes were then tilted to check for gelatin hydrolysis. If gelatin was hydrolyzed, inoculated tubes would remain liquid after ice bath.

2.2.6. Gram Stain

The Gram staining procedure outlined in the U.S. Food and Drug Administration BAM R32 method was followed (FDA, 2017a). If cells were stained purple, they were labeled Gram positive. If cells were stained pink/red, they were labeled Gram negative. Morphology was also evaluated under the microscope after Gram staining procedure.

2.2.7. API 50CH

The procedure outlined in API 50 CH test strips manual were followed (BioMérieux, 2019). A positive result was indicated by a color change from red to yellow in the tubes. For the esculin test, the color change was from red to black.

2.2.8. Phylogenetic Tree

A phylogenetic tree was created using the Mega7 software (Kumar, Stecher, & Tamura, 2016). Analysis was done with maximum likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993). The draft genome sequence for *Bacillus coagulans* Unique IS-2 was deposited at DDBJ/ENA/GenBank with the accession number JZDH00000000 (Upadrasta, Pitta, & Madempudi, 2016). The 16S strain for *Bacillus coagulans* Unique IS-2 was found within this whole genome sequence. A phylogenetic tree was created to compare how *B. coagulans* Unique IS-2 relates to other strains of *B. coagulans* and other common strains of *Bacillus, Lactobacillus and Bifidobacterium* used as probiotics. *Paenibacillus dendritiforis* was used to root the tree. All 16S sequences for comparison were retrieved from NCBI.

2.2.9. Thermal D-Values

A spore suspension was prepared by adding lyophilized spores of *Bacillus coagulans* Unique IS-2 (~2.0 x 10^{11} CFU/g) into phosphate-buffered saline (PBS) (FDA, 2017b) for a target bacterial population of 1.0 x 10^{8} CFU/ml. The spore suspension was heat treated at 80˚C for 12 minutes and immediately cooled in an ice bath to ensure only spores were present. Once cooled, 0.15 ml of the spore suspension were transferred to sterile capillary tubes and then sealed with heat.

One capillary tube was sealed with a calibrated Omega model TJ36-CPSS-116G-6 thermocouple placed through the top of the capillary tube. This thermocouple was used to accurately monitor temperature with the help of the Omega Logging Software for

Windows release 5.20.6 (Picto Technology LTD, England, GB) program. Temperature recordings were done every second, in order to create temperature vs. time plots.

Thermal death time of *B. coagulans* Unique IS-2 spores were evaluated at 90˚C, 110˚C, and 125˚C. At each temperature, thermal death time was evaluated in PBS, skim milk, whole milk and drinkable yogurt. All temperature and matrix combination were done in triplicate. A silicon oil bath was used as the heating medium. Heat sealed capillary tubes, along with the temperature monitoring capillary tube, were placed simultaneously into the oil bath. A capillary tube was pulled at designated intervals, which varied with the temperature being tested, and was immediately placed into an ice bath to cool suspension down quickly. Designated intervals did not begin until the desired temperature was reached inside the temperature monitoring ampule. Surviving spores were enumerated after ampules were cooled down to room temperature. Enumeration was done by serial dilutions in Butterfield's phosphate-buffered dilution water and then spread-plated on TSA. TSA plates were incubated aerobically at 55˚C for 48 hours. Results were used to create survival curves for each temperature and from these curves D-values were determined.

2.2.10. Statistical Analysis

Analysis of Variance was used to compare the D-values of each matrix at each temperature at the 5% level of significance. Statistics analysis was conducted using SAS 9.4 (SAS, INC, Cary, NC).

2.3. Results and Discussion

2.3.1. Biochemical Tests

Strain identification and characterization is important for two main reasons. First, not all probiotic properties are specific to a species of bacteria, as some are strain specific. Some research has shown evidence that there are certain sub-species, species or genus specific health benefits (Sanders et al., 2018). For example, species from the genus *Bifidobacterium* are known for their production of short chain fatty acids, which have been shown to have beneficial effects on the gastrointestinal tract (Sanders et al., 2018). However, it is recommended that each probiotic strain needs to be tested and characterized (Araya et al., 2002). Second, characterization tests can be used as a quicker method to differentiate species. Some tests may be unique to certain species and can differentiate them from others. *Bacillus coagulans* has been known to be a species with variation among different strains (De Clerck et al., 2004). Therefore, careful characterization of the strain of interest is even more important when evaluating its probiotic potential. 16S sequences were retrieved from NCBI to observe how *B. coagualans* Unique IS-2 is related to other *Bacillus* species regarded as potential probiotics and other popular bacteria strains used as probiotics from the *Lactobacillus* and *Bifidobacterium* genera (Figure 3).

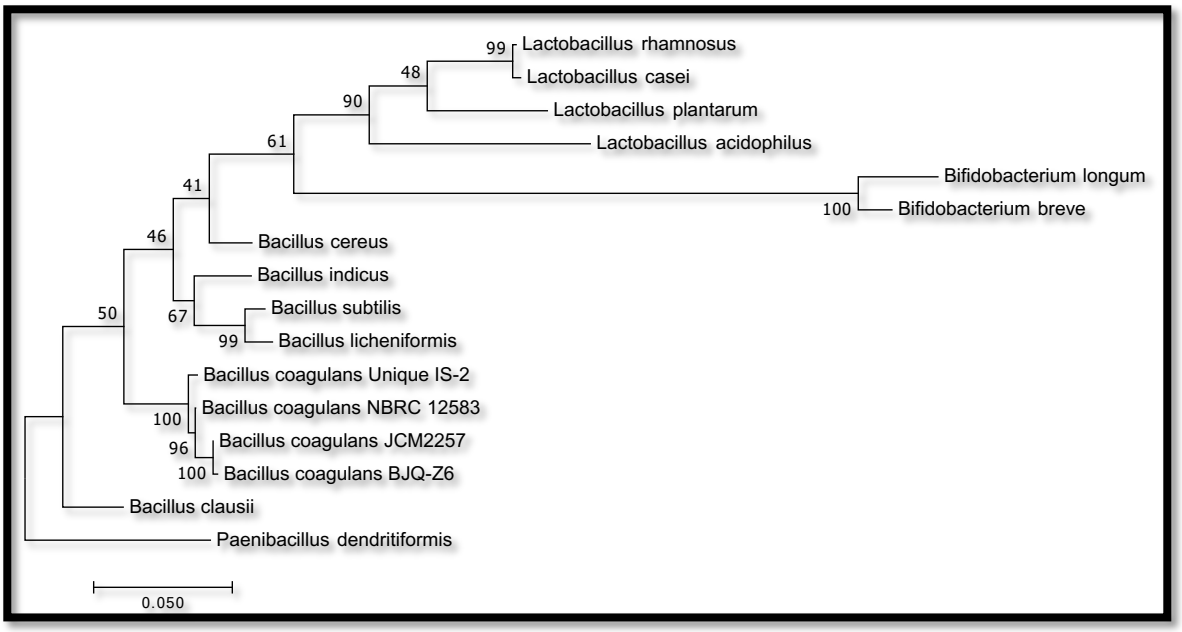

Figure 3. Phylogenetic tree using the maximum likelihood method of analysis for 16S sequences. Tree is drawn to scale. *Paenibacillus dendritiformis* was used to root the tree.

The biochemical test results for *Bacillus coagulans* Unique IS-2 are summarized in Table 1. This strain had a positive result during the catalase and oxidase test. Therefore, *B. coagulans* Unique IS-2 contains the catalase enzyme to neutralize hydrogen peroxide and possesses cytochrome C in its respiratory chain. However, negative results occurred during the indole and gelatin hydrolysis tests, indicating *B. coagulans* is unable to breakdown tryptophan to form indole and hydrolyze gelatin. These results compare with other strains of *B. coagulans* in the literature (Endres et al., 2011; Keller, Farmer, McCartney, & Gibson, 2010; Ratna Sudha, Chauhan, Dixit, Babu, & Jamil, 2010).

Table 1. Biochemical Test Results for *B. coagulans* Unique IS-2

Test	Result
Catalase	Positive
Oxidase	Positive
Indole	Negative
Gelatin Hydrolysis	Negative

B. coagulans is different from traditional probiotic strains because it is oxidase positive. Most probiotic strains are oxidase negative because they are anaerobic microorganisms. However, oxidase positive bacteria are able to utilize oxygen in their respiratory chain, which clarify them as facultative or aerobic species. *B. coagulans* is known as a facultative organism but grows best in the presence of oxygen, which could cause problems during germination in the gut as a probiotic due to the lack of oxygen in that environment.

Gram staining showed that *B. coagulans* Unique IS-2 are Gram positive rods typically by themselves or in short chains. The average length of the vegetative cell rods is 4.33 μm, which was measured using a light microscope (Figure 4). Additionally, the endospores are subterminally located, which is different than some other *Bacillus* species that are central or terminally located. However, this compares to other strains of *B. coagulans* (De Clerck et al., 2004). Endospores are important to this organism as it provides a built-in defense mechanism against harsh environments, such as heat, cold, acid, and desiccation.

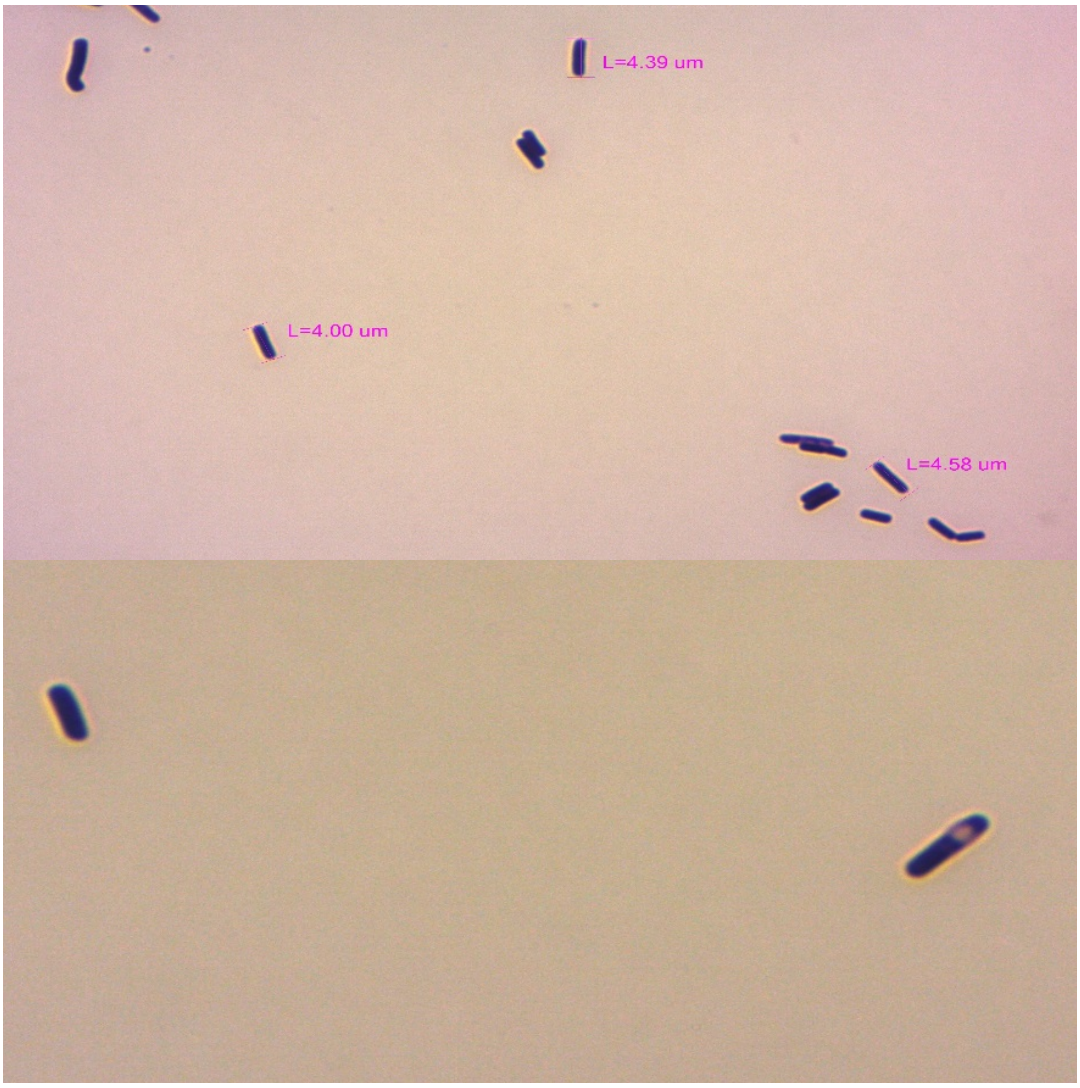

Figure 4. Gram staining of *Bacillus coagulans* Unique IS-2 showing Gram positive rods with lengths varying from 4.00 µm to 4.58 µm (top) and subterminally located endospores (bottom).

Bacillus coagulans is known to have physiological differences between strains (De Clerck et al., 2004; Majeed, Nagabhushanam, Natarajan, Sivakumar, Eshuis-de Ruiter, et al., 2016). Therefore, the API 50 CH test was used to determine which carbohydrates that *B. coagulans* strain Unique IS-2 is able to utilize. This strain was able to use 25 carbohydrates and was variable on 9 more (Table 2). These utilized carbohydrates were different than other strains of *B. coagulans* in the literature; L-arabinose, D-Ribose, D-

xylose, D-mannitol, Methyl-alphaD-glucopyranoside, amygdalin, arbutin, esculin, salicin, D-celiobiose, D-Lactose, D-saccharose, gentiobiose, D-tagatose, potassium gluconate, potassium 2-KetoGluconate, and potassium 5-ketogluconate. This data can be related to De Clerck et al. (2004) where they characterized 31 strains of *B. coagulans* with the intent to evaluate the diversity among them. In their research, 85% of the tested strains were also able to produce acid from D-galactose, D-fructose, D-glucose, glycerol, maltose, D-mannose, D-melibiose, N-acetylglucosamine and D-trehalose (De Clerck et al., 2004). However, there were also differences among the strains they tested and the Unique IS-2 strain reported here. Further emphasizing diversity among strains of this species and importance of proper strain characterization.

Table 2. API 50CH Results for *Bacillus coagulans* Unique IS-2

No.	Carbohydrates		Unique IS-2
0		Control	-
1	GLY	Glycerol	+
2	ERY	Erythritol	V
3	DARA	D-Arabinose	V
4	LARA	L-Arabinose	+
5	RIB	D-Ribose	+
6	DXYL	D-Xylose	+
7	LXYL	L-Xylose	-
8	ADO	D-Adonitol	-
9	MDX	Methyl-βD-Xylopyranoside	-
10	GAL	D-Galactose	V
11	GLU	D-Glucose	+
12	FRU	D-Fructose	+
13	MNE	D-Mannose	+
14	SBE	L-Sorbose	-
15	RHA	L-Rhamnose	V
16	DUL	Dulcitol	-
17	INO	Inositol	-
18	MAN	D-Mannitol	+
19	SOR	D-Sorbitol	-
20	MDM	Methyl-αD-Mannopyranoside	-
21	MDG	Methyl-αD-Glucoopyranoside	+
22	NAG	N-AcetylGlucosamine	+
23	AMY	Amygdalin	+
24	ARB	Arbutin	+
25	ESC	Esculin	+
26	SAL	Salicin	+
27	CEL	D-Celiobiose	+
28	MAL	D-Maltose	+
29	LAC	D-Lactose (bovine origin)	+
30	MEL	D-Melibiose	+
31	SAC	D-Saccharose (sucrose)	+
32	TRE	D-Trehalose	+
33	INU	Inulin	-
34	MLZ	D-Melezitose	-
35	RAF	D-Raffinose	V
36	AMD	Amidon (starch)	V
37	GLYG	Glycogen	V
38	XLT	Xylitol	-
39	GEN	Gentiobiose	+
40	TUR	D-Turanose	V
41	LYX	D-Lyxose	V
42	TAG	D-Tagatose	+
43	DFUC	D-Fucose	-
44	LFUC	L-Fucose	-
45	DARL	D-Arabitol	-
46	LARL	L-Arabitol	-
47	GNT	Potassium Gluconate	+
48	2KG	Potassium 2-KetoGluconate	+
49	5KG	Potassium 5-Ketogluconate	+

*V = Variable results among replications

2.3.3. Thermal D-Values

Thermal death times of *Bacillus coagulans* Unique IS-2 spores were observed at three different temperatures in four different matrices. One matrix was a buffer to observe the behavior of the spores in response to temperature alone as a control, while three food matrices were chosen to evaluate how different products with different fat and pH levels may affect the thermal death time of the organism of interest. Previous research indicates that thermal death times may be affected by the content of fat in the matrix due to a protective effect (Fain et al., 1991; Murphy, Osaili, Duncan, & Marcy, 2004). In this research, skim milk with no notable fat content, and whole milk with 3% fat content were evaluated. In addition, yogurt was also used as a matrix for determination of D-values. Milk has a neutral pH while yogurt has a pH of 4.6. Based on information available in the literature, a higher fat content may protect the spores and extend the thermal death times, while the lower pH is thought to shorten the thermal death time.

Survival curves for *Bacillus coagulans* IS-2 in the phosphate buffered solution (PBS), skim milk, whole milk and yogurt are shown in Figures 5 – 8, respectively. For all matrices tested, the greater the temperature the lower the D-value was, which was expected. The D-Values for *B. coagulans* at 90˚C were calculated as 29.9, 35.7, 39.9 and 7.7 minutes in PBS, skim milk, whole milk and yogurt respectively. At the lowest temperature tested, *B. coagulans* Unique IS-2 had the longest D-value in whole milk, followed by skim milk, showing slightly lower values. This indicated that during a pasteurization time/temperature combination of 60˚C for 30 minutes used for fluid milk the strain under evaluation could survive well (Desai & Varadaraj, 2010). Additionally,

the high temperature short time pasteurization of milk is 72˚C for 15 seconds, which the strain under evaluation could also survive well in (U.S. Department of Health and Human Services, Public Health Service, & Food and Drug Administration, 2017). Under these circumstances, this strain could be added before the pasteurization processing step and it would still be viable in the final product.

The biggest difference in D-values at 90˚C was in the yogurt matrix. The D-value of yogurt was statistically different from the D-value of whole milk ($p<0.05$), but not statistically different from the D-values of PBS or skim milk. The low D-value observed in this product can be attributed to its low pH. The acidic conditions contribute as a stress factor on the spore in addition to the thermal treatment, therefore the spores are inactivated faster. Palop et al., (1999) also found that heat resistance was lowered with a lower pH, and additionally they observed that the pH effect was greater at lower temperatures. This agrees with the observations reported here, because the greatest D-value difference among matrices was recorded at the lowest temperature tested. This would be an important note for manufacturers wishing to use this strain as a probiotic associated with a low pH product. They may have to account for these losses during processing by adjusting their product formulation.

The D-values calculated where the thermal treatment was conducted at 110˚C were 0.63, 1.25, 1.96, and 0.48 minutes in PBS, skim milk, whole milk and yogurt respectively. With a 20 degree increase in temperature, the survival of *B. coagulans* Unique IS-2 spores were significantly lower. The buffer system had the largest decrease in D-values,

dropping 47 times lower compared to the D-value at 90˚C. The rest of the D-values were 28 times, 20 times, and 16 times lower at 110˚C than 90˚C for skim milk, whole milk and yogurt, respectively. This trend is similar to the research done by Palop et al., (1999), where they saw that increased temperature lowered the D-values, and they observed greater differences in D-values in more neutral pH ranges. At 110˚C, the highest D-value for the spores was in whole milk, while the yogurt had the lowest D-value similar to what was seen at 90˚C. However, at 110˚C there is no significant difference between the D-values of PBS, skim milk, whole milk and yogurt ($p<0.05$).

At 125˚C the calculated D-values were 0.23, 0.54, 0.31 and 0.23 minutes in PBS, skim milk, whole milk and yogurt respectively. These D-values were lowered by 2 times in PBS, skim milk and yogurt from the D-value at 110˚C, while the D-value of whole milk had been lowered 6 times compared to the D-value at 110˚C. At the highest temperature tested, PBS and yogurt had the lowest D-value, while skim milk had the highest D-value. However, at 125˚C there was no significant difference between the D-values of PBS, skim milk, whole milk and yogurt ($p<0.05$). Some of this variation could be due to the higher standard deviation between replications at this temperature. These differences could be partially due to the close proximity between time points needed to establish the thermal death curve. Overall, the trend is similar to 110˚C, where the D-value was again shortened but it did not decrease as much as the decrease between 90˚C and 110˚C.

The regulated parameters for ultra-pasteurization for milk is a heat treatment at 138˚C for 2 seconds (U.S. Department of Health and Human Services et al., 2017). This

temperature is greater than the highest evaluated temperature, 125˚C, however the spores' lowest D-value (0.23 min), could indicate the potential for survival for a few seconds at 138˚C.

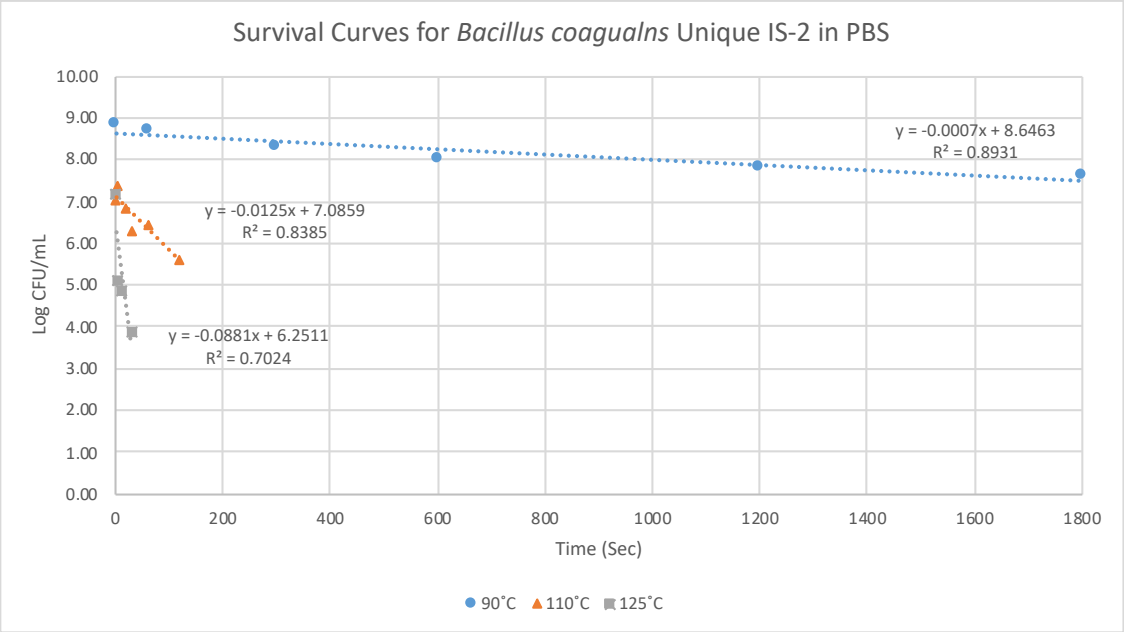

Figure 5. Survival curves for *Bacillus coagulans* Unique IS-2 spores in Phosphate Buffered Solution when treated at 90, 110, and 125˚C.

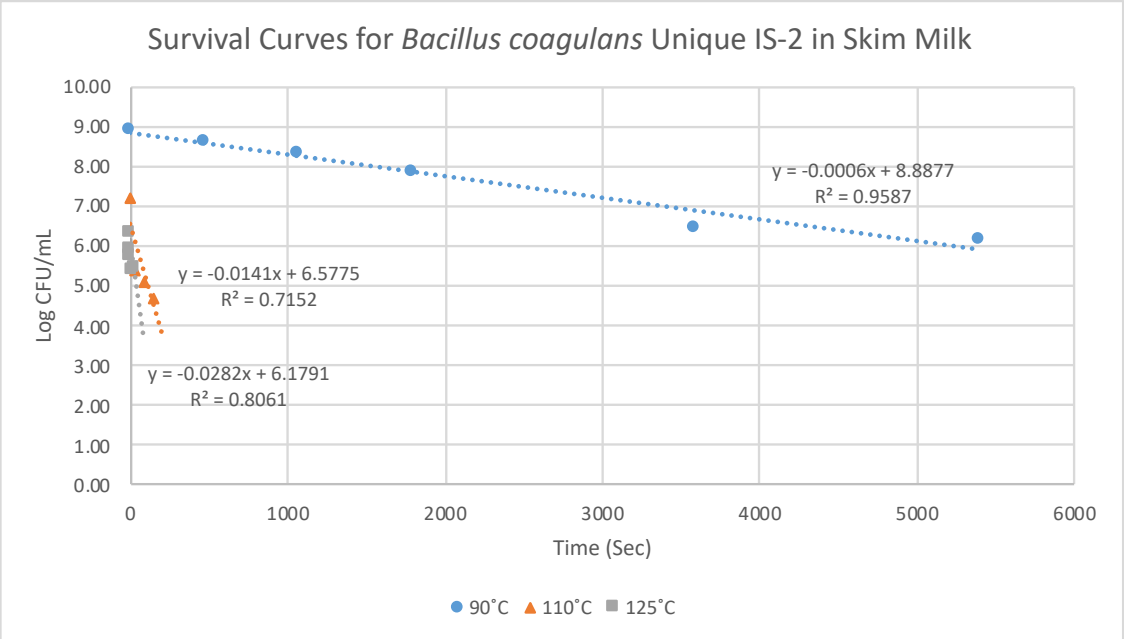

Figure 6. Survival curves for *Bacillus coagulans* Unique IS-2 spores in skim milk when treated at 90, 110, and 125˚C.

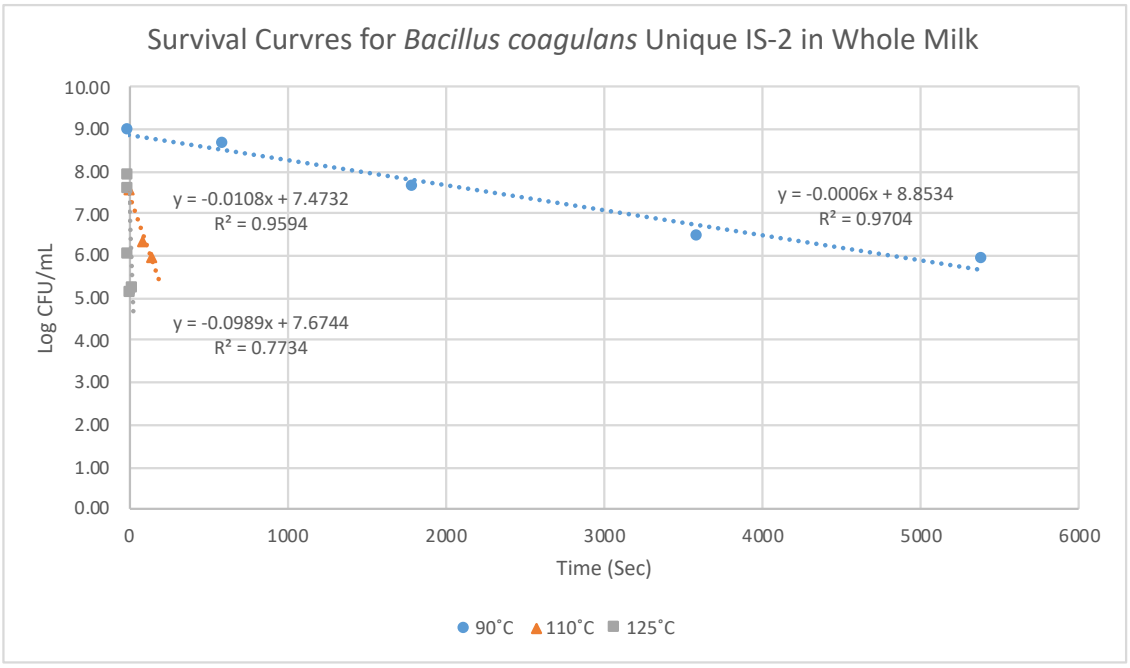

Figure 7. Survival Curves for *Bacillus coagulans* Unique IS-2 spores in whole milk when treated at 90, 110, and 125˚C.

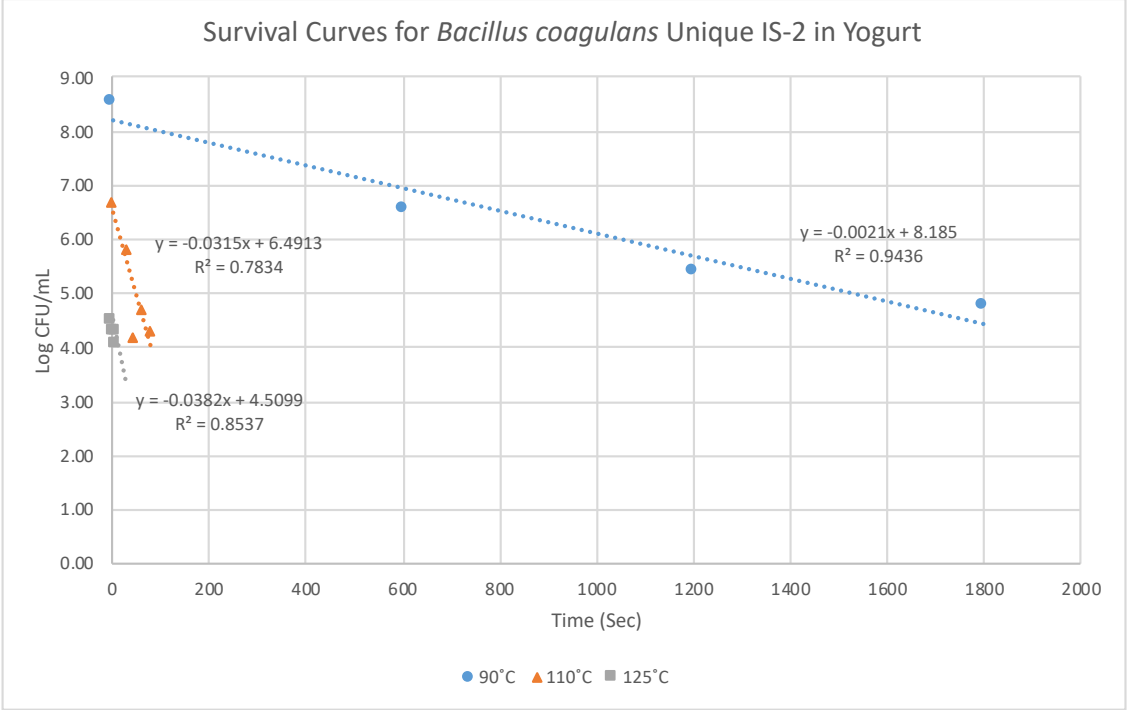

Figure 8. Survival Curves for *Bacillus coagulans* Unique IS-2 spores in drinkable yogurt when treated at 90, 110, and 125˚C.

The D-values observed for *B. coagulans* Unique IS-2 spores in this research can be compared to other information available for *Bacillus* spores. *B. cereus* spores have been found to have D-values of 19.45 minutes and 4.4 minutes at 85˚C and 95˚C respectively (Desai & Varadaraj, 2010). Another *B. coagulans* strain was found to have calculated D-values in milk D_{95} = 4 minutes and D_{110} = 0.20 (Janštová & Lukášová, 2001). These calculated D-values are lower than those obtained in this research. Additionally, Janštová and Lukášová, (2001) evaluated different *Bacillus* spores. Among those studied, *B. licheniformis* had the greatest heat resistance with D-values of 4.51, 0.68 and 0.18 minutes at 95˚C, 110˚C and 120˚C respectively. In general, based on information available in the literature, the *B. coagulans* Unique IS-2, appears to have higher thermal resistance than other *Bacillus* spores.

From the survival curves of *B. coagulans* Unique IS-2 in the four matrices, D-values were able to be determined. Those D-values were then plotted against time (Figure 9) in order to predict D-values at different temperatures than those tested here. For whole milk, the R^2 value was 0.9969 (Table 3), which indicates a strong correlation between D-value and temperature. This will allow for the estimation of D-values at different temperatures, within those tested here, with confidence. For all other matrices evaluated, the R^2 value was equal to or lower than 0.9429, indicating that the association between D-values and temperature was not linear within the interval evaluated. If the interval is reduced to included data for the thermal death between 90˚C and 110˚C then the correlation seems to be linear. The leveling of the curve beyond 110˚C may reflect intrinsic characteristics associated with the spores, indicating that higher temperatures do

not further contribute to thermal death. However, without further experiments in this temperature range, conclusions could not be made. In further work, additional testing of temperatures should be considered between 90˚C and 110˚C and between 110˚C and 125˚C to improve the confidence on this data.

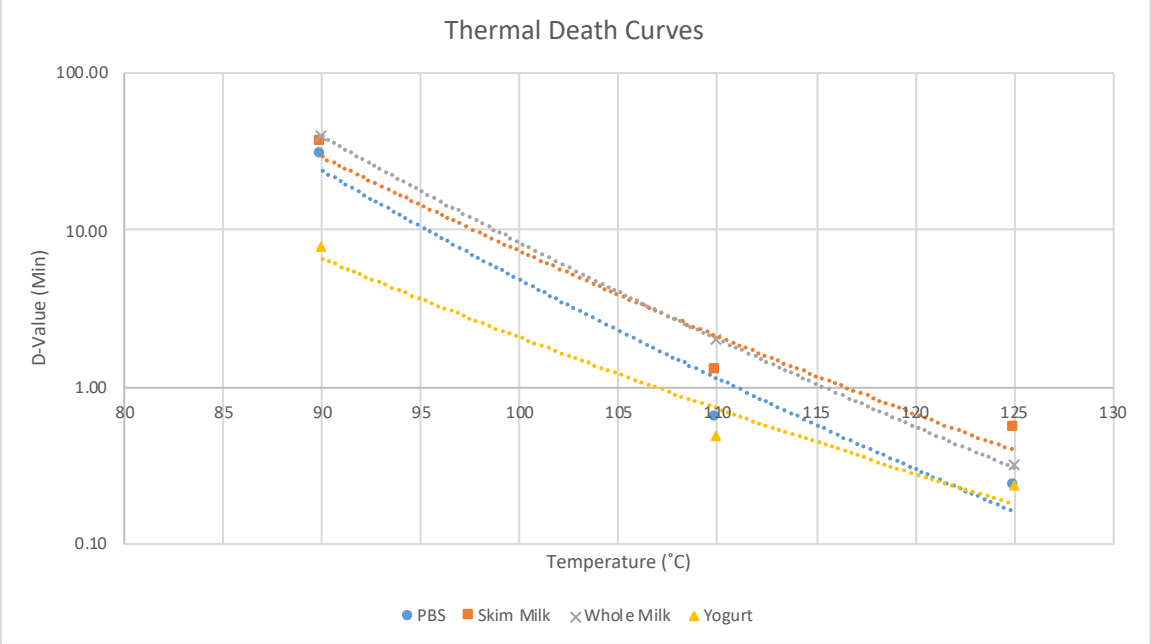

Figure 9. Thermal death curves

Table 3. Summary of D-Values for each temperature/matrix combination and linear regression lines with R^2 values.

	D-Value (Min)[1]				
Product	90˚C	110˚C	125˚C	Linear Regression Line	R^2 Value
PBS	29.94[a,b]	0.63	0.23	y=-0.0617x+6.8946	0.9416
Skim Milk	35.74[a,b]	1.25	0.54	y=-0.0531x+6.2183	0.9383
Whole Milk	39.91[a]	1.96	0.31	y=-0.0606x+7.0219	0.9969
Yogurt	7.70[b]	0.48	0.23	y=-0.0445x+4.7938	0.9429

[1]Treatments with different letters within the same column are significantly different ($p<0.05$)

2.4. Conclusion

Bacillus coagulans is a very diverse species with varying characteristics among strains (De Clerck et al., 2004). This reason reveals the importance of evaluating the biochemical tests of each strain of interest when evaluating probiotic properties. In this research, *B. coagulans* Unique IS-2 was determined to be a Gram-positive rod, spore-former, catalase positive, oxidase positive, indole negative and gelatin hydrolysis negative. These results are similar to other *B. coagulans* strains in the literature, though the evaluation of biochemical test is also important for potential probiotic strains (Araya et al., 2002; De Clerck et al., 2004; Keller et al., 2010; Ratna Sudha et al., 2010).

According to these experiments, *Bacillus coagulans* Unique IS-2 spores have high thermal resistance. At neutral pH, spores have a D-value of at least 29 minutes at 90˚C, which is significantly greater than parameters usually used for pasteurization in the food industry. These D-values are of great importance to industry as they help indicate which products could have *B. coagulans* Unique IS-2 incorporated into their formulation with the potential probiotic strain surviving the thermal processing. The D-values are also useful in the determination of any formulation adjustment that may be necessary to ensure probiotic levels in the final product, based on the processing parameters of thermal treatments. Another important piece of information obtained from these experiments was the effect of pH on the D-value of *B. coagulans* Unique IS-2 spores. Extra care should be taken with acidified products as the lower the pH, the faster the spores are inactivated. To ensure desired bacterial levels in the final product, the product formulation would have to account for the loss due to any heat treatment process applied to the product.

These experiments evaluated three temperatures and four different matrices. Therefore, the D-values calculated here may only be applied to these specific conditions. Further work should incorporate more temperatures within the range of 90-125˚C for skim milk and yogurt, as well as other food matrices. One factor to consider would be to test food matrices with a higher variation of fat content. Results showed there was no significant difference in D-values between the 3% fat difference of skim milk and whole milk. It may be beneficial to evaluate the effect of fat content levels varying between 10% - 20% , or higher, for the potential inclusion of *B. coagulans* spores into more products (Fain et al., 1991; Murphy et al., 2004). The information associated with thermal inactivation of spores of *B. coagulans* Unique IS-2 reported here is a good indication of its thermal resistance and that it could be a potential probiotic of choice for products that must go through a thermal process.

2.5. References

Araya, M., Morelli, L., Reid, G., Sanders, M. E., Stanton, C., Pineiro, M., & Ben Embarek, P. (2002). Guidelines for the Evaluation of Probiotics in Food. *Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food*, 1–11. https://doi.org/10.1111/j.1469-0691.2012.03873

Ashraf, R., & Shah, N. P. (2011). Selective and differential enumerations of *Lactobacillus delbrueckii* subsp. *bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus casei* and *Bifidobacterium* spp. in yoghurt - A review. *International Journal of Food Microbiology*, *149*(3), 94–208. https://doi.org/10.1016/j.ijfoodmicro.2011.07.008

BioMérieux. (2019). API50CH Manual. Retrieved from https://www.biomerieux.com

Bora, P. S., Puri, V., & Bansal, A. K. (2009). Physicochemical Properties and Excipient Compatibility studies of Probiotic *Bacillus coagulans* Spores. *Scientia Pharmaceutica*, *77*, 625–637. https://doi.org/10.3797/scipharm.0904-01

De Clerck, E., Rodriguez-Diaz, M., Forsyth, G., Lebbe, L., Logan, N. A., & DeVos, P. (2004). Polyphasic Characterization of *Bacillus coagulans* Strains, Illustrating Heterogeneity within this Species, and Emended Description of the Species. *Systematic and Applied Microbiology*, *27*(1), 50–60. https://doi.org/10.1078/0723-2020-00250

Desai, S. V., & Varadaraj, M. C. (2010). Behavioural pattern of vegetative cells and spores of *Bacillus* cereus as affected by time-temperature combinations used in processing of Indian traditional foods. *Journal of Food Science and Technology*, *47*(5), 549–556. https://doi.org/10.1007/s13197-010-0099-9

Endres, J. R., Qureshi, I., Farber, T., Hauswirth, J., Hirka, G., Pasics, I., & Schauss, A. G. (2011). One-year chronic oral toxicity with combined reproduction toxicity study of a novel probiotic, *Bacillus coagulans*, as a food ingredient. *Food and Chemical Toxicology*, *49*(5), 1174–1182. https://doi.org/10.1016/j.fct.2011.02.012

Fain, A. R., Line, E. J., Moran, A. B., Martin, M. L., Lechowich, R. V., Carosella, J. M., & Brown, W. L. (1991). Lethality of Heat to *Listeria monocytogenes* Scott A: D-Value and Z-Value Determinations in Ground Beef and Turkey. *Journal of Food Protection*, *54*(10), 756–761. https://doi.org/10.4315/0362-028x-54.10.756

Fakhry, S., Sorrentini, I., Ricca, E., De Felice, M., & Baccigalupi, L. (2008). Characterization of spore forming Bacilli isolated from the human gastrointestinal tract. *Journal of Applied Microbiology*, *105*(6), 2178–2186. https://doi.org/10.1111/j.1365-2672.2008.03934.x

FDA. (2017a). BAM R32: Gram Stain. Retrieved from https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm062229.htm

FDA. (2017b). BAM R59: Phosphate-Buffered Saline (PBS), pH 7.4. Retrieved from https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm062268.htm

Gotcheva, V., Hristozova, E., Hristozova, T., Guo, M., Roshkova, Z., & Angelov, A. (2002). Assessment of Potential Probiotic Properties of Lactic Acid Bacteria and Yeast Strains. *Food Biotechnology*, *16*(3), 211–225. https://doi.org/10.1081/FBT-120016668

Haberbeck, L. U., Alberto da Silva Riehl, C., de Cássia Martins Salomão, B., & Falcão

de Aragão, G. M. (2012). *Bacillus coagulans* spore inactivation through the application of oregano essential oil and heat. *LWT - Food Science and Technology*, *46*(1), 267–273. https://doi.org/10.1016/j.lwt.2011.09.021

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C., & Sanders, M. E. (2014). The International Scientific Association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *National Review Gastroenterol Hepatol*, *11*(11), 506–514. https://doi.org/10.1038/nrgastro.2014.66

Janštová, B., & Lukášová, J. (2001). Heat resistance of *bacillus* spp. spores isolated from cow's milk and farm environment. *Acta Veterinaria Brno*, *70*(2), 179–184.

Keller, D., Farmer, S., McCartney, A., & Gibson, G. (2010). *Bacillus coagulans* as a probiotic. *Food Science and Technology Bulletin: Functional Foods*, *7*, 103–109. https://doi.org/10.1616/1476-2137.16015.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, *33*, 1870–1874.

Majeed, M., Nagabhushanam, K., Natarajan, S., Sivakumar, A., Eshuis-de Ruiter, T., Booij-Veurink, J., de Vries, Y. P., & Ali, F. (2016). Evaluation of genetic and phenotypic consistency of *Bacillus coagulans* MTCC 5856: a commercial probiotic strain. *World Journal of Microbiology and Biotechnology*, *32*(4), 1–12. https://doi.org/10.1007/s11274-016-2027-2

Murphy, R. Y., Osaili, T., Duncan, L. K., & Marcy, J. A. (2004). Effect of Sodium Lactate on Thermal Inactivation of *Listeria monocytogenes* and *Salmonella* in

Ground Chicken Thigh and Leg Meat. *Poultry Science*, *83*, 1218–1225. https://doi.org/10.4315/0362-028x-67.7.1403

Nicholson, W. L., Munakata, N., Horneck, G., Setlow, P., Melosh, H. J., & Setlow, P. (2003). Resistance of *Bacillus* Endospores to Extreme Terrestrial and Extraterrestrial Environments. *Microbiology and Molecular Biology Reviews*, *64*(3), 548–572. https://doi.org/10.1128/mmbr.64.3.548-572.2000

Orrù, L., Salvetti, E., Cattivelli, L., Lamontanara, A., Michelotti, V., Capozzi, V., Spano, G., Keller, D., Cash, H., Martina, A., Torriani, S., & Felis, G. E. (2014). Draft Genome Sequence of *Bacillus coagulans* GBI-30, 6086, a Widely Used Spore-Forming Probiotic Strain. *Genome Announcement*, *2*(6), 6–7. https://doi.org/10.1128/genomeA.01080-14.Copyright

Palop, A., Raso, J., Pagán, R., Condón, S., & Sala, F. J. (1999). Influence of pH on heat resistance of spores of *Bacillus coagulans* in buffer and homogenized foods. *International Journal of Food Microbiology*, *46*(3), 243–249. https://doi.org/10.1016/S0168-1605(98)00199-8

Ratna Sudha, M., Chauhan, P., Dixit, K., Babu, S., & Jamil, K. (2010). Molecular typing and probiotic attributes of a new strain of *Bacillus coagulans* - Unique IS-2: A potential biotherapeutic agent. *Genetic Engineering and Biotechnology Journal*, *2010*(1986), 1–20.

Sanders, M. E., Benson, A., Lebeer, S., Merenstein, D. J., & Klaenhammer, T. R. (2018). Shared mechanisms among probiotic taxa: implications for general probiotic claims. *Current Opinion in Biotechnology*, *49*(Figure 1), 207–216. https://doi.org/10.1016/j.copbio.2017.09.007

Tamura, K., & Nei, M. (1993). Estimation of the number of necleotide substitutions in the control region of mitrochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, *10*, 512–526.

U.S. Department of Health and Human Services, Public Health Service, & Food and Drug Administration. (2017). Grade "A" Pasteurized Milk Ordinance (Includes provisions from the Grade "A" Condensed and Dry Milk Products and Condensed and Dry Whey-Supplement I to the Grade "A" PMO) 2017 Revision. Retrieved from https://www.fda.gov/downloads/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Milk/UCM612027.pdf

Upadrasta, A., Pitta, S., & Madempudi, R. S. (2016). Draft Genome Sequence of the Spore-Forming Probiotic Strain *Bacillus coagulans* Unique IS-2 . *Genome Announcements*, *4*(2), 7–8. https://doi.org/10.1128/genomea.00225-16

CHAPTER 3: SURVIVAL AND GERMINATION OF BACILLUS COAGULANS UNIQUE IS-2 IN THE GASTROINTESTINAL TRACT

3.1. Introduction

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). While there is no official intake level of probiotics for inclusion in food, it is generally accepted that probiotic foods should be formulated to contain between $10^7 – 10^9$ CFU/g of a product (Ashraf & Shah, 2011; Konuray & Erginkaya, 2018). At these levels, probiotics are able to confer health benefits upon reaching the colon by increasing the growth of good bacteria and hindering the growth of undesirable pathogenic bacteria (Sanders, 2000). As probiotics are becoming more popular for inclusion into both animal and human food products, to improve gut health, probiotic research has increased. The main properties researchers are focusing on are the microorganisms' tolerance to acid and bile conditions, growth and propagation in the gut, and the viability during processing and storage (Kerry et al., 2018).

Tolerance to acidic and bile conditions are important properties of a probiotic microorganism. For probiotics consumed orally, the target organ is the gut, which is where they will grow and proliferate to provide a health benefit. After consumption of the probiotic organism, it will travel through the gastrointestinal tract encountering adverse conditions that may prevent it from reaching the gut, such as the acidic conditions of the stomach and the presence of bile in the small intestine. While the consumption of food can lower the acidity of the stomach, its pH is most commonly between pH 2.5 – 3.5; however it can be between pH 1.5 – 6.0 at its extremes (Holzapfel, Haberer, Snel, Schillinger, & Huis In'T Veld, 1998; Huang & Adams, 2004). These are

potentially harmful conditions to microorganisms and the tolerance to these conditions is characteristic of probiotic strains. Popular probiotics strains from the *Lactobacillus* and *Bifidobacterium* genera have been chosen because of their higher tolerance to acid and bile conditions (Dunne et al., 2001). However, research has shown that some lactic acid bacteria have a significant decrease in populations at pH 2.0, which means they may survive but could benefit from some protection during transit through the stomach (Gotcheva et al., 2002). Typically, their percent survival has been observed anywhere from 1-15%, which is relatively low (Keller et al., 2019).

Due to the variable survivability of common probiotics, a more recent trend in the probiotic industry is the use of sporeforming bacteria as probiotics. Spores are dormant life forms that are resistant to a number of harsh environments, such as heat, cold, acid, and dryness (Nicholson et al., 2003). During the vegetative life cycle, the cell is continually monitoring the environment for unfavorable conditions. When nutrients start to deplete or the environment changes to undesirable conditions, the cells can detect these and switch to the production of endospores (Nicholson et al., 2003). When this happens, the mother cell creates a protein rich coat, that surrounds a dehydrated chromosome as protection (Cutting, 2011; Henriques & Moran, Jr., 2007). This spore is then almost completely metabolically inactive, but can survive for years in this dormant state. Then upon reaching a suitable environment, the spore can germinate to produce vegetative cells again (Moir, 2006).

One popular sporeformer being considered for its probiotic properties is *Bacillus coagulans*. This organism is Gram-positive, facultative, rod-shaped, lactic acid producing, motile and sporeforming bacteria (Payot, Chemaly, & Fick, 1999). Therefore, the spore form of *B. coagulans* should be a more resistant structure than its vegetative cell and would theoretically provide further protection against acid conditions and bile presence. Some strains of *Bacillus* vegetative cells have been observed to have resistance to low pH environments and bile presence (Lee et al., 2012). After survival through the upper gastrointestinal tract, *B. coagulans* spores will be expected to reach the gut and then begin to germinate.

There have been studies done showing that *B. coagulans* spores have increased growth performance effects in chickens and promote growth of healthy microorganisms in the gut of chickens and pigs (Hung et al., 2012; Wu et al., 2018; Zhou, Wang, Gu, & Li, 2010). *Bacillus coagulans* is also capable of producing lactic acid and coagulin, an antibacterial substance, which has been shown to inhibit the growth of *Listeria, Enterococcus* and *Leuconostoc* (Hyronimus, Le Marrec, & Urdaci, 1998). Baron (2009) also observed that the consumption of *B. coagulans* led to an increase in T-cell production of TNF-alpha, a type of messenger protein, when exposed to adenovirus and influenza A (Baron, 2009). More specifically associated with the requirements for probiotic cultures, Tam and colleagues were able to use a molecular approach to observe germination, growth and resporulation of *Bacillus subtilis* spores using a mouse model (Tam et al., 2006). Because *B. subtilis* shares some similar characteristics with *B.*

coagulans, the results by Tam et al. (2006) may indicate an ability by *B. coagulans* to also germinate, grow and resporulate in the gut.

Therefore, the purpose of this research was to evaluate the acid and bile tolerance of the spores of *B. coagulans* Unique IS-2 in a simulated stomach environment and the germination of these spores in an *in vitro* gut environment. These are both important properties that an organism used as a probiotic should have.

3.2. Materials and Methods

3.2.1. Bacterial Culture

A lyophilized powder containing spores of *Bacillus coagulans* Unique IS-2 was obtained from Nebraska Cultures, LLC, which is now UAS Laboratories, LLC (Wausau, WI). Lyophilized culture was stored in 4˚C until use.

3.2.2. *In vitro* Stomach pH and Bile Salts Stability

A modified version of Hyronimus et al. (2000) was used for pH and bile salts stability studies. Tubes of 10 ml Tryptic Soy Broth (TSB) were adjusted to pH 2.0, 2.5, and 3.0 using 3M HCl. The 10 ml TSB tubes containing 0.3% bile salts were also prepared along with control tubes, which included 10 ml of TSB without the addition of acid or bile salts.

A spore suspension was prepared by adding lyophilized spores of *Bacillus coagulans* Unique IS-2, concentration of ~2.0 x 10^{11} CFU/g, into phosphate-buffered saline to achieve a concentration of 10^{10} CFU/ml. The spore suspension was heat treated at 80˚C

for 12 minutes and was then immediately chilled in an ice bath. A 10 µL aliquot of spore suspension was inoculated into each modified TSB tube to achieve a bacterial suspension of approximately 1 x 10^8 CFU/ml.

Modified TSB tubes were incubated at 37˚C and pulled for enumeration at 0, 0.5, 1, 2 and 4 hours. Samples were serially diluted using Butterfield's phosphate-buffered dilution water. Dilutions were spread-plated on Tryptic Soy Agar (TSA) in duplicate and incubated aerobically at 55˚C for 48 hours. Survival curves and survival rates (percent of initial concentration recovered after 4 hours) were calculated and compared to control.

3.2.3. *In vitro* Digestion

To determine germination of *B. coagulans* Unique IS-2 *in vitro*, a fecal slurry was used to simulate the gut environment. Preparation of the fecal slurry followed a modified version of the experiment outlined in Hartzell et al. (2013). Fecal samples were obtained from adult volunteers within the Department of Food Science and Technology at the University of Nebraska-Lincoln (IRB # 20171017517EP). Volunteers did not take probiotics regularly and had not been recently ill. Fecal samples were collected using specimen collection kits. Upon receipt of fecal sample, all processing of the sample was done in the anaerobic chamber. Ten g of the fecal sample was added to 90 ml of phosphate-buffered saline plus 10% glycerol. The mixture was hand blended until completely mixed, then filtered through 4 layers of cheese cloth. Filtered fecal slurry was then stored in sterile conical tubes at -80˚C until use.

Fermentation broth was prepared by mixing 1 g peptone, 1 g yeast extract, 0.05 g NaCl, 0.25 g cysteine HCl, 0.25 g bile salts, 1 mL tween 80, 5 μL vitamin K (added after autoclaving), 2 mL 0.025% (w/v) resazurin solution, 0.5 mL $MgSO_4 \bullet 7H_2O$ (10 mg/ml), 0.5 mL $CaCl_2 \bullet 2H_2O$ (10 mg/ml), 0.5 mL hemin (5 mg/ml) dissolved in Dimethyl sulfoxide (DMSO), 0.04g K_2HPO_4, and 1 g $NaHCO_3$ into 400 ml of distilled water. The pH of the broth was adjusted to pH 7 using 3M HCl, then the volume was adjusted to 500 mL with additional distilled water followed by autoclaving for sterility.

A spore suspension was prepared by adding 1 g of lyophilized spores of *Bacillus coagulans* Unique IS-2, concentration of ~2.0 x 10^{11} CFU/g, into 99 ml of phosphate-buffered saline. To keep conditions anaerobic, the preparation of the experiment was done in an anaerobic chamber. To create the gut environment, the fermentation broth was mixed with the filtered fecal slurry in a 6:3 ratio. The fecal slurry provided by each volunteer served as experimental replicates. The fermentation broth and fecal slurry mixture was then split in half to provide one portion as control and another in which the spore suspension was added to achieve a bacterial concentration of 10^8 CFU/ml.

Both the inoculated and control fecal slurry was further split into sterile conical tubes (at least 5) to allow for sampling over time. For each fecal slurry, one control and inoculated samples were set aside to be enumerated for spores at time zero while the remaining samples were anaerobically incubated at 37˚C in a MaxQ 4000 benchtop incubator shaker (Thermo Scientific, Marietta, OH), shaking at 130 rpm. From each volunteer, inoculated and control samples were split into four sealed containers added with an anaeropack

(Mitshubishi Gas Chemical Company, Inc, New York, NY) to create an anaerobic environment. Each container was removed from incubation at 4, 8, 12 and 24 hours.

For each time point, control and inoculated samples were heat treated at 80˚C for 12 minutes, immediately chilled in an ice bath, serially diluted using Butterfield's Phosphate-Buffered Dilution Water and spread-plated on TSA. Enumeration plates were incubated aerobically at 55˚C for 48 hours.

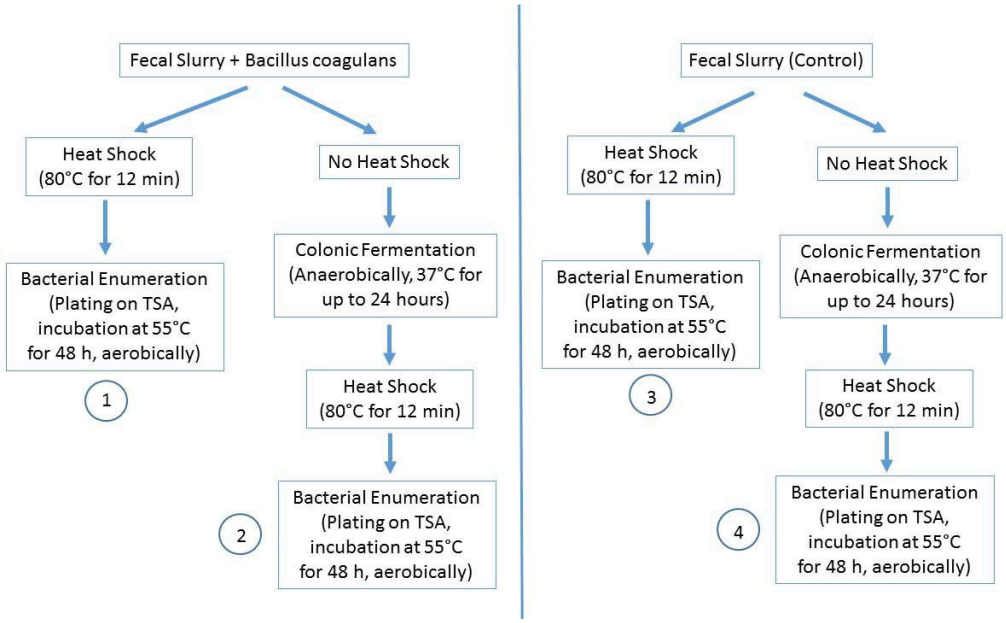

Figure 10. Diagram describing the experiments used to determine the amount of *B. coagulans* Unique IS-2 that may germinate under colonic conditions.

By subtracting the plate counts from "3" from "1" in figure 10, the initial amount of spores of *B. coagulans* Unique IS-2 added will be determined. Then by subtracting the plate counts from "4" from "2" in figure 10, the amount of spores that did not germinate will be determined. Finally, by subtracting the amount of spores that did not germinate from the initial amount of spores added to the slurries, the amount of *B. coagulans* Unique IS-2 that germinated will be determined. The higher germination in these colonic

conditions that is observed is the desired outcome. Germination percentage was determined by subtracting the *B. coagulans* Unique IS-2 spore counts at the time point 24 hours from the initial amount of added *B. coagulans* Unique IS-2 spore count at time 0, divided by the initial counts of *B. coagulans* Unique IS-2 and multiplied by 100.

3.2.4. Statistical Analysis

Analysis of variance was used to compare the results obtained from the different treatments in the acid and bile salts tolerance experiment and to compare germination over time in the *in vitro* gut environment. Statistics were run on the survival percentage for the acid and bile salts tolerance and on the average counts per time for the *in vitro* gut environment at the 5% level of significance. Statistical analysis was conducted using SAS 9.4 (SAS, INC, Cary, NC).

3.3. Results and Discussion

3.3.1 *In vitro* Stomach pH and Bile Salts Stability

For a probiotic organism to provide health benefits, it first must reach the area in the gastrointestinal tract that will be colonized. Therefore, it must first survive transit through the stomach and small intestine. Throughout transit, the organism will encounter the acidic condition of the stomach and the bile salts in the small intestine. A good probiotic organism will be able to withstand these adverse conditions and will reach the lower intestine where its positive impact would be accomplished. Thus, evaluating the stability of *B. coagulans* spores in the presence of simulated gastric acid conditions and bile salt becomes an essential part of characterizing this strain as a probiotic organism.

The survivability of *B. coagulans* Unique IS-2 in these adverse conditions was evaluated in a broth for up to 4 hours. Survival curves for each treatment are shown in figure 11. The average percent survival for each treatment was calculated and compared to the control (Table 4).

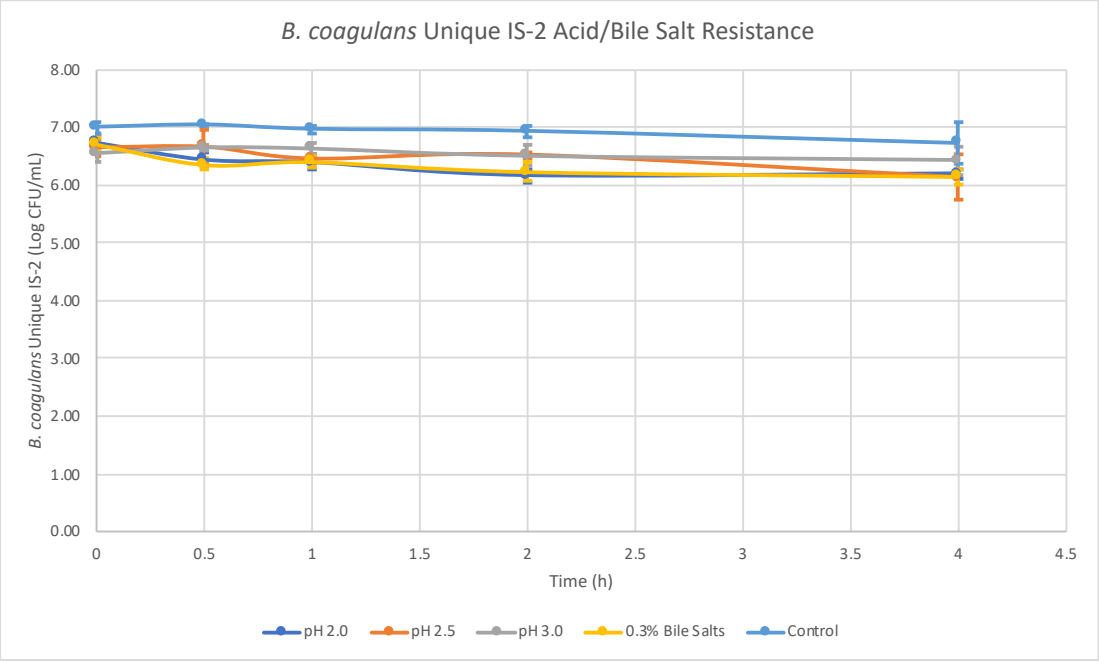

Figure 11. Acid and Bile Salts Tolerance of *Bacillus coagulans* Unique IS-2 spores over 4 hours at 37˚C.

B. coagulans spores were able to survive in all conditions tested with only slight reductions in counts for up to 4 hours. The average survival ranged from 91.7% to 98.1% in 0.3% bile salts to pH 3.0, respectively. As expected, lower survivability of spores was observed in the lower pH conditions and in the presence of bile salts. Previous research with the same strain Unique IS-2 has showed similar acid and bile resistance results where, at pH 2.0, pH 3.0 and 1% bile salts, there was only a 1 log reduction over 3 hours (Ratna Sudha et al., 2010). These results are comparable to Hyronimus et al. (2000),

where *B. coagulans* showed a strong resistance to bile presence. Majeed and colleagues, also observed a 2 log reduction at pH 1 after 4 hours and growth of *B. coagulans* in the presence of 0.3% and 0.5% bile presence (Majeed, Nagabhushanam, Natarajan, Sivakumar, Eshuis-de Ruiter, et al., 2016). Le Duc and colleagues, studied the survival of *Bacillus subtilis* spores in a simulated gastrointestinal tract environment and observed 97% and 94% survival of *B. subtilis* spores in a pH 2.0 environment and in the presence of bile salts, respectively, which is comparable to the survival percentages for *B. coagulans* spores observed here (Le Duc et al., 2004).

Ganeden Biotech used an *in vitro* model of the stomach and small intestine, called a TIM-1, to observe a 70% survival of their *B. coagulans* probiotic strain, GanedenBC^{30}, in simulated stomach conditions (Maathuis et al., 2010). Based on the results reported here and by others in the scientific literature, there is strong evidence that *B. coagulans* is resistant to low pH and bile conditions, which indicates it would be able to survive transit through the upper gastrointestinal tract as expected of a good probiotic strain.

Table 4. Summary of *Bacillus coagulans* Unique IS-2 Survival in Acid and Bile Salts

Treatment	Average Survival (% ± SD)[1]
Control	96.28 ± 3.01^{a,b}
pH 2.0	91.87 ± 2.16^{b}
pH 2.5	91.98 ± 3.90^{b}
pH 3.0	98.06 ± 2.18^{a}
0.3% Bile Salts	91.65 ± 0.29^{b}

[1]Treatments with different letters within the same column are significantly different ($p<0.05$)

3.3.2. *In Vitro* Digestion

The most important probiotic property of an organism is its ability to grow in the lower gastrointestinal tract because this is where it will confer the health benefit to the host. Therefore, fecal samples were used to simulate the gut environment in order to observe the ability of *Bacillus coagulans* Unique IS-2 spores to germinate. Seven volunteers provided fecal samples to this experiment and the concentration of spores that were able to germinate in the sample during incubation simulating conditions encountered in the gut at each time point can be seen in Figure 12. There was no significant difference in germination among time points ($p<0.05$).

Each individual had a slightly different effect on the germination of added spores. The percentage of germination was variable with the highest at 19.85% and lowest at -3.95% for samples A and D, respectively (Table 5). Of the seven slurry samples, three had a negative germination percentage. This outcome could be a result of some of the spores germinating and replicating as vegetative cells, but then resporulating throughout the experimental period (24 hours). This behavior has been observed in previous research where *Bacillus subtilis* spores were observed to germinate, grow and resporulate in the gut (Tam et al., 2006). This could also be an explanation for the increasing trend of germination within the first 8 hours and followed by a decrease after 8 hours.

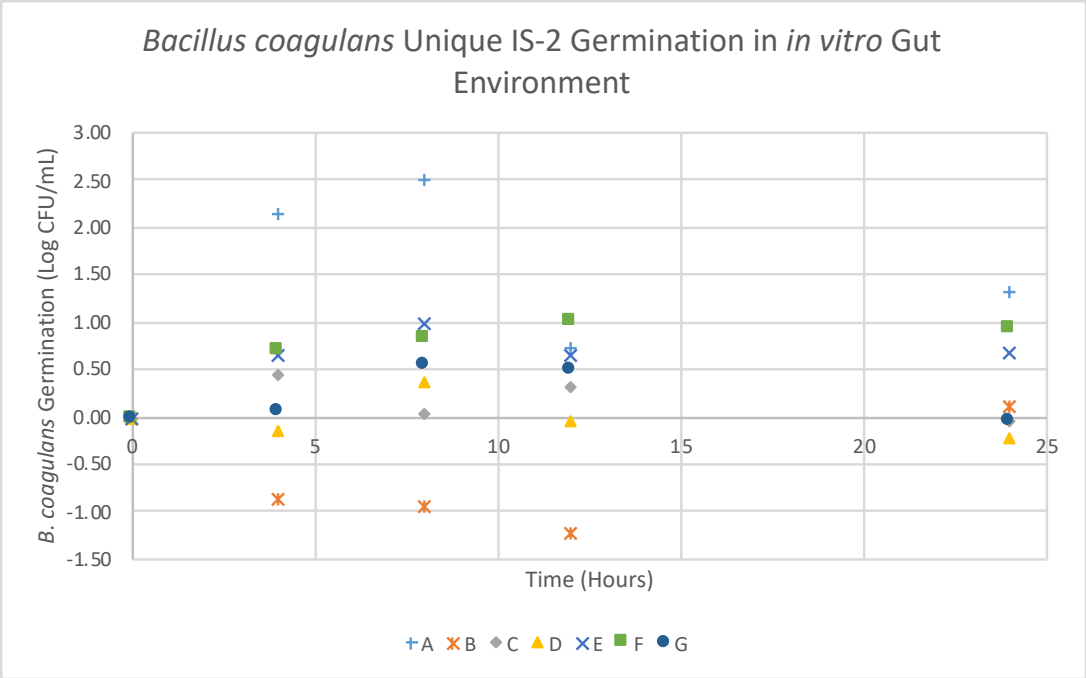

Figure 12. *Bacillus coagulans* Unique IS-2 survival in *in vitro* gut environment. Each volunteer is designated with a letter A-G. There is no significance between population of spores at each time point ($p<0.05$).

Variation among fecal slurries result from the differences in the resident microbiota of the intestinal tract of the volunteers participating in the study. Due to these differences, microorganisms that are introduced into this environment may be more or less able to compete with the resident microbiota in order to establish themselves. The results presented here show a germination percentage range of -3.95% to 19.9% with an average of 6.32% (Table 5) for *B. coagulans* Unique IS-2 in the gut environment. This may be considered a very low percentage of germination. However, in 2010, Maathuis et al, using an *in vitro* stomach and small intestine model (TIM-1), also observed a 10% germination for the *B. coagulans* GanedenBC^{30}. More recently, Keller et al. (2019) used a dynamic, computer-controlled model of the adult human gastrointestinal tract to reevaluate germination rates for GanedenBC^{30} in the gut. In this new system a meal was

added along with the probiotic organism while evaluating its germination (Keller et al., 2019). According to the authors, due to the added meal factor in this study, there were more germination triggers which increased the observed germination of *B. coagulans* spores to greater than 90%.

Table 5. Percentage of *Bacillus coagulans* Unique IS-2 spore germination in each of the fecal slurries under evaluation.

Sample	A	B	C	D	E	F	G	Average
Germination (%)	19.85	2.20	-0.77	-3.95	10.28	13.75	-0.47	6.32

The results from this experiment showed that within an *in vitro* gut environment only a small percentage of *B. coagulans* spores germinated. The germination observed here was on average 6%, however, there is no research available on how much germination is actually required to promote a health benefit. However, there is research showing that while the consumption of probiotics may help alleviate certain health issues, the removal of the probiotic treatment also showed a relapse of that health issue (Tamboli, Caucheteux, Cortot, Colombel, & Desreumaux, 2003). Therefore, the beneficial organisms may not need to colonize the gut long term, but instead be able to temporarily display the resident microbiota. Further, there are multiple human clinical studies describing that oral intake of *B. coagulans* has increased resident populations of beneficial bacteria in the elderly, lowered cholesterol and has improved symptoms of rheumatoid arthritis, irritable bowel syndrome, major depressive disorder associated with irritable bowel syndrome and intestinal gas symptoms (Hun, 2009; Kalman et al., 2009; Majeed, Majeed, et al., 2018; Majeed, Nagabhushanam, Natarajan, Sivakumar, Ali, et al., 2016; Majeed, Nagabhushanam, Arumugam, Majeed, & Ali, 2018; Mandel et al., 2010; Nyangale, Farmer, Keller, Chernoff, & Gibson, 2014). These clinical studies, in addition

to other available research and the results reported in this study, low germination of *B. coagulans* between 6-10% may be enough to provide the desired effect. Further research is needed to evaluate what level of germination may truly be required to observe the desired health benefits.

3.4. Conclusion

Bacillus coagulans Unique IS-2 displayed strong survivability through simulated stomach and small intestine conditions. Even at the lowest pH level tested and in the presence of bile salts, *B. coagulans* Unique IS-2 spores had greater than 91% survivability. These results indicate its ability to survive the adverse conditions encountered in the upper gastrointestinal tract of humans. However, the germination of spores in simulated gut conditions was, on average 6% germination, with the fecal slurries of some individuals showing a negative overall germination. This indicates that although the spores can survive travel to reach the gut, only a small percentage of the spores will be able to germinate.

Therefore, further clinical testing in humans and animals is needed to evaluate more conclusively what happens to the *B. coagulans* spores once they reach the gut. This research has shown that *B. coagulans* Unique IS-2 is capable of germinating in an *in vitro* gut environment but more research is needed to validate the actual effect of this germination, specifically regarding any potential health benefit that may be experienced by the host.

3.5. References

Ashraf, R., & Shah, N. P. (2011). Selective and differential enumerations of *Lactobacillus delbrueckii* subsp. *bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus casei* and *Bifidobacterium* spp. in yoghurt - A review. *International Journal of Food Microbiology*, *149*(3), 94–208. https://doi.org/10.1016/j.ijfoodmicro.2011.07.008

Baron, M. (2009). A patented strain of *Bacillus coagulans* increased immune response to viral challenge. *Postgraduate Medicine*, *121*(2), 114–118. https://doi.org/10.3810/pgm.2009.03.1971

Cutting, S. M. (2011). *Bacillus* probiotics. *Food Microbiology*, *28*, 214–220. https://doi.org/10.1016/j.fm.2010.03.007

Dunne, C., O'Mahony, L., Murphy, L., Thornton, G., Morrissey, D., O'Halloran, S., Feeney, M., Flynn, S., Fitzgerald, G., Daly, C., Kiely, B., O'Sullivan, G. C., Shanahan, F., & Collins, J. K. (2001). In vitro selection criteria for probiotic bacteria of human origin : correlation with *in vivo* findings 1 – 4, *73*, 386–392.

Gotcheva, V., Hristozova, E., Hristozova, T., Guo, M., Roshkova, Z., & Angelov, A. (2002). Assessment of Potential Probiotic Properties of Lactic Acid Bacteria and Yeast Strains. *Food Biotechnology*, *16*(3), 211–225. https://doi.org/10.1081/FBT-120016668

Hartzell, A. L., Maldonado-Gómez, M. X., Hutkins, R. W., & Rose, D. J. (2013). Synthesis and in vitro digestion and fermentation of acylated inulin. *Bioactive Carbohydrates and Dietary Fibre*, *1*(1), 81–88. https://doi.org/10.1016/j.bcdf.2013.01.004

Henriques, A. O., & Moran, Jr., C. P. (2007). Structure, Assembly, and Function of the Spore Surface Layers. *Annual Review of Microbiology*, *61*(1), 555–588. https://doi.org/10.1146/annurev.micro.61.080706.093224

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C., & Sanders, M. E. (2014). The International Scientific Association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *National Review Gastroenterol Hepatol*, *11*(11), 506–514. https://doi.org/10.1038/nrgastro.2014.66

Holzapfel, W. H., Haberer, P., Snel, J., Schillinger, U., & Huis In'T Veld, J. H. J. (1998). Overview of gut flora and probiotics. *International Journal of Food Microbiology*, *41*(2), 85–101. https://doi.org/10.1016/S0168-1605(98)00044-0

Huang, Y., & Adams, M. C. (2004). In vitro assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. *International Journal of Food Microbiology*, *91*(3), 253–260. https://doi.org/10.1016/j.ijfoodmicro.2003.07.001

Hun, L. (2009). Original Research: *Bacillus coagulans* Significantly Improved Abdominal Pain and Bloating in Patients with IBS. *Postgraduate Medicine*, *121*(2), 119–124. https://doi.org/10.3810/pgm.2009.03.1984

Hung, A. T., Lin, S.-Y., Yang, T.-Y., Chou, C.-K., Liu, H.-C., Lu, J.-J., Wang, B., Chen, S, -Y., & Lien, T.-F. (2012). Effects of *Bacillus coagulans* ATCC 7050 on growth performance, intestinal morphology, and microflora composition in broiler chickens. *Animal Production Science*, *52*(9), 874–879. https://doi.org/10.1071/an11332

Hyronimus, B., Le Marrec, C., Sassi, A. H., & Deschamps, A. (2000). Acid and bile tolerance of spore-forming lactic acid bacteria. *International Journal of Food*

Microbiology, *61*, 193–197.

Hyronimus, B., Le Marrec, C., & Urdaci, M. C. (1998). Coagulin, a bacteriocin-like-inhibitory substance produced by *Bacillus coagulans* I. *Journal of Applied Microbiology*, *85*(1), 42–50.

Kalman, D. S., Schwartz, H. I., Alvarez, P., Feldman, S., Pezzullo, J. C., & Krieger, D. R. (2009). A prospective, randomized, double-blind, placebo-controlled parallel-group dual site trial to evaluate the effects of a *Bacillus coagulans*-based product on functional intestinal gas symptoms. *BMC Gastroenterology*, *9*(1), 85. https://doi.org/10.1186/1471-230X-9-85

Keller, D., Verbruggen, S., Cash, H., Farmer, S., & Venema, K. (2019). Spores of *Bacillus coagulans* GBI-30, 6086 show high germination, survival and enzyme activity in a dynamic, computer-controlled *in vitro* model of the gastrointestinal tract. *Beneficial Microbes*, *10*(1), 77–87. https://doi.org/10.3920/BM2018.0037

Kerry, R. G., Patra, J. K., Gouda, S., Park, Y., Shin, H. S., & Das, G. (2018). Benefaction of probiotics for human health: A review. *Journal of Food and Drug Analysis*, *26*(3), 927–939. https://doi.org/10.1016/j.jfda.2018.01.002

Konuray, G., & Erginkaya, Z. (2018). Potential Use of *Bacillus coagulans* in the Food Industry. *Foods*, *7*(6), 92. https://doi.org/10.3390/foods7060092

Le Duc, H., Hong, H. a, Barbosa, T. M., Henriques, A. O., & Cutting, S. M. (2004). Characterization of *Bacillus* Probiotics Available for Human Use Characterization of *Bacillus* Probiotics Available for Human Use. *Applied and Environmental Microbiology*, *70*(4), 2161–2171. https://doi.org/10.1128/AEM.70.4.2161

Lee, J., Park, I., Choi, Y., & Cho, J. (2012). *Bacillus* strains as feed additives: *In vitro*

evaluation of its potential probiotic properties, 577–585.

Maathuis, A. J. H., Keller, D., & Farmer, S. (2010). Survival and metabolic activity of the GanedenBC30 strain of *Bacillus coagulans* in a dynamic *in vitro* model of the stomach and small intestine. *Beneficial Microbes*, *1*(1), 31–36. https://doi.org/10.3920/BM2009.0009

Majeed, M., Majeed, S., Nagabhushanam, K., Arumugam, S., Beede, K., & Ali, F. (2018). Evaluation of the *in vitro* cholesterol-lowering activity of the probiotic strain *Bacillus coagulans* MTCC 5856. *International Journal of Food Science and Technology*, 212–220. https://doi.org/10.1111/ijfs.13926

Majeed, M., Nagabhushanam, K., Arumugam, S., Majeed, S., & Ali, F. (2018). *Bacillus coagulans* MTCC 5856 for the management of major depression with irritable bowel syndrome: A randomised, double-blind, placebo controlled, multi-centre, pilot clinical study. *Food and Nutrition Research*, *62*, 1–15. https://doi.org/10.29219/fnr.v62.1218

Majeed, M., Nagabhushanam, K., Natarajan, S., Sivakumar, A., Ali, F., Pande, A., Majeed, S., & Karri, S. K. (2016). *Bacillus coagulans* MTCC 5856 supplementation in the management of diarrhea predominant Irritable Bowel Syndrome: a double blind randomized placebo controlled pilot clinical study. *Nutrition Journal*, *15*(1), 21. https://doi.org/10.1186/s12937-016-0140-6

Majeed, M., Nagabhushanam, K., Natarajan, S., Sivakumar, A., Eshuis-de Ruiter, T., Booij-Veurink, J., de Vries, & Ali, F. (2016). Evaluation of genetic and phenotypic consistency of *Bacillus coagulans* MTCC 5856: a commercial probiotic strain. *World Journal of Microbiology and Biotechnology*, *32*(4), 1–12.

https://doi.org/10.1007/s11274-016-2027-2

Mandel, D. R., Eichas, K., & Holmes, J. (2010). *Bacillus coagulans*: a viable adjunct therapy for relieving symptoms of rheumatoid arthritis according to a randomized, controlled trial. *BMC Complementary and Alternative Medicine*, *10*, 1–7.

Moir, A. (2006). How do spores germinate? *Journal of Applied Microbiology*, *101*(3), 526–530. https://doi.org/10.1111/j.1365-2672.2006.02885.x

Nicholson, W. L., Munakata, N., Horneck, G., Setlow, P., Melosh, H. J., & Setlow, P. (2003). Resistance of *Bacillus* Endospores to Extreme Terrestrial and Extraterrestrial Environments. *Microbiology and Molecular Biology Reviews*, *64*(3), 548–572. https://doi.org/10.1128/mmbr.64.3.548-572.2000

Nyangale, E. P., Farmer, S., Keller, D., Chernoff, D., & Gibson, G. R. (2014). Effect of prebiotics on the fecal microbiota of elderly volunteers after dietary supplementation of *Bacillus coagulans* GBI-30, 6086. *Anaerobe*, *30*, 75–81. https://doi.org/10.1016/j.anaerobe.2014.09.002

Payot, T., Chemaly, Z., & Fick, M. (1999). Lactic acid production by *Bacillus coagulans*—kinetic studies and optimization of culture medium for batch and continuous fermentations. *Enzyme and Microbial Technology*, *24*(3–4), 191–199. https://doi.org/10.1016/S0141-0229(98)00098-2

Ratna Sudha, M., Chauhan, P., Dixit, K., Babu, S., & Jamil, K. (2010). Molecular typing and probiotic attributes of a new strain of *Bacillus coagulans* - Unique IS-2: A potential biotherapeutic agent. *Genetic Engineering and Biotechnology Journal*, *2010*(1986), 1–20.

Sanders, M. E. (2000). Considerations for Use of Probiotic Bacteria to Modulate Human

Health. *Journal of Nutrition*, 384–390.

Tam, N. K. M., Uyen, N. Q., Hong, H. A., Duc, L. H., Hoa, T. T., Claudia, R., Henriques, A. O., & Cutting, S. M. (2006). The Intestinal Life Cycle of *Bacillus subtilis* and Close Relatives †. *Journal of Bacteriology*, *188*(7), 2692–2700. https://doi.org/10.1128/JB.188.7.2692

Tamboli, C. P., Caucheteux, C., Cortot, A., Colombel, J. F., & Desreumaux, P. (2003). Probiotics in inflammatory bowel disease: A critical review. *Bailliere's Best Practice and Research in Clinical Gastroenterology*, *17*(5), 805–820. https://doi.org/10.1016/S1521-6918(03)00076-3

Wu, T., Zhang, Y., Lv, Y., Li, P., Yi, D., Wang, L., Zhao, D., Chen, H., Gong, J., & Hou, Y. (2018). Beneficial impact and molecular mechanism of *Bacillus coagulans* on piglets' intestine. *International Journal of Molecular Sciences*, *19*(7), 1–17. https://doi.org/10.3390/ijms19072084

Zhou, X., Wang, Y., Gu, Q., & Li, W. (2010). Effect of dietary probiotic, *Bacillus coagulans*, on growth performance, chemical composition, and meat quality of Guangxi Yellow chicken. *Poultry Science*, *89*(3), 588–593. https://doi.org/10.3382/ps.2009-00319

CHAPTER 4: EVALUATION OF STABILITY OF *BACILLUS COAGULANS* UNIQUE IS-2 IN FOOD PRODUCTS

4.1. Introduction

There has been an increasing trend among consumers preferences for healthier and more beneficial foods. Consumers are interested in eating foods that have no additives and preservatives; as well as foods that are health promoting in order to achieve a healthier lifestyle. This trend has led the food industry create the concept of functional foods. These are foods that are consumed as part of an everyday diet that provides beneficial effects on human health, such as reducing cholesterol or high blood pressure (Charalampopoulos et al., 2002). Foods that fall under the umbrella term, functional foods, are ones that have added synthesized food ingredients, naturally occurring bioactive substances or added bioactive substances (Grajek et al., 2005). Among the most commonly added ingredients are probiotics, which are microorganisms, that when consumed in adequate amounts, confer a health benefit on the host (Hill et al., 2014).

The idea of health promoting organisms has been around for over 100 years when Döderlein and Metchnikoff observed health benefits after the consumption of lactic acid producing bacteria (De Vecchi & Drago, 2006). Now, as of 2017, the probiotic market is over $1.8 billion USD and the food and beverage sector, as the largest sector in this market, was greater than $125 million USD (Ahuja & Deb, 2018). As this market continues to grow, so does the amount of research conducted on probiotics to evaluate their viability in products and product application for new probiotic cultures and alternative ways to expand the use of probiotics in food products.

The most commonly used probiotics are members from the *Lactobacillus* and *Bifidobacterium* genera and they are most frequently included in refrigerated products, such as milk, cheese, and yogurt among others. Currently, there are no industry requirements associated with the amount of probiotic organisms needed for a health benefit, however it is generally accepted that it should be 10^7-10^9 CFU/g of the product (Ashraf & Shah, 2011; Konuray & Erginkaya, 2018). However, reports in the literature have shown loss of viability of these organisms during the shelf life of the products. During testing of yogurts, some were found to have viable probiotic counts that slowly decreased throughout the shelf life of the product, with some containing less than the levels claimed by the label by the last day of shelf life (Jayamanne & Adams, 2006). This is an issue because there has to be a consumption of adequate amounts of probiotic organisms for the host to receive health benefits. To address this problem, the probiotic industry has invested in micro-encapsulation of probiotics, such as *Lactobacillus rhamnosus, L. acidophilus* and *Bifidobacterium lactis*, to help protect the organism during the shelf life of the product (Weinbreck et al., 2010). However, even micro-encapsulation has its limitations and there is still much to be researched on new technologies to improve the overall process (Martín et al., 2015).

A solution to viability issues has been the use of sporeforming bacteria as potential probiotics. Namely, *Bacillus coagulans* is one sporeforming bacteria that has received interest from the probiotic industry. *Bacillus coagulans* is a Gram positive, facultative, rod and is capable of producing lactic acid and spores. This organism has optimum temperature growth between 30-55˚C and an optimal pH growth between 5.5 – 6.5 (De

Vecchi & Drago, 2006). The sporeforming properties of *B. coagulans* is of interest to the probiotic industry as the spore is a natural protection to extreme environments, such as heat, cold, dryness and acid (Nicholson et al., 2003). The resistant nature of the spore could be a benefit to the industry as it may allow the organism to survive harsh processing and storage conditions better than vegetative cells.

The health promoting benefits of *B. coagulans* have been studied by researchers. Animals studies have shown that ingestion of *B. coagulans* had antidiarrheal affects, helped remedy hypercholesterolemia, improved some symptoms of *Clostridium difficile* induced colitis and increased the population of lactic acid bacteria in the gut (Aminlari et al., 2018; Bomko et al., 2017; Fitzpatrick et al., 2011; Majeed, Natarajan, et al., 2016; Wu et al., 2018). In addition, this organism is considered safe for human consumption and has found success in human clinical studies as an anti-diarrheal and secondary treatment to antibiotic therapy (Endres et al., 2011; Majeed, Nagabhushanam, Natarajan, Sivakumar, Ali, et al., 2016; Majeed & Nagabhushanam, 2016). It has also shown positive results as a treatment for high cholesterol, irritable bowel syndrome and rheumatoid arthritis (Majeed, Majeed, et al., 2018; Majeed, Natarajan, et al., 2016; Mandel et al., 2010). Others have described it as potentially able to increase the immune response against some respiratory infections (Baron, 2009).

None the less, *B. coagulans* will only be able to provide these health benefits if it can survive processing and remain stable in the product during shelf life until consumption by the host. As a sporeforming probiotic, *B. coagulans* must not germinate in the product

during the shelf life so that the protective spore coat is still present upon consumption. Thus, the viability of the probiotic must extend through the shelf life of the product and remain at a concentration that is equal to or higher than what is generally accepted as required in order for the host to obtain any health benefit. The viability of the probiotic will mostly depend on the composition of the food product being used as the carrier for the organism. Some product characteristics that may affect the viability of probiotic organisms are water activity, nutrient profile, pH, oxygen presence and the temperature of storage (Song et al., 2012). It is also important that added spores do not negatively affect the quality of the final product. Therefore, organoleptic properties of the product should be monitored with the attributes of the product with probiotics being similar to commercial products without probiotics (Song et al., 2012).

The goal of this research was to evaluate the viability of *B. coagulans* Unique-IS 2 spores over time at different pH levels, while evaluating the effect of different acids normally used by the food industry for product formulation. In addition, the viability of *B. coagulans* Unique-IS 2 spores and product stability were also evaluated when the probiotic culture was added to commercial products.

4.2. Materials and Methods

4.2.1. Bacterial Cultures

A lyophilized powder containing spores of *Bacillus coagulans* Unique IS-2 was obtained from Nebraska Cultures, LLC, which is now UAS Laboratories, LLC (Wausau, WI). Lyophilized culture was stored at 4˚C until use.

4.2.2. Viability of *B. coagulans* During Storage at Different pHs

Tryptic Soy Broth (TSB) was prepared in flasks. Individual flasks of TSB had their pH adjusted to pH levels 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5. To adjust the pH of the broth four different acids were used: 3.0M hydrochloric acid (HCl), 85% (w/w) lactic acid (LA), 99.8% acetic acid (AA), and 1.0M citric acid (CA). Additionally, a TSB flask with a pH level of 8.0 was also prepared by adjusting its pH with 0.1N sodium hydroxide (NaOH). Once the pH of the TSB flasks was adjusted, they were autoclaved for sterility.

A spore suspension was prepared by adding lyophilized *B. coagulans* spores into Butterfields Phosphate-Buffered dilution water to achieve a concentration of 10^{10} CFU/ml. The spore suspension was heat treated at 80˚C for 12 minutes and then immediately chilled in an ice bath. The spore suspension was used to inoculate each pH adjusted TSB flask to achieve a bacterial concentration of 10^8 CFU/ml. The inoculated pH adjusted TSB was then separated into sterile conical tubes (10 ml/tube) with enough tubes prepared to allow testing for each time point of interest, which varied depending on the temperature of storage.

Sterile conical tubes were stored at both 4˚C and 25˚C, for at least 120 and 30 days, respectively. Time points used for evaluation of samples stored at 4˚C were 0, 1, 2, 3, and 4 months. Time points for samples stored at 25˚C were 0, 0.5, 1, 2, 3, 6, 9, 12, 18, 27, and 30 days when the acids and bases used for pH adjustment were HCl, LA and NaOH. For samples where the pH was adjusted using CA and AA, the time points for samples stored at 25˚C were 0, 0.5, 1, 2, 3, 5, 10, 15, 20, 25, and 30 days. At each time

point, a sample was removed from storage and divided into three parts for total plate counts, spore counts and a pH measurement. The sub-sample to be used for spore count was heat treated at 80˚C for 12 minutes to inactivate any vegetative cells present. The sub-sample for total plate count received no additional treatment. For enumeration of total plate counts and heat-treated spore counts, samples were serially diluted using Butterfield's Phosphate-Buffered dilution water. Dilutions were spread-plated on Tryptic Soy Agar (TSA) and plates were incubated aerobically at 55˚C for 48 hours. The pH of the samples was evaluated at each time point using a pH meter (Accument AE150, Fisher Scientific, NH) to observe if any changes occurred during storage. Graphs showing the total plate counts, spore counts and pH at each time point were created for each pH level of each acid.

4.2.3. Viability of *B. coagulans* Spores and Product Stability During Product Shelf Life

Four dairy products, milk, buttermilk, Ensure® protein shake and drinkable yogurt, were evaluated for quality during their shelf life after the addition of *B. coagulans* Unique IS-2. Each product was laboratory pasteurized at 80˚C for 12 minutes to eliminate any background microorganisms. After pasteurization, lyophilized powder of *B. coagulans* Unique IS-2 was added to each product for a bacterial concentration of about 10^7 CFU/ml. Products were then divided into sterile conical tubes and stored at 4˚C for the life of the product. Product evaluation was done at time points that were determined based on the normal shelf life of the product: time points for the drinkable yogurt and Ensure® protein shake was 0, 16, 30, 45, 60, and 90 days; time points for buttermilk

were 0, 3, 10, 16, 20, 25, and 30 days; and time points for milk were 0, 3, 6, 9, 12, 17, and 21 days.

Throughout storage, the quality of the product was evaluated by testing pH, color, acidity, and rancidity. The pH measurement was done using a pH meter (Accument AE150, Fisher Scientific, NH). Color was evaluated using the L, a, b color score with a colorimeter (CR-300 Chroma Meter, Konica Minolta, NJ). Acidity tests was conducted according to Wehr and Frank (2004). Rancidity was evaluated with peroxide value and thiobarbituric acid reactive substances (TBARS). The TBA tests were done according to AOCS Method DC 19-90. The peroxide value tests were done according to ("Spectrophotometric determination of lamb tissue peroxide value," 2001). The fat was extracted with 2:1 chloroform: methanol (v:v).

In addition to quality tests, the stability of the probiotic *B. coagulans* Unique IS-2 to remain in the product in its spore form throughout the shelf life was also evaluated. Samples were removed at each time point and divided into two portions for total plate counts and spore counts. The sub-sample to be used for spore count was heat treated at 80˚C for 12 minutes to inactivate any vegetative cells present. The sub-sample for total plate count received no additional treatment. For enumeration of total plate counts and heat-treated spore counts, samples were serially diluted using Butterfield's Phosphate-Buffered dilution water. Dilutions were spread-plated on Tryptic Soy Agar (TSA) and plates were incubated aerobically at 55˚C for 48 hours.

4.2.4. Statistical Analysis

Analysis of Variance was used to compare how the pH adjusted tryptic soy broths and products changed over the storage time. Parameters under evaluation included the viability of *B. coagulans* spores in different pH levels, product, and the quality of products with *B. coagulans* spores. Statistical analysis was done on the total plates counts, spore counts and pH for the experiments evaluating viability of *B. coagulans* spores in different pH levels. For those experiments involving food products, the total counts, spores counts, pH, titratable acidity, and LAB color scores were used to evaluate product quality in the presence of *B. coagulans* spores. All statistical tests were done at the 5% level of significance. Statistical analysis was conducted using SAS 9.4 (SAS, INC, Cary, NC).

4.3. Results and Discussion

4.3.1 Viability of *B. coagulans* Unique IS-2 during storage at 4˚C in broth at different pH levels

When determining probiotic product formulations, it is important to ensure the viability of the organism throughout the shelf life of the product. This is to guarantee that the desired amount of probiotic is able to reach the gut of the host to confer health benefits (Bora et al., 2009). *Bacillus* species, when added to food products, have been observed to be more stable during processing and shelf life of products compared to other probiotics that have only a vegetative life cycle (Elshaghabee et al., 2017). Therefore, the survivability of *B. coagulans* Unique IS-2 spores was evaluated in different pH ranges at two different temperatures. Hydrochloric acid (HCl), lactic acid (LA), acetic acid (AA)

and citric acid (CA) were the four acids used to adjust the pH of the broth used for this study. The survivability of *B. coagulans* was evaluated at pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5. These acids were chosen because they are commonly used in the food industry. Additionally, sodium hydroxide (NaOH) was used to evaluate the survivability of the spores at pH 8.0.

For each of the pH conditions, similar trends for the viability of *B. coagulans* were seen during storage at 4˚C for a total of 4 months. In general, no significant differences ($p<0.05$) on spore counts over 4 months at each of the pH values evaluated regardless of the acid or base used for the pH adjustment. These results indicate that the spores of *B. coagulans* Unique IS-2 are very stable at 4˚C in different pHs ranging from 3.0 – 8.0. Figure 13 shows the results observed for broths, with pH adjusted to 5.0 with LA as an example of *B. coagulans* Unique IS-2 survivability during 4 months of storage at 4˚C. Additional data for other pHs and acids/base can be found in appendix A.

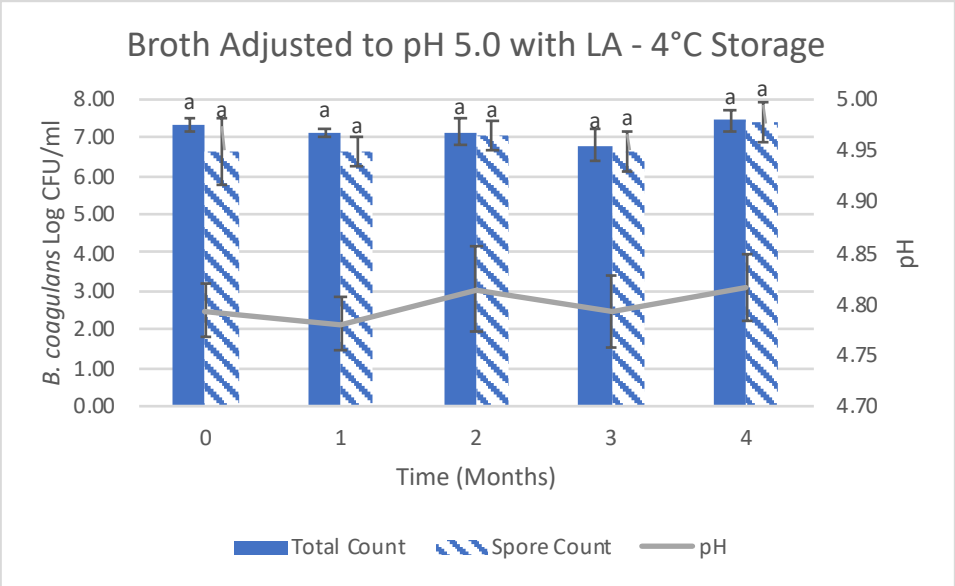

Figure 13. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 5.0 with LA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

For the total counts, there was some variability across the storage time which led to statistically significant differences among the time points ($p<0.05$). However, since the inclusion of this probiotic into products, and viability over product shelf life, should be related to its spore form, a decrease in total counts would not compromise the efficacy of the product. Additionally, as long as any increase in total cells do not contribute to spoilage of the product, it also would not be of concern.

During the storage time, the pH of the broths was also monitored and results showed some variation during the 4 months of storage. Specifically, the broths where the pH was adjusted with LA, no differences were observed. For the other acids, the pH levels varied by less than 0.10, 0.22, and 0.14 pH units for HCl, AA and CA, respectively. The biggest change in the pH level of the broth was observed for pH 8.0, which decreased over the storage period by 0.35 pH units. However, the true significance of these differences would be best evaluated by manufacturers using this probiotic strain based on the specifications and characteristics of their products.

Overall, the results of these experiments show that the spores of *B. coagulans* Unique IS-2 are resilient during storage at 4˚C in a range of pHs from 3.0 – 8.0, regardless of the acid or base used to adjust the pH. Other probiotic cultures, such as *Lactobacillus plantarum* have also shown ability to survive at low pH values. More specifically *L. plantarum* survived well in juices with pH ranging from 2.5 – 4.0, showing only a decrease of 1.1 log CFU/ml at the lowest pH, after storage at 4˚C for 6 weeks (Nualkaekul & Charalampopoulos, 2011).

4.3.2. Viability of *B. coagulans* Unique IS-2 storage at 25˚C in broths at different pH levels

The same acids (HCl, LA, AA, CA) and base (NaOH) previously used to evaluate the effect of pH on the survivability of *B. coagulans* Unique IS-2 when stored at 4˚C were also used for studies conducted at 25˚C. Once again broths had their pH adjusted to 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 8.0. During storage at 25˚C, similar trends were observed among the acids tested; however, the results showed greater variation than those observed at 4˚C. Figure 14 shows the results observed for broths, with pH adjusted to 5.0 with LA as an example, of *B. coagulans* Unique IS-2 survivability during 30 days of storage at 25˚C. Additional data for other pHs and acids/base can be found in appendix A.

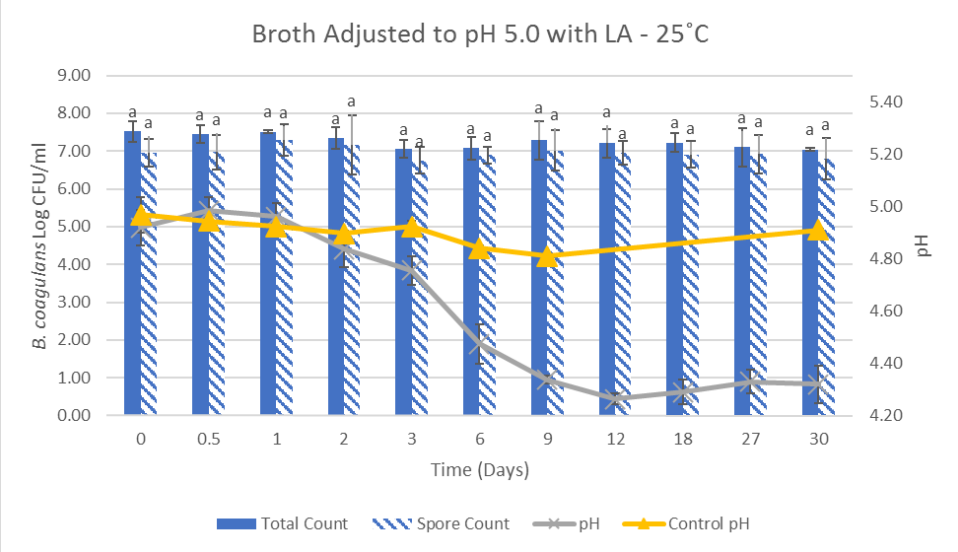

Figure 14. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 5.0 with LA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

In general, no significant differences ($p<0.05$) on spore counts were observed over 30 days for each of the pHs tested, regardless of the acid or base used for pH adjustment. However, the total counts, showed significant differences ($p<0.05$) over the storage period, especially for those broths with pH adjusted to values of 5.0 or higher. These variations in total counts could indicate germination of the spores in pH levels greater than pH 5.0, during storage at 25˚C. The optimum temperature for growth of *B. coagulans* is between 40-47˚C, but they can grow at temperature as low as 30˚C and some strains have been found to grow in acidic conditions, at pH levels as low as 4.0 (De Clerck et al., 2004; Haberbeck et al., 2012; Keller et al., 2010). This potential germination of *B. coagulans* Unique IS-2 could explain the observed reduction in pH levels during storage of those broths initially adjusted to 5.0 or higher. The germination of *B. coagulans* spores lead to vegetative cells that produce acids, which may explain the aforementioned reduction in pH of the broths (Figure 14). In these experiments, the change in pH can most likely be attributed to the activity of vegetative cells of *B. coagulans* Unique IS-2 as the pH of all non-inoculated broths (control) remained stable for the entirety of the shelf life.

In general, for the pH levels below pH 4.0, the pH of the broth remained stable during storage with less than 0.13, 0.24, 0.08 and 0.11 variability in pH units for LA, HCl, CA, and AA, respectively. These results are similar to those observed during storage at 4˚C, where there were no changes in spore or total counts over the storage period and slight variations in pH level. For these situations, the significance of the pH variation would once again be dependent upon the product specifications and characteristics.

However, the results from those broths with pH adjusted to levels higher than 5.0 indicated that *B. coagulans* Unique IS-2 may not be as stable under these conditions. Potential germination leading to production of acids by the probiotic strain caused considerable variation in the pH of the broth. This would be detrimental to the quality of the products that fit this profile (pH > 5.0; storage at 25˚C) and are added with *B. coagulans* Unique IS-2. Therefore, this strain of *B. coagulans* may potentially be used as a probiotic organism in products that are stored at 25˚C and have pH lower than 5.0, but it would not be a good option for those with higher pH levels.

4.3.3. Quality Evaluation of Products Added with *Bacillus coagulans* Unique IS-2

To meet the definition of a probiotic, the organism must provide a health benefit on the host. However, to reach the location in the host's body where the health benefit may be achieved, it must survive in the delivery product for the duration of its shelf life. It also should not cause undesirable changes in the quality of the delivery product. Dairy products, such as milk, yogurt, cheeses, and ice cream, are desirable delivery products for probiotics for their composition and storage conditions (Canning, Bandyopadhyay, Biswas, & Aslund, 2012). Since these products are stored and held under refrigeration, the cold chain helps preserve probiotic organisms that may be associated with these products. For inclusion of probiotic organisms into a product, several factors must be evaluated to ensure a good final product. Product composition, processing temperature, process duration, and post processing can have an effect on the probiotic viability (Foligné et al., 2013). Additionally, product should be observed for the viability of the

probiotic organism, while physical and organoleptic properties should not change during the shelf life of the product. Essentially, the sensory attributes of the probiotic product should be similar to commercial products without probiotics (Canning et al., 2012; Song et al., 2012).

The survival of probiotic organisms during food processing, during the storage of the product, and during its transit to the lower gastrointestinal tract are the most important factors for functional foods included with probiotics (Jafari et al., 2017; Keller et al., 2010; Konuray & Erginkaya, 2018). Pasteurization processes are commonly used by the food industry to kill vegetative cells and increase the shelf life of products. Because *Bacillus* species spores are known to be heat resistant and survive these processes, it is expected that they would be an adequate probiotic culture to be used in products that require pasteurization if the inclusion of the probiotic was to occur before the thermal treatment (Desai & Varadaraj, 2010). Indeed, the Ganeden strain, *Bacillus coagulans* GanedenBC^{30}, is already being included in products that undergo heat treatments during their processing (Cutting, 2011). In this study, the ability of *B. coagulans* Unique IS-2 in surviving pasteurization and its effects on product quality during their storage was evaluated.

4.3.3.1. Milk

Milk with added *B. coagulans* Unique IS-2 was evaluated over a 21-day period, which is the expected normal shelf life for this product. Over the 21 days, there was no significant difference ($p<0.05$) in spore counts, with only the last time period showing a decrease

compared to the initial levels (Figure 15). However, this decrease was only 0.60 Log CFU/ml, which may not adversely affect the ability of the product to provide a probiotic effect. However, the pH of the product towards the end of the storage period was also significantly different from those levels measured initially, with an increase from pH 6.64 to pH 6.93.

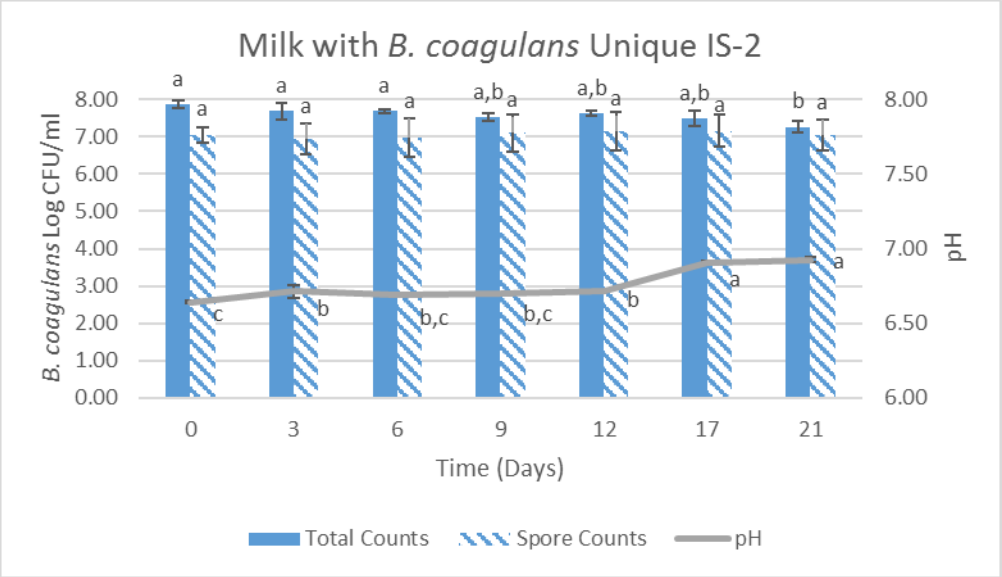

Figure 15. Stability of *B. coagulans* Unique IS-2 in milk as denoted by total and spore counts. Stability test were done during storage at 4˚C with the pH of the product monitored throughout 21 days of storage.

For the quality evaluation, color, percent acidity, and rancidity were measured. When compared to the commercial milk, the milk with added *B. coagulans* was slightly darker, and after probiotic addition the spores settled to the bottom of the container due to their powder composition (visual observation). However, throughout the evaluated shelf life, there was no significant changed in the values of L, a, and b color scores, which means that after the initial change in color caused by the added spores, the color remained stable. Additionally, the acidity of the product did not change over the entire shelf life and remained stable at an average value of 0.15 (Table 6).

The rancidity of the product was determined by peroxide value and thiobarbituric acid reactive substances (TBARS). The peroxide value represents the active oxygen in lipids that can oxidize potassium iodine, which these values increase during the propagation phase and then decrease during the termination phase of lipid oxidation as it reads the primary oxidation products (Laguerre, Lecomte, & Villeneuve, 2007). Thus, TBARS are used in conjunction with peroxide values because it is able to use thiobarbituric acid's reaction with secondary oxidation products to observe further oxidation of the product (Laguerre et al., 2007). Lipid oxidation is a common chemical reaction in food products and it usually results in undesirable flavors (O'Connor & O'Brien, 2006). Table 6 also shows the peroxide and TBARS value during storage. The initial peroxide values were reduced by day 3 of storage followed by an increase up to day 12 of the shelf life of the product before decreasing. The TBARS values continuously decreased during storage until the 21st day. The literature reports increasing trend in peroxide values over the shelf life of milk and cheeses (Mortensen, Sørensen, & Stapelfeldt, 2002; Park, 2001; Zygoura et al., 2004). The peroxide values follow their natural trend of slowing increasing until they reach a peak and then declining (O'Connor & O'Brien, 2006). As oxidation becomes advanced, the primary oxidation products will decrease to more secondary oxidation products, which could be indicated by the low stability of TBARS throughout the shelf life until the last time point evaluated. By the end of the shelf life of milk, the secondary oxidation products are starting to increase, which would show a more advanced stage of rancidity. Therefore, the rancidity values reported here show an increase towards the end of the natural shelf life, thus *B. coagulans* Unique IS-2 may not contribute a noticeable effect on the rancidity of the product.

Table 6. Quality characteristics of milk with *B. coagulans* Unique IS-2 evaluated over a 21-day shelf life at 4°C[1]

		Color			Rancidity	
Time (days)	% Acidity	L	a	b	Peroxide Value (mmol iodine equiv./kg sample)	TBARS (uM TBARS/g sample)
0	0.14 ± 0.01[a]	80.46 ± 6.19[a]	-2.65 ± 0.49[a]	-0.50 ± 3.33[a]	4.60 ± 0.95	3.00 ± 0.10
3	0.15 ± 0.01[a]	84.60 ± 0.37[a]	-3.21 ± 0.06[a]	1.23 ± 0.66[a]	1.51 ± 0.19	1.40 ± 0.90
6	0.14 ± 0.03[a]	80.89 ± 1.58[a]	-2.71 ± 0.32[a]	-1.24 ± 0.75[a]	2.82 ± 0.33	1.40 ± 0.30
9	0.15 ± 0.00[a]	83.68 ± 0.10[a]	-3.19 ± 0.06[a]	1.58 ± 0.29[a]	7.54 ± 0.19	1.00 ± 0.20
12	0.16 ± 0.01[a]	84.00 ± 0.73[a]	-4.08 ± 0.15[a]	1.98 ± 0.21[a]	8.30 ± 1.22	1.00 ± 0.30
17	0.15 ± 0.01[a]	83.86 ± 0.31[a]	-4.14 ± 0.03[a]	1.45 ± 0.34[a]	4.44 ± 0.41	1.00 ± 0.10
21	0.15 ± 0.01[a]	83.64 ± 0.66[a]	-4.16 ± 0.04[a]	1.03 ± 0.62[a]	5.70 ± 0.54	1.50 ± 0.20

[1]Tests with different letters within the same column are significantly different ($p<0.05$).

4.3.3.2 Buttermilk

In fermented milks, acidity, pH, redox potential and added flavorings have been found to affect the viability of probiotic organisms (Canning et al., 2012). Therefore, buttermilk added with *B. coagulans* Unique IS-2 was evaluated over a period of 30 days, in alignment with the normal shelf life of this type of product. Results shown in Figure 16 indicate that the total and spore counts in buttermilk had no significant changes at any time point during its shelf life. The pH of the buttermilk showed a slight variability of 0.2 pH units during the evaluated period.

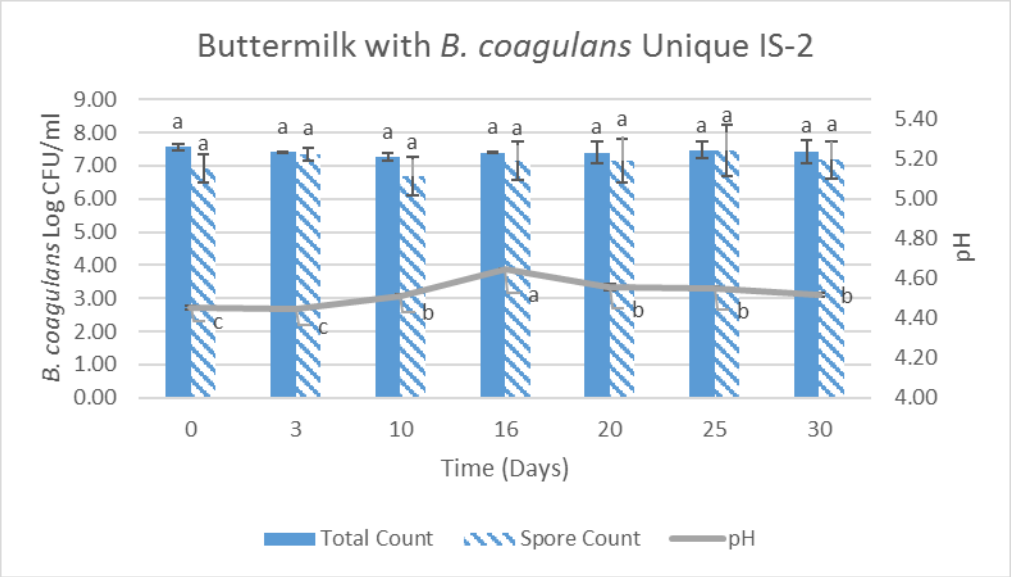

Figure 16. Stability of *B. coagulans* Unique IS-2 in buttermilk as denoted by total and spore counts. Stability test were done during storage at 4˚C with the pH of the product monitored throughout 30 days of storage.

When compared to the commercial buttermilk product, the one sample with added *B. coagulans* Unique IS-2 spores was darker in color (visual observation). However, there were no further changes in the L and b color over the length of the shelf life period (Table 7). The a color score showed a significant difference between the first two time points and the other ones. This indicates that the color moved farther along the green scale after the first 3 days of storage. The acidity of the product did not change during the period evaluated, with an average value of 0.86 (Table 7). The evaluation of product rancidity showed some changes over the storage period (Table 7). The peroxide values increased from the 3rd day until the 20th day, and decreased for the remainder of the storage time. The TBARS values remained somewhat stable while the peroxide values increased until the 25 days of storage when values started to increase. Compared to the milk, the peroxide values in buttermilk reached a much higher peak before decreasing. There are many factors that affect the lipid oxidation of milk and milk products. Some of these

factors are light, oxygen, metals, antioxidants, enzymes, ascorbic acid, storage conditions and water activity (O'Connor & O'Brien, 2006). However, both milk and buttermilk, reached their peroxide value peak over halfway through their shelf life, with the TBARS value only increasing near the very end of the shelf life, which was expected.

Table 7. Quality characteristics of buttermilk with *B. coagulans* Unique IS-2 evaluated over a 30-day shelf life at 4°C[1]

		Color			Rancidity	
Time (days)	% Acidity	L	a	b	Peroxide Value (mmol iodine equiv./kg sample)	TBARS (uM TBARS/g sample)
0	0.87 ± 0.00[a]	79.19 ± 3.54[a]	-2.30 ± 0.33[a]	2.88 ± 1.27[a]	4.41 ± 0.73	1.40 ± 0.10
3	0.89 ± 0.01[a]	81.72 ± 3.54[a]	-2.38 ± 0.30[a]	5.05 ± 1.44[a]	1.64 ± 0.80	0.90 ± 0.20
10	0.87 ± 0.03[a]	80.67 ± 3.26[a]	-3.52 ± 0.34[b]	5.32 ± 0.22[a]	8.80 ± 1.73	0.70 ± 0.10
16	0.88 ± 0.02[a]	81.49 ± 3.04[a]	-3.51 ± 0.23[b]	4.99 ± 0.23[a]	20.28 ± 2.77	1.00 ± 0.10
20	0.84 ± 0.06[a]	81.90 ± 2.00[a]	-3.71 ± 0.13[b]	4.43 ± 0.18[a]	24.62 ± 0.11	1.00 ± 0.20
25	0.84 ± 0.04[a]	81.64 ± 1.17[a]	-3.50 ± 0.36[b]	4.55 ± 0.30[a]	2.53 ± 0.48	5.00 ± 0.10
30	0.83 ± 0.01[a]	81.69 ± 1.59[a]	-3.32 ± 0.14[b]	5.06 ± 1.42[a]	3.00 ± 0.28	9.00 ± 0.11

[1]Tests with different letters within the same column are significantly different ($p<0.05$).

4.3.3.3. Ensure® Protein Shake

Ensure® protein shake with added *B. coagulans* Unique IS-2 was evaluated over 3 months. Figure 17 shows the results obtained for the stability of the probiotic strain during product storage. In general, no significant differences were observed in total and spore counts during the period evaluated. The pH of the product varied by only 0.13 pH units over the shelf life, indicating no microbial activity over 3 months of storage.

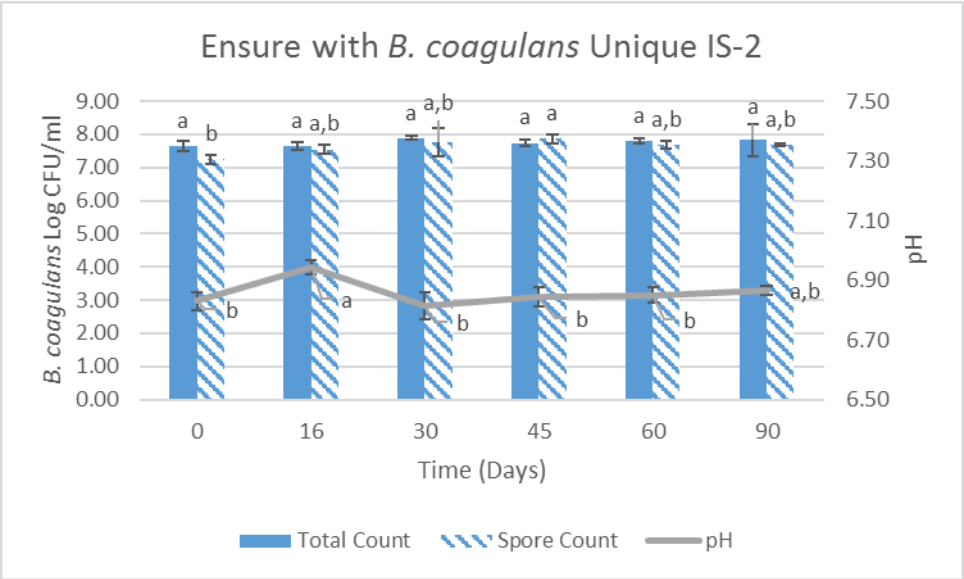

Figure 17. Stability of *B. coagulans* Unique IS-2 in Ensure® protein shake as denoted by total and spore counts. Stability test were done during storage at 4˚C with the pH of the product monitored throughout 90 days of storage.

When compared to the commercial Ensure® product, the sample with added *B. coagulans* Unique IS-2 spores was darker in color and added spores settled to the bottom of the container (visual observation). Throughout storage, there were some differences in the color profile of the protein shake added with *B. coagulans* Unique IS-2 (Table 8). The product turned slightly lighter and moved farther along the yellow scale as the product aged. This, however, was not perceived by visual observation. Therefore, it may not lead to a significant impact on consumer's acceptance of the product. The acidity of the product remained stable, with an average value of 0.17, during the whole shelf life, with no significant differences observed (Table 8). The peroxide and TBARS values generally increased throughout the shelf life of this product (Table 8). Both the peroxide and TBARS value for Ensure® followed a different trend than was observed in the milk or buttermilk products. There is a lack of information in the literature about the natural rancidity profile of this product. From the values reported here, it is shown that the

peroxide value is generally increasing throughout the evaluated shelf life. As these values will hit a peak and start decreasing, these results could indicate that this product is still in the beginning stages of lipid oxidation. Additionally, the TBARS may increase slightly, but they are relatively stable, which may indicate this product has a naturally high TBARS value before it increases when secondary oxidation products start becoming formed. Additional studies evaluating a longer shelf life may make these results clearer.

Table 8. Quality characteristics of Ensure protein shake with *B. coagulans* Unique IS-2 evaluated over a 90-day shelf life at 4°C[1]

		Color			Rancidity	
Time (days)	% Acidity	L	a	b	Peroxide Value (mmol iodine equiv./kg sample)	TBARS (uM TBARS/g sample)
0	0.15 ± 0.01[a]	74.20 ± 1.33[b]	0.26 ± 0.34[a]	14.04 ± 0.37[b]	7.17 ± 2.03	7.70 ± 0.20
16	0.16 ± 0.10[a]	75.01 ± 0.13[a,b]	0.42 ± 0.47[a]	15.34 ± 0.47[a]	3.10 ± 0.99	9.80 ± 0.02
30	0.18 ± 0.02[a]	75.57 ± 0.09[a,b]	0.71 ± 0.45[a]	15.93 ± 0.09[a]	9.97 ± 0.77	12.00 ± 0.30
45	0.18 ± 0.03[a]	75.35 ± 0.05[a,b]	0.74 ± 0.53[a]	15.96 ± 0.32[a]	10.32 ± 1.16	11.83 ± 0.46
60	0.20 ± 0.03[a]	75.71 ± 0.17[a,b]	0.86 ± 0.06[a]	16.13 ± 0.18[a]	16.49 ± 8.74	9.04 ± 1.17
90	0.16 ± 0.08[a]	76.22 ± 0.26[a]	0.53 ± 0.57[a]	16.10 ± 0.74[a]	17.19 ± 1.65	11.44 ± 2.52

[1]Tests with different letters within the same column are significantly different ($p<0.05$).

4.3.3.4. Drinkable Yogurt

Yogurt is one of the most popular food products used as a vehicle for probiotic organisms, with the ones usually included in this product being *Lactobacillus acidophilus* and *Bifidobacterium* species (Ashraf & Shah, 2011). However, studies have shown a decrease in viability in the probiotic organisms by the time of product consumption, which emphasizes the importance of evaluating probiotic viability over the shelf life of products (Ashraf & Shah, 2011; Shah, 2000). The viability of these organisms can be affected by product pH, acidity, dissolved oxygen, buffering capacity of the media and

survival has been found to be strain specific (Canning et al., 2012). Therefore, drinkable yogurt with added *B. coagulans* Unique IS-2 was evaluated over 3 months. Over the period evaluated, there was no significant difference ($p<0.05$) in total counts, and only slight variation in spore counts (Figure 18). However, the levels of *B. coagulans* Unique IS-2 spores in the product never decreased compared to the initial levels. The pH of the product varied by 0.18 pH units over the shelf life, indicating very little changes in the product throughout storage. Overall, it appears that the added spores were very stable in this product over 3 months.

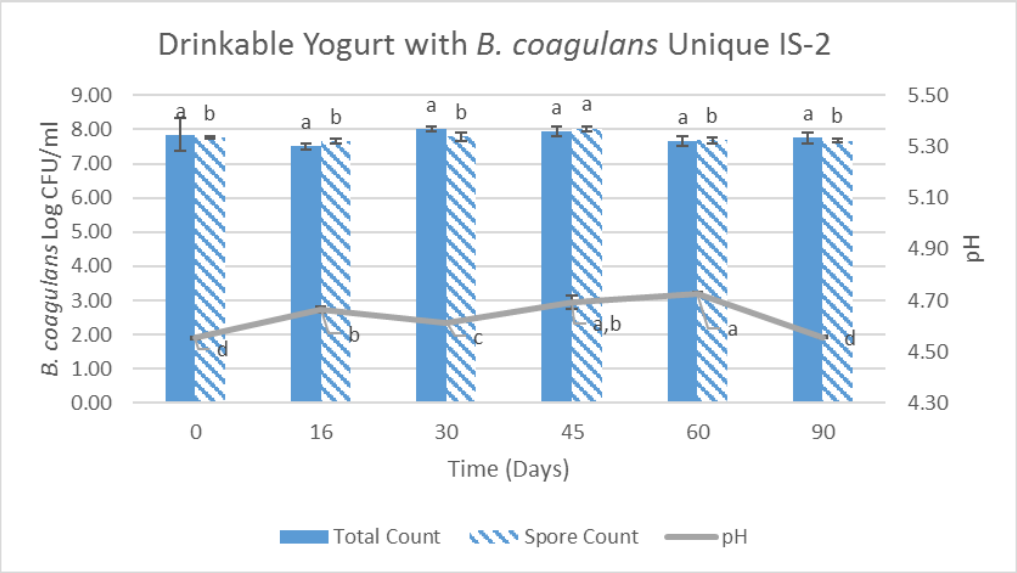

Figure 18. Stability of *B. coagulans* Unique IS-2 in drinkable yogurt as denoted by total and spore counts. Stability test were done during storage at 4˚C with the pH of the product monitored throughout 90 days of storage.

When compared to the commercial drinkable yogurt, the sample with added *B. coagulans* Unique IS-2 spores was visually similar without much change to the eye. During storage, there were slight differences in the color profile of the drinkable yogurt (Table 9). The product moved farther along the green scale as the product aged. However, this was not perceived by visual observation. Therefore, it may not lead to a significant impact on

consumer's acceptance of the product. Similar to the other products tested, the acidity of the product remained stable, with an average value of 0.80, during its shelf life, with no significant differences observed (Table 9). The peroxide and TBARS values for the drinkable yogurt varied throughout the shelf life of this product (Table 9). These values follow the same trend observed with the Ensure® product, where there were variations among the evaluated shelf life. However, other research in the literature has reported peroxide values increasing over the shelf life of a yogurt product, which is the common result of peroxide values (O'Sullivan et al., 2016). With the variability of both peroxide and TBARS values over the evaluated shelf life, this could indicate an early stage of rancidity or low rancidity of this product.

Table 9. Quality characteristics of drinkable yogurt with *B. coagulans* Unique IS-2 evaluated over a 90-day shelf life at 4°C[1]

		Color			Rancidity	
Time (days)	% Acidity	L	a	b	Peroxide Value (mmol iodine equiv./kg sample)	TBARS (uM TBARS/g sample)
0	0.81 ± 0.03[a]	86.69 ± 1.22[a]	-2.41 ± 0.18[a]	5.03 ± 0.44[a]	5.63 ± 1.08	2.60 ± 0.90
16	0.79 ± 0.02[a]	86.07 ± 0.52[a]	-3.41 ± 0.21[b]	5.52 ± 0.54[a]	4.80 ± 0.91	1.70 ± 0.40
30	0.79 ± 0.02[a]	87.23 ± 0.53[a]	-3.30 ± 0.26[b]	6.51 ± 0.19[a]	8.30 ± 1.22	4.70 ± 1.20
45	0.77 ± 0.04[a]	85.56 ± 2.00[a]	-3.35 ± 0.28[b]	5.02 ± 1.82[a]	4.24 ± 0.12	2.50 ± 0.50
60	0.80 ± 0.02[a]	86.60 ± 0.23[a]	-3.36 ± 0.28[b]	6.20 ± 0.64[a]	5.90 ± 0.16	2.33 ± 0.32
90	0.81 ± 0.05[a]	86.87 ± 0.71[a]	-3.14 ± 0.30[b]	6.16 ± 0.60[a]	6.56 ± 0.57	4.03 ± 0.73

[1]Tests with different letters within the same column are significantly different ($p<0.05$).

4.4. Conclusion

The purpose of this research was to evaluate the viability of the spores of *B. coagulans* Unique IS-2 in broths adjusted to different pHs levels when stored at 4˚C and 25˚C. Overall, the spores of *B. coagulans* were stable when stored at 4˚C in broths with pH 3.0

– 8.0 for up to 4 months. The losses in counts observed over the shelf life could be overcome by adding extra spores to probiotic products. However, the viability of the spores was not as promising when samples were stored at 25˚C. When the pH of the broth was 4.5 or greater, the spores appeared to have germinated during storage. This germination lead to a significant pH drop in some samples during storage, which would be an undesirable change in commercial products. Based on the results reported here, shelf stable products with a pH of 4.0 or lower, could have *B. coagulans* Unique IS-2 added to them, as under these conditions the spores were stable during storage for up to 30 days.

Additionally, the viability of *B. coagulans* Unique IS-2 when added to commercial products and their quality throughout storage were also evaluated. In general, *B. coagulans* showed good viability and stability in the commercial products tested. Overall, spore counts remained stable in the products over their shelf life without negatively changing the acidity or pH of the products. Another quality factor evaluated was the color of the product. While the color was not altered significantly during the shelf life of the products, the initial addition of *B. coagulans* Unique IS-2 caused the products to display a darker color. This was most likely due to the color of the lyophilized probiotic which was a brown hue. Additionally, it is worth mentioning that in some products the added spores settled to the bottom of the product containers. With the milk and buttermilk products, the rancidity values showed a natural trend of an increasing peroxide value to a peak before decreasing, and then a subsequent increase in TBARS after the peak, which shows the change from primary to secondary oxidation products.

However, the Ensure® protein shake and yogurt rancidity values did not follow that trend. There is a lack of conclusive information in the literature about true acceptability values of rancidity compared to the acceptability of the product. Additional research on acceptability of measured rancidity values in dairy products and comparison of control products would strengthen the results reported here. In addition to the tests done here, additional evaluation with a sensory panel could provide additional valuable information about the acceptability of inclusion of *B. coagulans* Unique IS-2 to food products as a probiotic culture.

4.5. References

Abhari, K., Shekarforoush, S. S., Sajedianfard, J., Hosseinzadeh, S., & Nazifi, S. (2015). The effects of probiotic, prebiotic and synbiotic diets containing *Bacillus coagulans* and inulin on rat intestinal microbiota. *Iranian Journal of Veterinary Research*, *16*(3), 267–273.

Ahuja, K., & Deb, S. (2018). Global Market Insights. Retrieved February 20, 2019, from https://www.gminsights.com/industry-analysis/probiotics-market

Aminlari, L., Shekarforoush, S. S., Hosseinzadeh, S., Nazifi, S., & Sajedianfard, J. (2018). Effect of Probiotics *Bacillus coagulans* and *Lactobacillus plantarum* on Lipid Profile and Feces Bacteria of Rats Fed Cholesterol-Enriched Diet. https://doi.org/10.1007/s12602-018-9480-1

Ashraf, R., & Shah, N. P. (2011). Selective and differential enumerations of *Lactobacillus delbrueckii* subsp. *bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus casei* and *Bifidobacterium* spp. in yoghurt - A review. *International Journal of Food Microbiology*, *149*(3), 94–208. https://doi.org/10.1016/j.ijfoodmicro.2011.07.008

Baron, M. (2009). A patented strain of *Bacillus coagulans* increased immune response to viral challenge. *Postgraduate Medicine*, *121*(2), 114–118. https://doi.org/10.3810/pgm.2009.03.1971

Bomko, T. V., Nosalskaya, T. N., Kabluchko, T. V., Lisnyak, Y. V., & Martynov, A. V. (2017). Immunotropic aspect of the *Bacillus coagulans* probiotic action. *Journal of Pharmacy and Pharmacology*, *69*(8), 1033–1040. https://doi.org/10.1111/jphp.12726

Bora, P. S., Puri, V., & Bansal, A. K. (2009). Physicochemical Properties and Excipient Compatibility studies of Probiotic *Bacillus coagulans* Spores. *Scientia Pharmaceutica*, *77*, 625–637. https://doi.org/10.3797/scipharm.0904-01

Canning, J., Bandyopadhyay, S., Biswas, P., & Aslund, M. (2012). Recent Application of Probiotics in Food and Agricultural Science. *Intech*. https://doi.org/10.5772/50570

Charalampopoulos, D., Wang, R., Pandiella, S. S., & Webb, C. (2002). Application of cereal and cereal components in functional foods: a review. *International Journal of Food Microbiology*, *79*(1–2), 131–141.

Cutting, S. M. (2011). *Bacillus* probiotics. *Food Microbiology*, *28*, 214–220. https://doi.org/10.1016/j.fm.2010.03.007

De Clerck, E., Rodriguez-Diaz, M., Forsyth, G., Lebbe, L., Logan, N. A., & DeVos, P. (2004). Polyphasic Characterization of *Bacillus coagulans* Strains, Illustrating Heterogeneity within this Species, and Emended Description of the Species. *Systematic and Applied Microbiology*, *27*(1), 50–60. https://doi.org/10.1078/0723-2020-00250

De Vecchi, E., & Drago, L. (2006). *Lactobacillus sporogenes* or *Bacillus coagulans*: misidentification or mislabelling? *International Journal of Probiotics and Prebiotics*, *1*(1), 3–10.

Desai, S. V., & Varadaraj, M. C. (2010). Behavioural pattern of vegetative cells and spores of *Bacillus cereus* as affected by time-temperature combinations used in processing of Indian traditional foods. *Journal of Food Science and Technology*, *47*(5), 549–556. https://doi.org/10.1007/s13197-010-0099-9

Elshaghabee, F. M. F., Rokana, N., Gulhane, R. D., Sharma, C., & Panwar, H. (2017).

Bacillus as potential probiotics: Status, concerns, and future perspectives. *Frontiers in Microbiology*, *8*(AUG), 1–15. https://doi.org/10.3389/fmicb.2017.01490

Endres, J. R., Qureshi, I., Farber, T., Hauswirth, J., Hirka, G., Pasics, I., & Schauss, A. G. (2011). One-year chronic oral toxicity with combined reproduction toxicity study of a novel probiotic, *Bacillus coagulans*, as a food ingredient. *Food and Chemical Toxicology*, *49*(5), 1174–1182. https://doi.org/10.1016/j.fct.2011.02.012

Fitzpatrick, L. R., Small, J. S., Greene, W. H., Karpa, K. D., & Keller, D. (2011). *Bacillus Coagulans* GBI-30 (BC30) improves indices of *Clostridium difficile*-Induced colitis in mice. *Gut Pathogens*, *3*(1), 16. https://doi.org/10.1186/1757-4749-3-16

Foligné, B., Daniel, C., & Pot, B. (2013). Probiotics from research to market: The possibilities, risks and challenges. *Current Opinion in Microbiology*, *16*(3), 284–292. https://doi.org/10.1016/j.mib.2013.06.008

Grajek, W., Olejnik, A., & Sip, A. (2005). Probiotics, prebiotics and antioxidants as functional foods. *Acta Biochimica Polonica*, *52*(3), 665–671. https://doi.org/10.1093/brain/awt108

Haberbeck, L. U., Alberto da Silva Riehl, C., de Cássia Martins Salomão, B., & Falcão de Aragão, G. M. (2012). *Bacillus coagulans* spore inactivation through the application of oregano essential oil and heat. *LWT - Food Science and Technology*, *46*(1), 267–273. https://doi.org/10.1016/j.lwt.2011.09.021

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C., & Sanders, M. E. (2014). The International Scientific Association for probiotics and prebiotics consensus

statement on the scope and appropriate use of the term probiotic. *National Review Gastroenterol Hepatol*, *11*(11), 506–514. https://doi.org/10.1038/nrgastro.2014.66

Jafari, M., Mortazavian, A. M., Hosseini, H., Safaei, F., Mousavi Khaneghah, A., & Sant'Ana, A. S. (2017). Probiotic *Bacillus*: Fate during sausage processing and storage and influence of different culturing conditions on recovery of their spores. *Food Research International*, *95*, 46–51. https://doi.org/10.1016/j.foodres.2017.03.001

Jayamanne, V. S., & Adams, M. R. (2006). Determination of survival, identity and stress resistance of probiotic bifidobacteria in bio-yoghurts. *Letters in Applied Microbiology*, *42*(3), 189–194. https://doi.org/10.1111/j.1472-765X.2006.01843.x

Keller, D., Farmer, S., McCartney, A., & Gibson, G. (2010). *Bacillus coagulans* as a probiotic. *Food Science and Technology Bulletin: Functional Foods*, *7*, 103–109. https://doi.org/10.1616/1476-2137.16015.

Konuray, G., & Erginkaya, Z. (2018). Potential Use of *Bacillus coagulans* in the Food Industry. *Foods*, *7*(6), 92. https://doi.org/10.3390/foods7060092

Laguerre, M., Lecomte, J., & Villeneuve, P. (2007). Evaluation of the ability of antioxidants to counteract lipid oxidation: existing methods. new trends and challenges. *Progress in Lipid Research*, (46), 244–282.

Majeed, M., Majeed, S., Nagabhushanam, K., Arumugam, S., Beede, K., & Ali, F. (2018). Evaluation of the *in vitro* cholesterol-lowering activity of the probiotic strain *Bacillus coagulans* MTCC 5856. *International Journal of Food Science and Technology*, 212–220. https://doi.org/10.1111/ijfs.13926

Majeed, M., & Nagabhushanam, K. (2016). A Double-Blind, Placebo-Controlled,

Parallel Study Evaluating the Safety of Bacillus coagulans MTCC 5856 in Healthy Individuals. *Journal of Clinical Toxicology*, *06*(01), 1–9. https://doi.org/10.4172/2161-0495.1000283

Majeed, M., Nagabhushanam, K., Natarajan, S., Sivakumar, A., Ali, F., Pande, A., Majeed, S., & Karri, S. K. (2016). *Bacillus coagulans* MTCC 5856 supplementation in the management of diarrhea predominant Irritable Bowel Syndrome: a double blind randomized placebo controlled pilot clinical study. *Nutrition Journal*, *15*(1), 21. https://doi.org/10.1186/s12937-016-0140-6

Majeed, M., Natarajan, S., Sivakumar, A., Ali, F., Pande, A., Majeed, S., & Karri, S. K. (2016). Evaluation of Anti-Diarrhoeal Activity of *Bacillus coagulans* MTCC 5856 and its Effect on Gastrointestinal Motility in Wistat Rats. *International Journal of Pharma and Bio Sciences*, *7*(1), 311–316.

Mandel, D. R., Eichas, K., & Holmes, J. (2010). *Bacillus coagulans*: a viable adjunct therapy for relieving symptoms of rheumatoid arthritis according to a randomized, controlled trial. *BMC Complementary and Alternative Medicine*, *10*, 1–7.

Martín, M. J., Lara-Villoslada, F., Ruiz, M. A., & Morales, M. E. (2015). Microencapsulation of bacteria: A review of different technologies and their impact on the probiotic effects. *Innovative Food Science and Emerging Technologies*, *27*, 15–25. https://doi.org/10.1016/j.ifset.2014.09.010

Mortensen, G., Sørensen, J., & Stapelfeldt, H. (2002). Light-induced oxidation in semihard cheeses. Evaluation of methods used to determine levels of oxidation. *Journal of Agricultural and Food Chemistry*, *50*(15), 4364–4370. https://doi.org/10.1021/jf025513z

Nicholson, W. L., Munakata, N., Horneck, G., Setlow, P., Melosh, H. J., & Setlow, P. (2003). Resistance of *Bacillus* Endospores to Extreme Terrestrial and Extraterrestrial Environments. *Microbiology and Molecular Biology Reviews*, *64*(3), 548–572. https://doi.org/10.1128/mmbr.64.3.548-572.2000

Nualkaekul, S., & Charalampopoulos, D. (2011). Survival of *Lactobacillus plantarum* in model solutions and fruit juices. *International Journal of Food Microbiology*, *146*(2), 111–117. https://doi.org/10.1016/j.ijfoodmicro.2011.01.040

O'Connor, T. P., & O'Brien, N. M. (2006). *Lipid Oxidation*. *Advanced Dairy Chemistry* (Vol. 2). https://doi.org/10.1016/0163-7827(80)90006-5

O'Sullivan, A. M., O'Grady, M. N., O'Callaghan, Y. C., Smyth, T. J., O'Brien, N. M., & Kerry, J. P. (2016). Seaweed extracts as potential functional ingredients in yogurt. *Innovative Food Science and Emerging Technologies*, *37*(PB), 293–299. https://doi.org/10.1016/j.ifset.2016.07.031

Park, Y. W. (2001). Proteolysis and Lipolysis of Goat Milk Cheese. *Journal of Dairy Science*, *84*, 84–92.

Shah, N. P. (2000). Probiotic Bacteria: Selective Enumeration and Survival in Dairy Foods. *Journal of Dairy Science*, *83*(4), 894–907. https://doi.org/10.3168/jds.S0022-0302(00)74953-8

Song, D., Ibrahim, S., & Hayek, S. (2012). Recent Application of Probiotics in Food and Agricultural Science. *Intech Open*, *2*, 3–36. https://doi.org/10.5772/32009

Spectrophotometric determination of lamb tissue peroxide value. (2001). Ohio State University Research Bulletin. Retreived from http:ohiolin.osu.edu/sc183/sc1836.hyml

Wehr, H. M., & Frank, J. F. (2004). *Standard Methods for the Examination of Dairy Products* (17th Edition).

Weinbreck, F., Bodnár, I., & Marco, M. L. (2010). Can encapsulation lengthen the shelf-life of probiotic bacteria in dry products? *International Journal of Food Microbiology*, *136*(3), 364–367. https://doi.org/10.1016/j.ijfoodmicro.2009.11.004

Wu, T., Zhang, Y., Lv, Y., Li, P., Yi, D., Wang, L., Zhao, D., Chen, H., Gong, J., & Hou, Y. (2018). Beneficial impact and molecular mechanism of *Bacillus coagulans* on piglets' intestine. *International Journal of Molecular Sciences*, *19*(7), 1–17. https://doi.org/10.3390/ijms19072084

Zygoura, P., Moyssiadi, T., Badeka, A., Kondyli, E., Savvaidis, I., & Kontominas, M. G. (2004). Shelf life of whole pasteurized milk in Greece: Effect of packaging material. *Food Chemistry*, *87*(1), 1–9. https://doi.org/10.1016/j.foodchem.2003.10.009

APPENDIX A. GRAPHS FOR THE STABILITY OF *BACILLUS COAGULANS* UNIQUE IS-2 IN pH ADJUSTED BROTHS

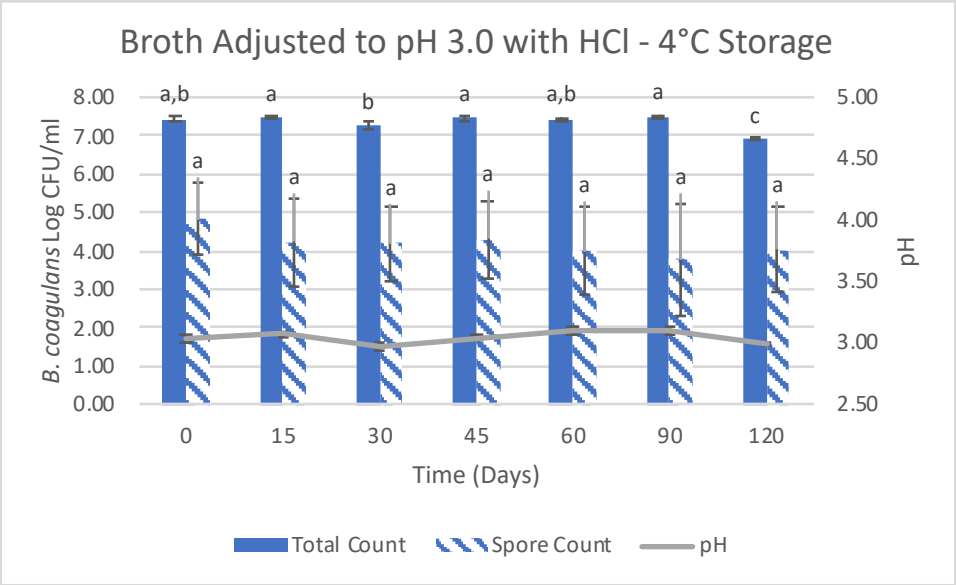

Figure 19. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 3.0 with HCl as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 120 days of storage.

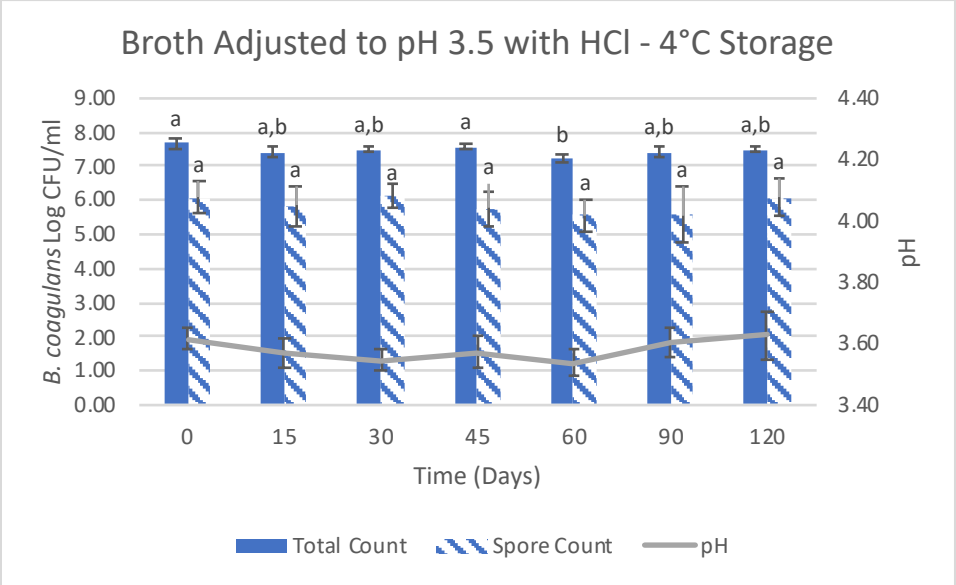

Figure 20. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 3.5 with HCl as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 120 days of storage.

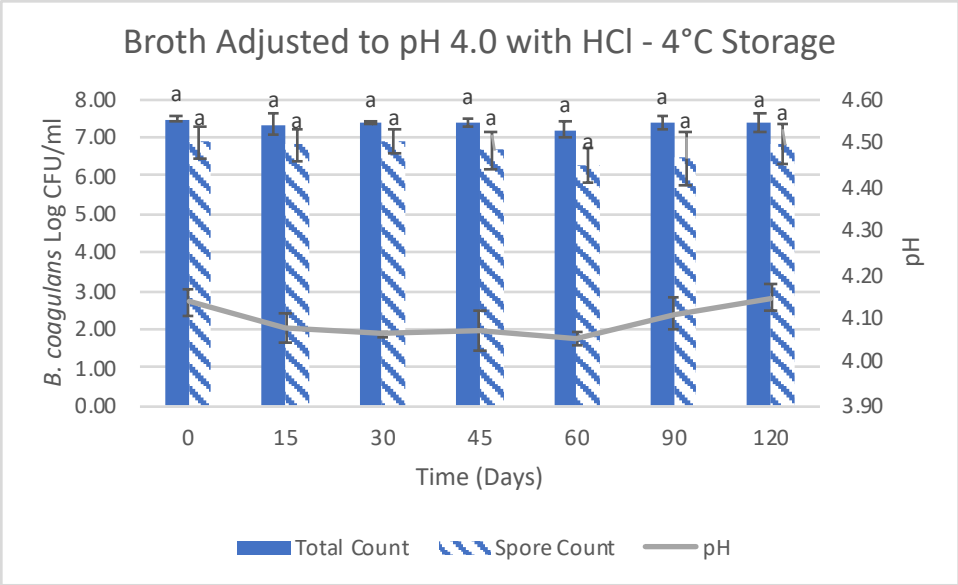

Figure 21. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 4.0 with HCl as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 120 days of storage.

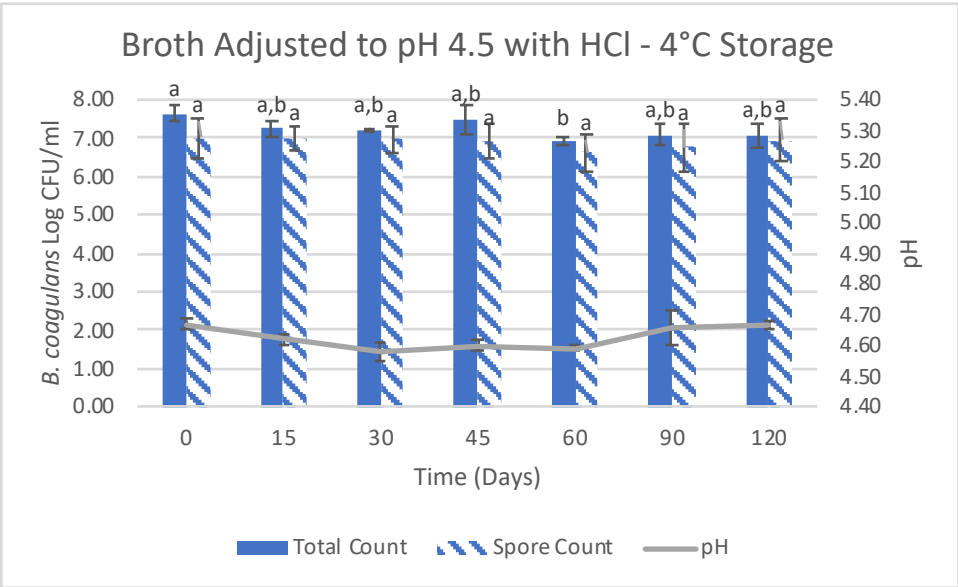

Figure 22. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 4.5 with HCl as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 120 days of storage.

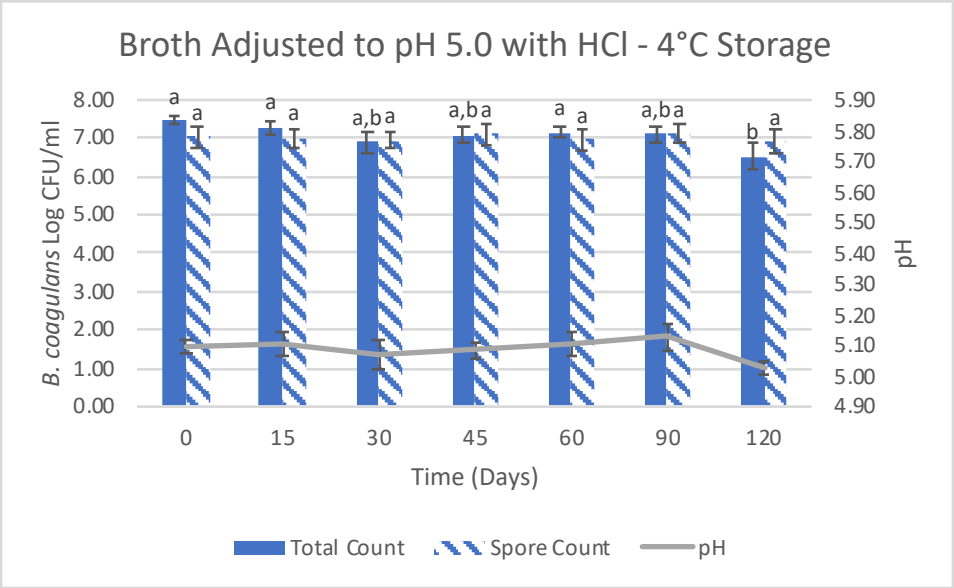

Figure 23. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 5.0 with HCl as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 120 days of storage.

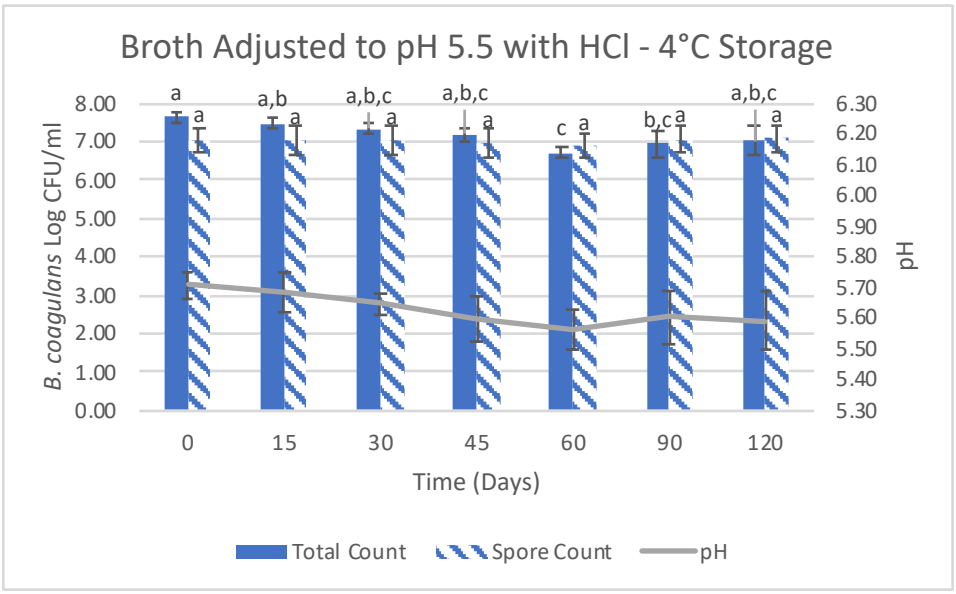

Figure 24. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 5.5 with HCl as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 120 days of storage.

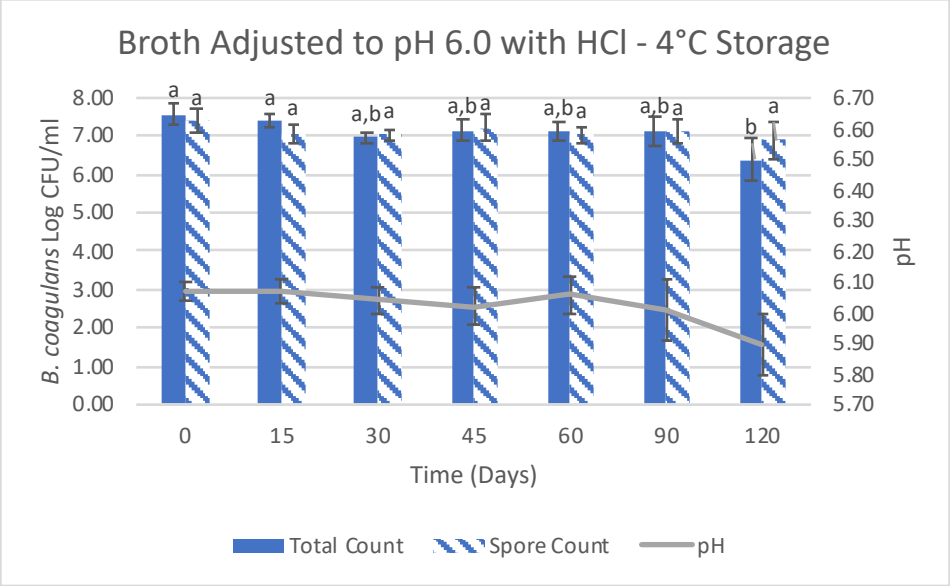

Figure 25. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 6.0 with HCl as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 120 days of storage.

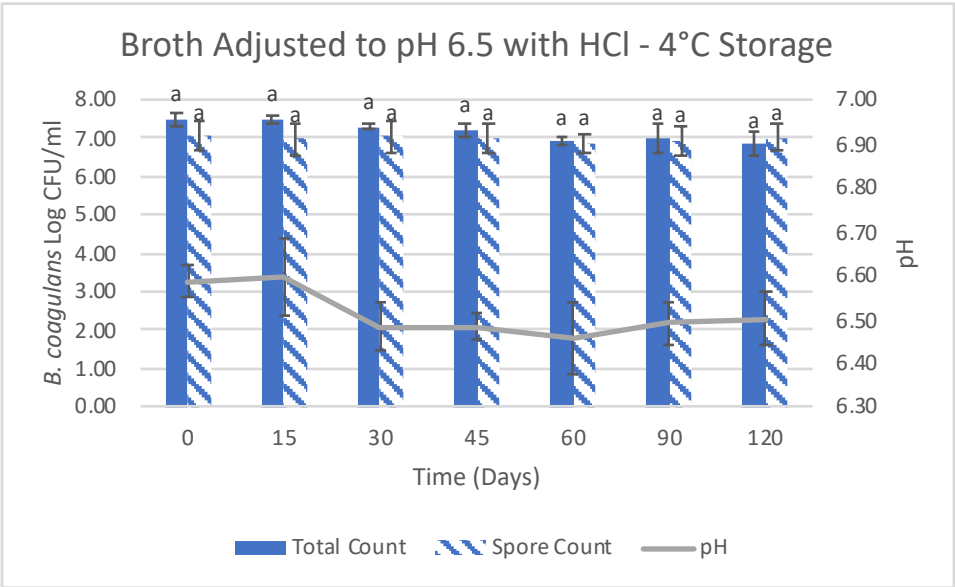

Figure 26. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 6.5 with HCl as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 120 days of storage.

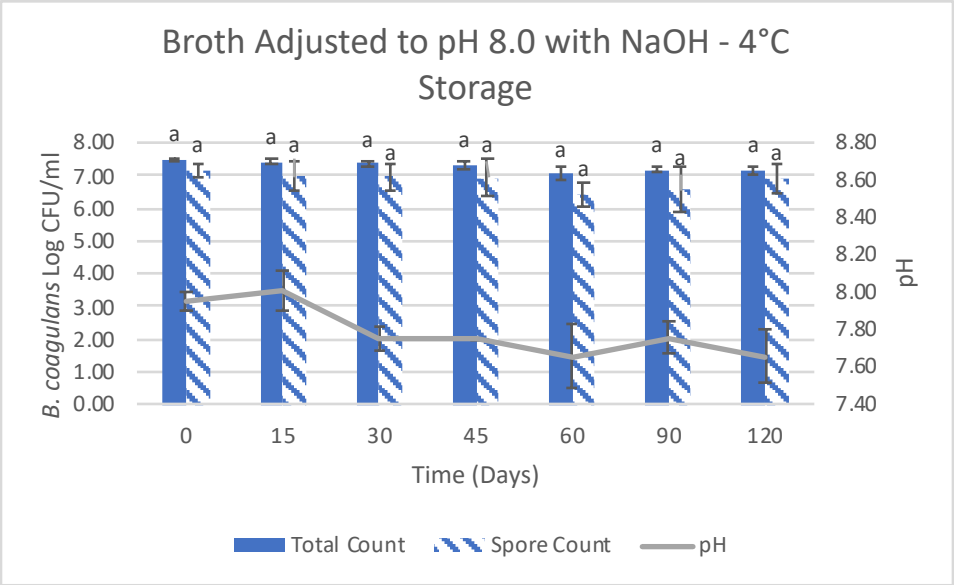

Figure 27. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 8.0 with NaOH as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 120 days of storage.

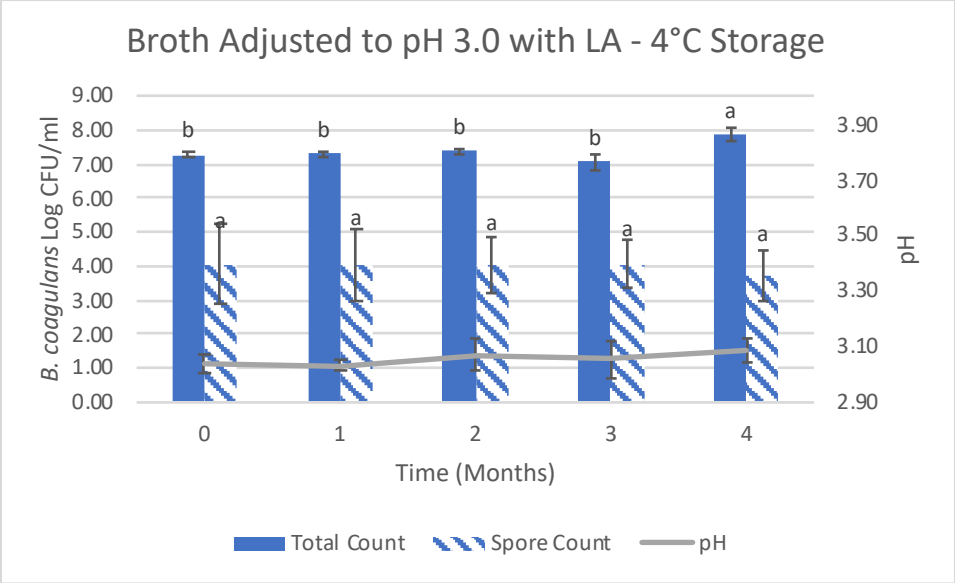

Figure 28. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 3.0 with LA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

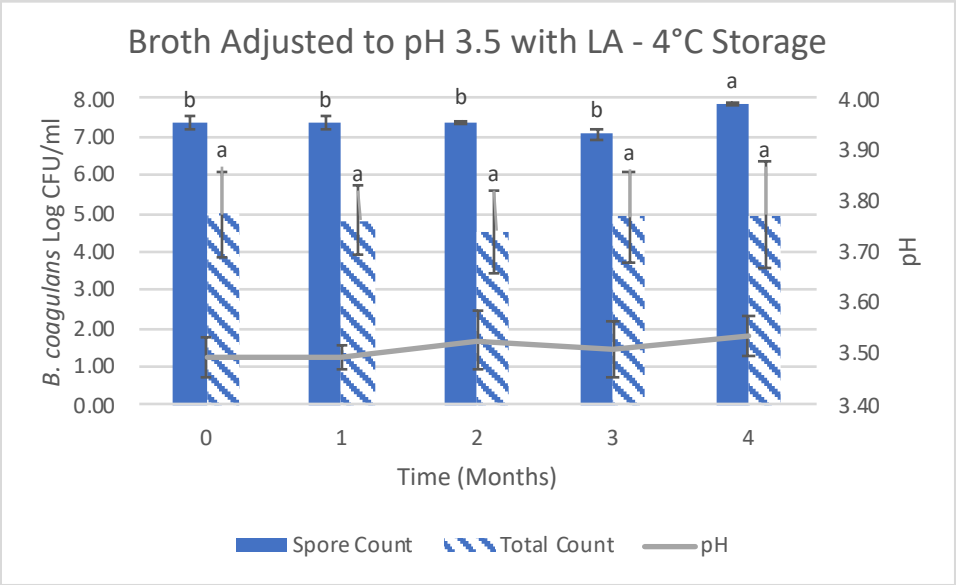

Figure 29. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 3.5 with LA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

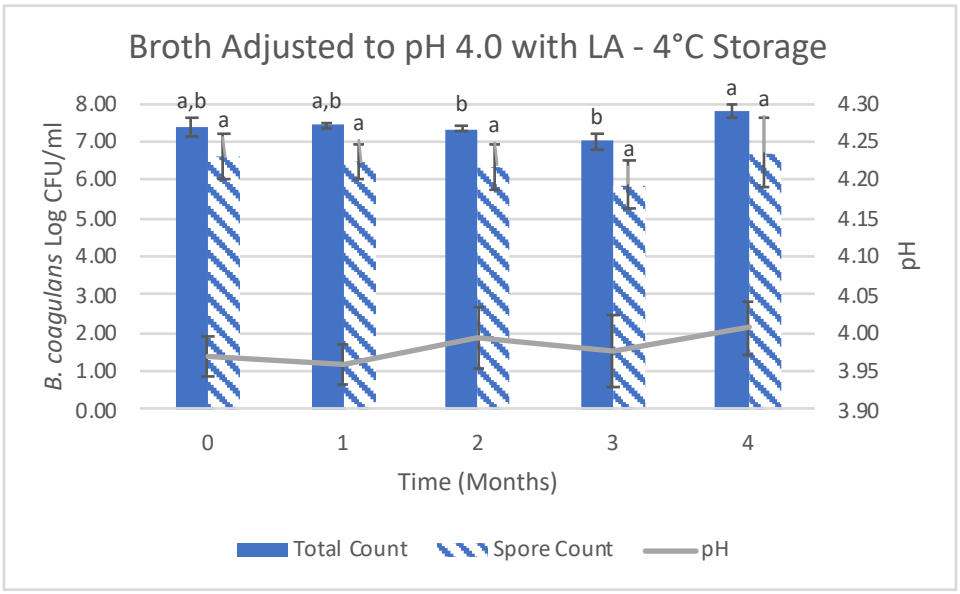

Figure 30. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 4.0 with LA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

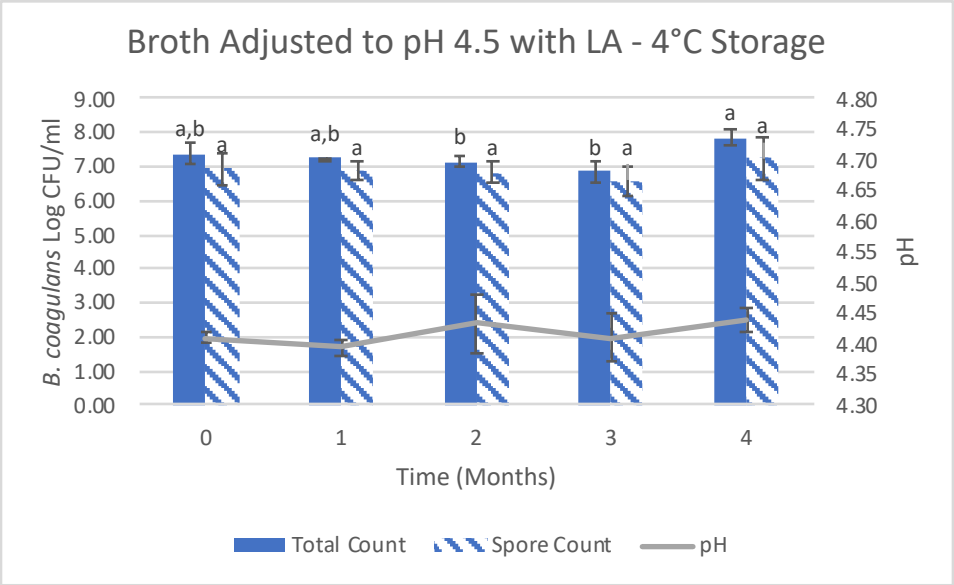

Figure 31. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 4.5 with LA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

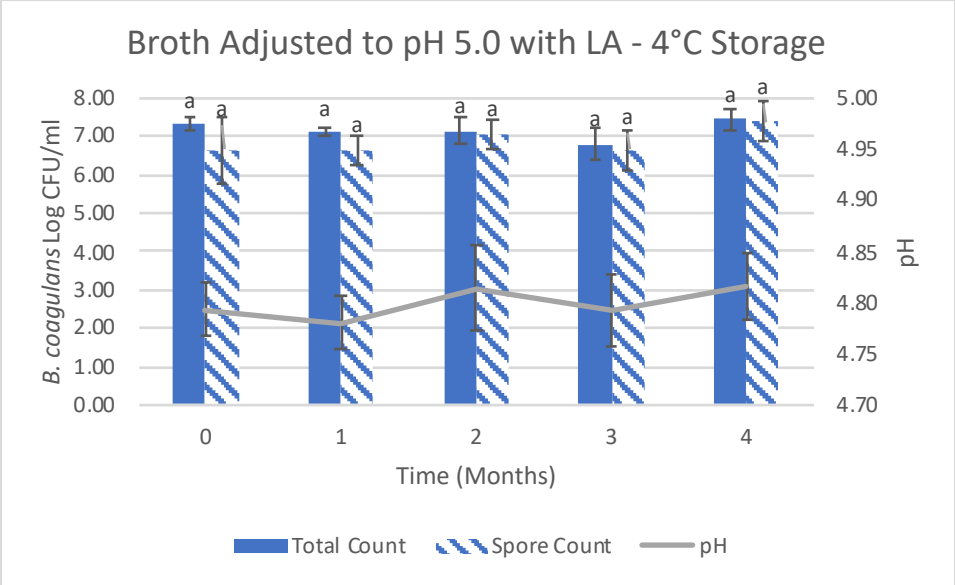

Figure 32. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 5.0 with LA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

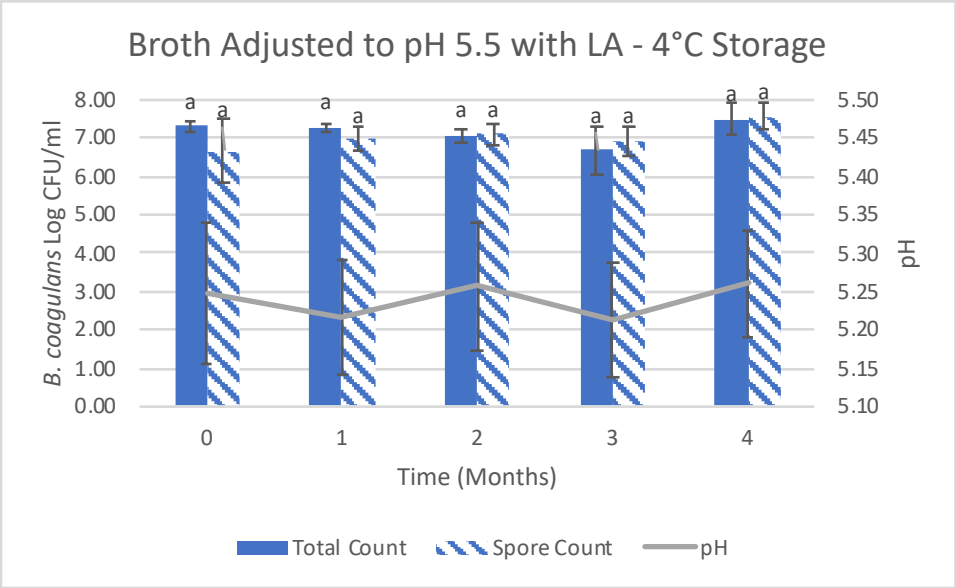

Figure 33. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 5.5 with LA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

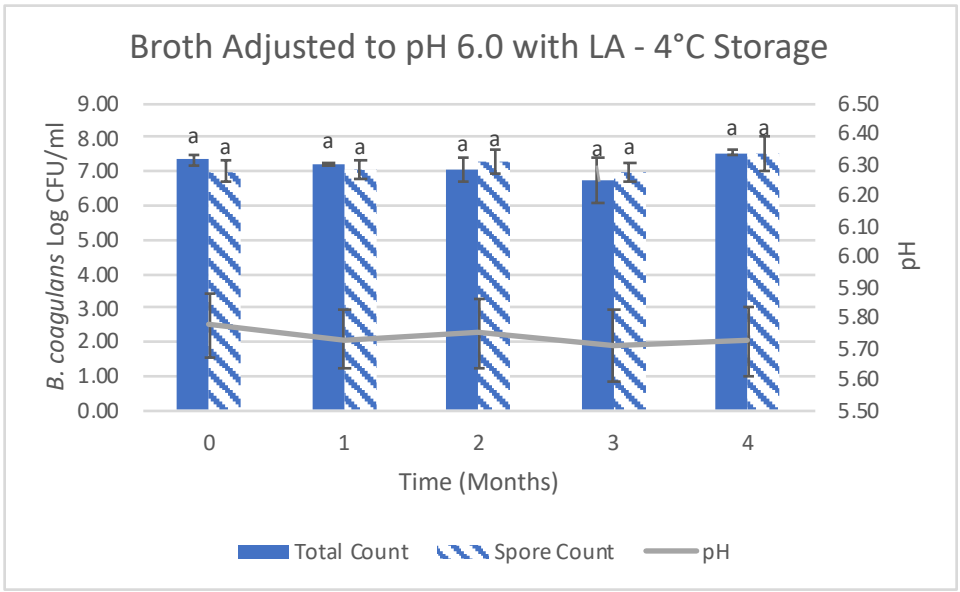

Figure 34. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 6.0 with LA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

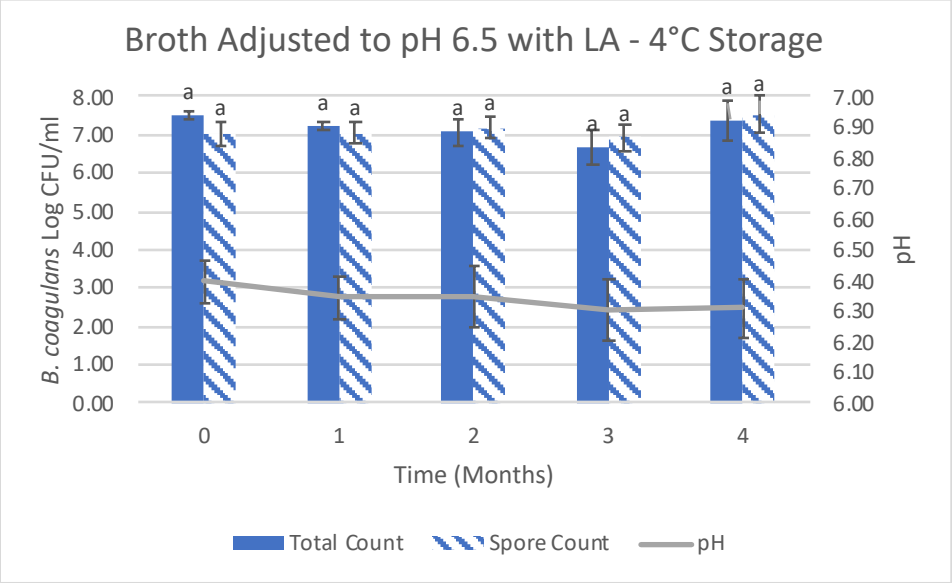

Figure 35. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 6.5 with LA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

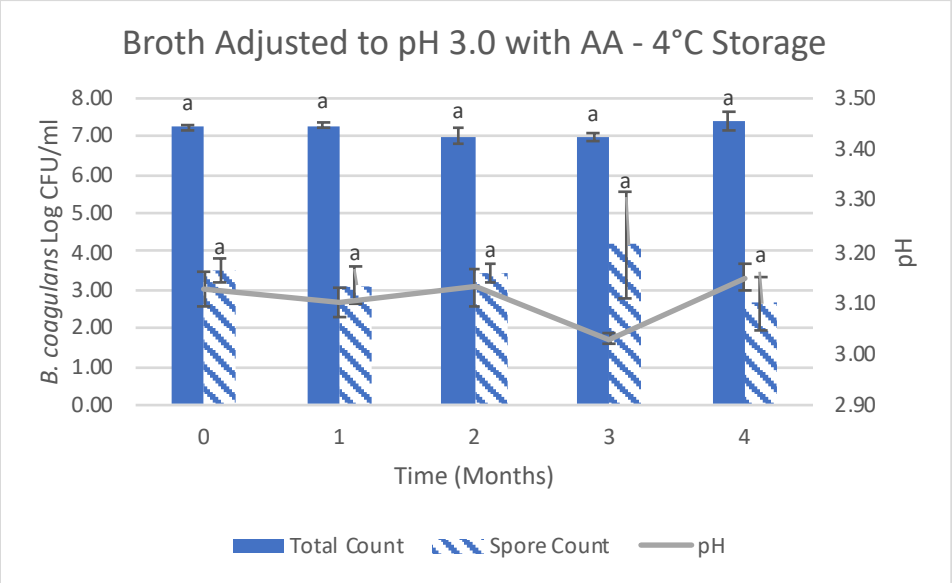

Figure 36. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 3.0 with AA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

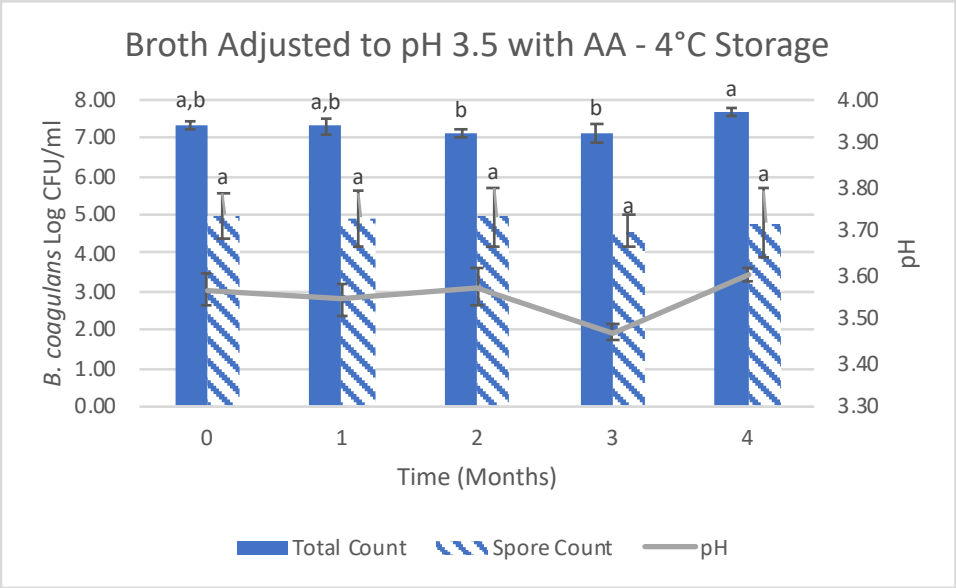

Figure 37. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 3.5 with AA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

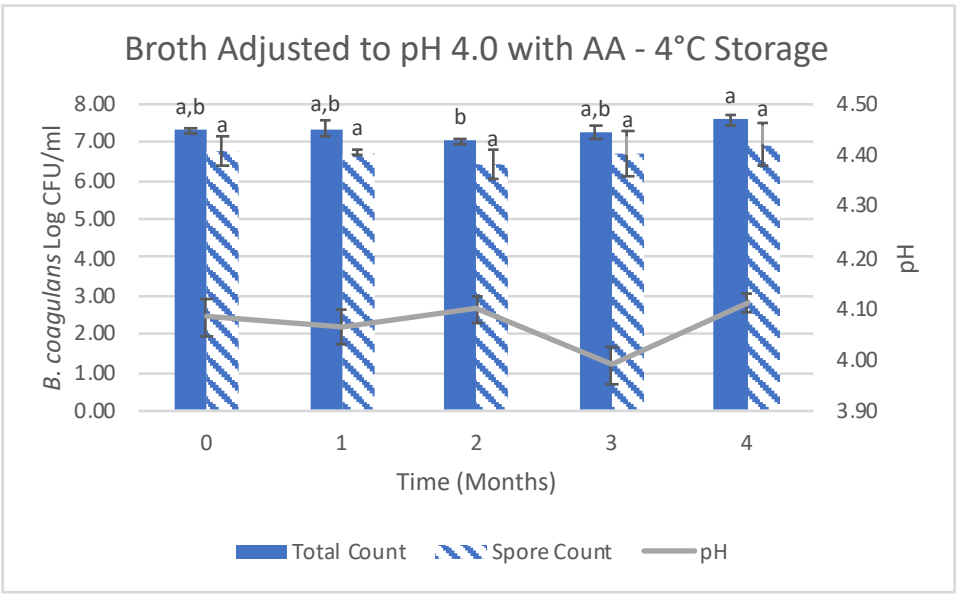

Figure 38. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 4.0 with AA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

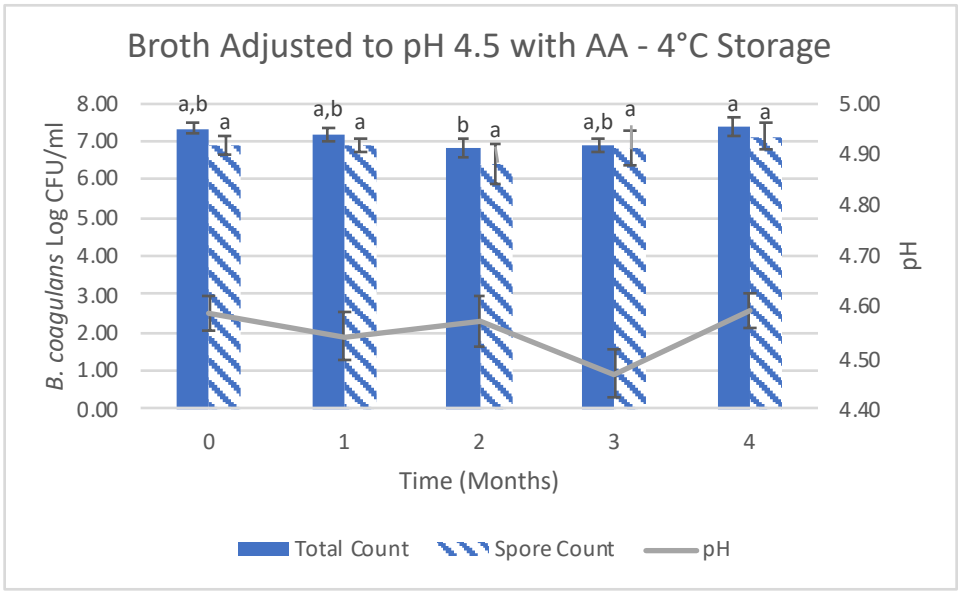

Figure 39. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 4.5 with AA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

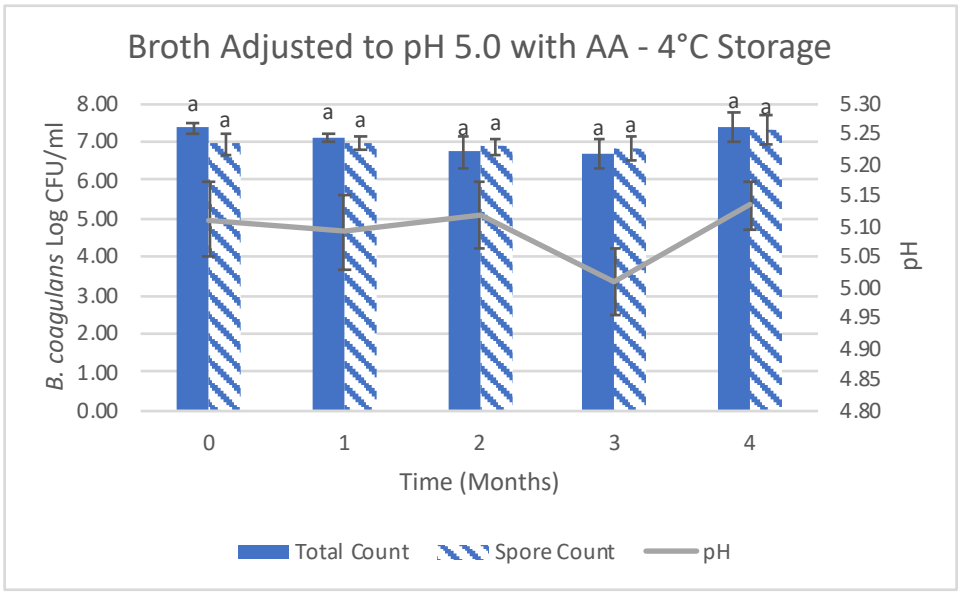

Figure 40. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 5.0 with AA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

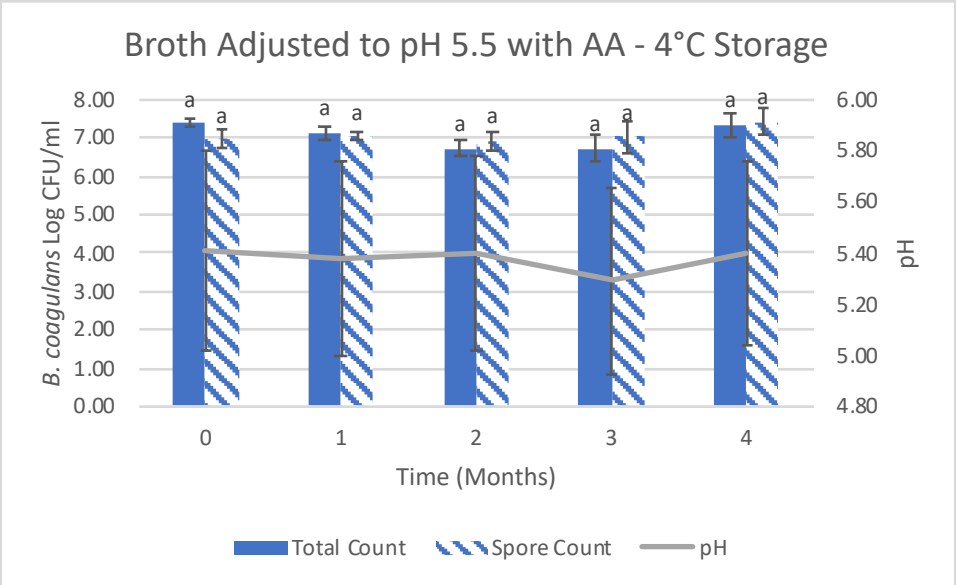

Figure 41. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 5.5 with AA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

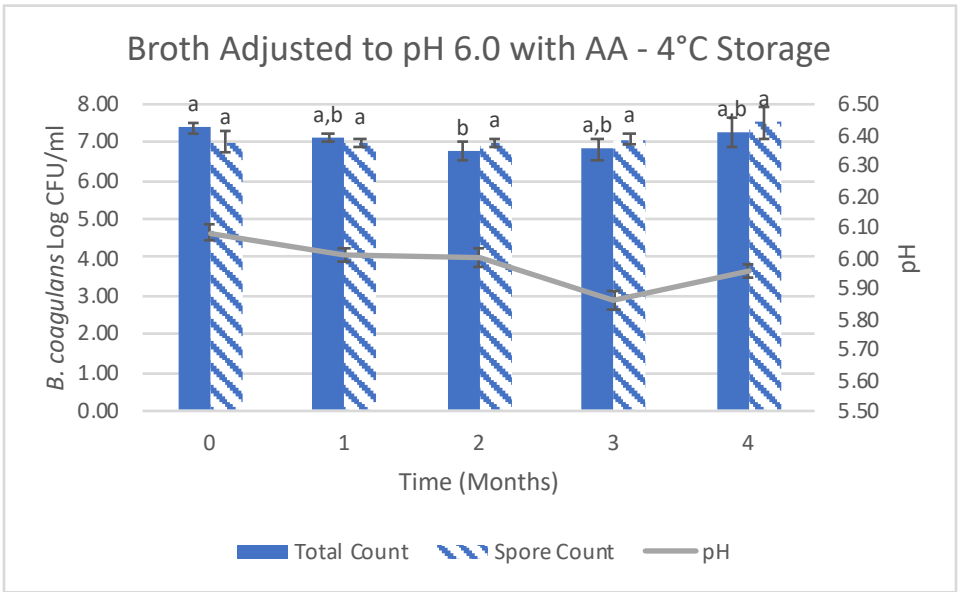

Figure 42. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 6.0 with AA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

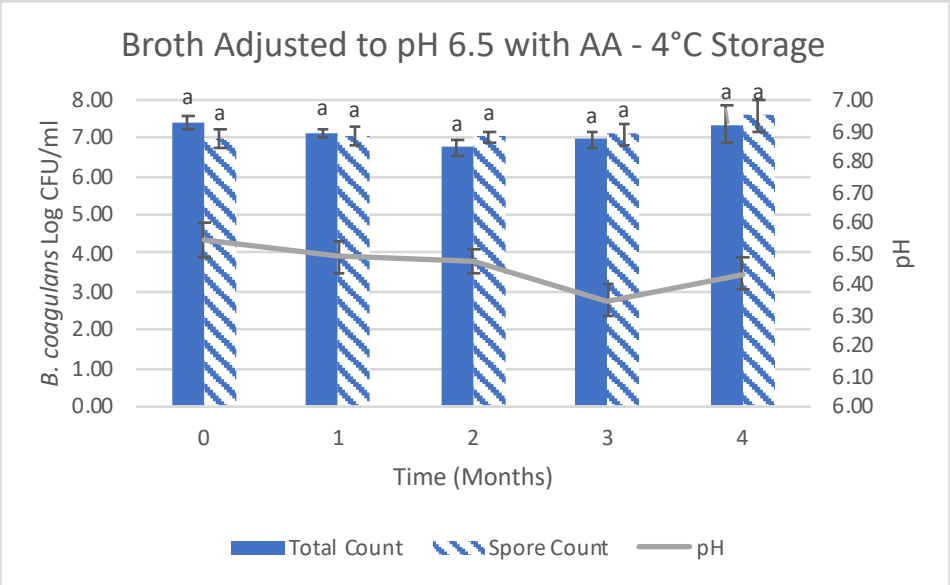

Figure 43. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 6.5 with AA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

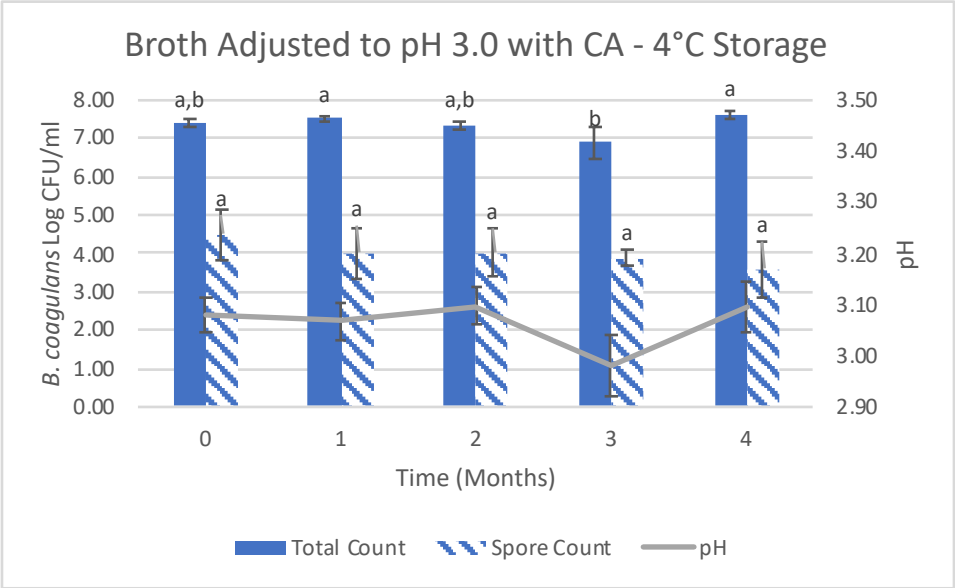

Figure 44. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 3.0 with CA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

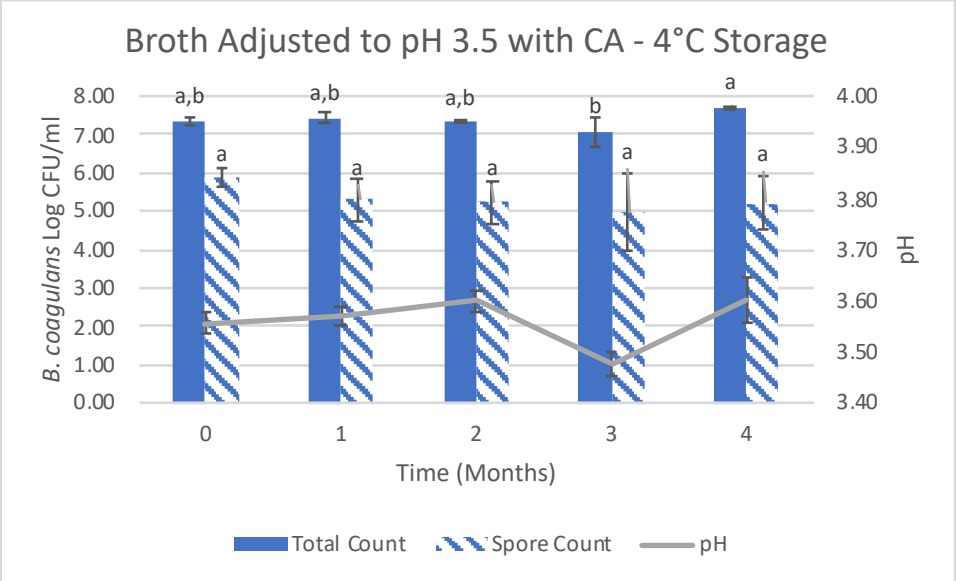

Figure 45. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 3.5 with CA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

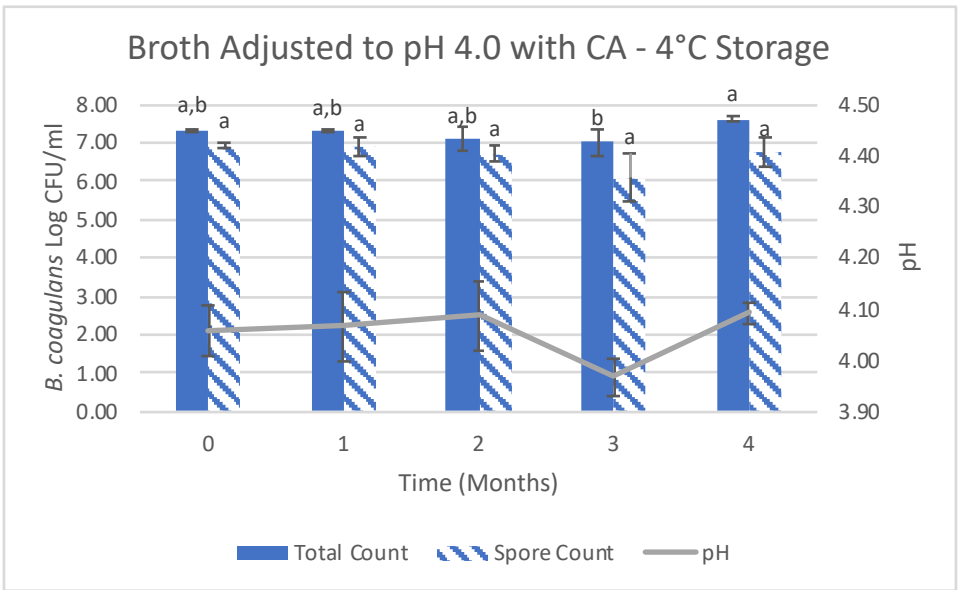

Figure 46. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 4.0 with CA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

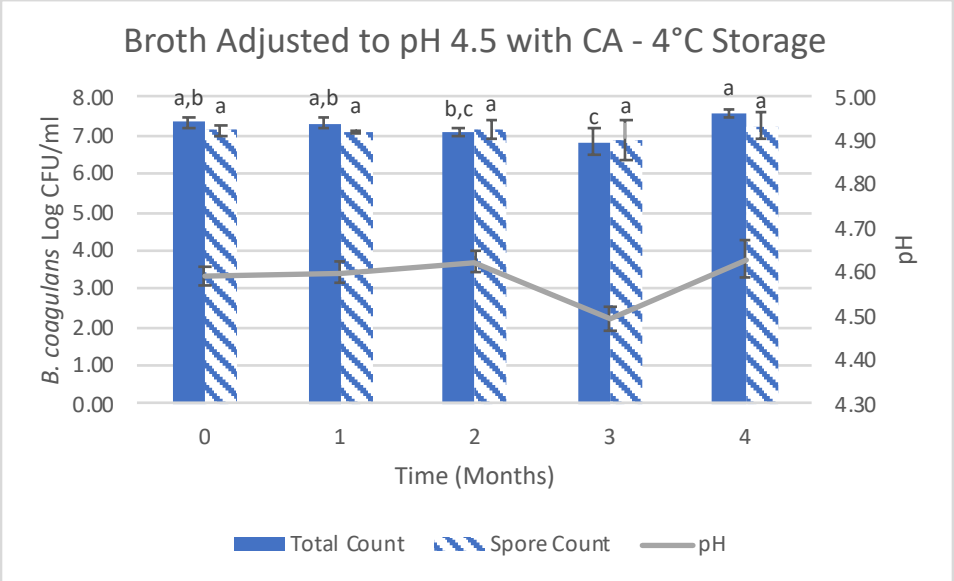

Figure 47. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 4.5 with CA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

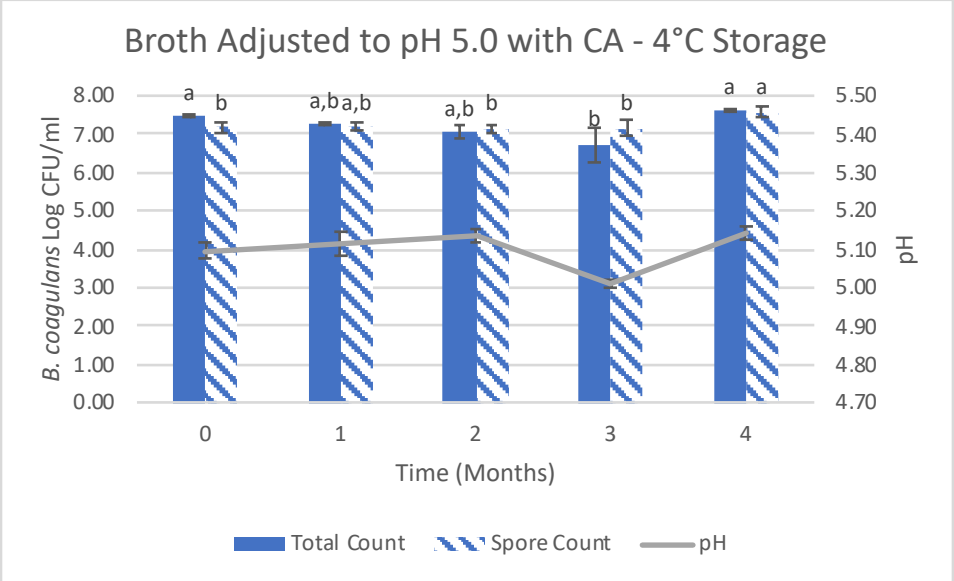

Figure 48. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 5.0 with CA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

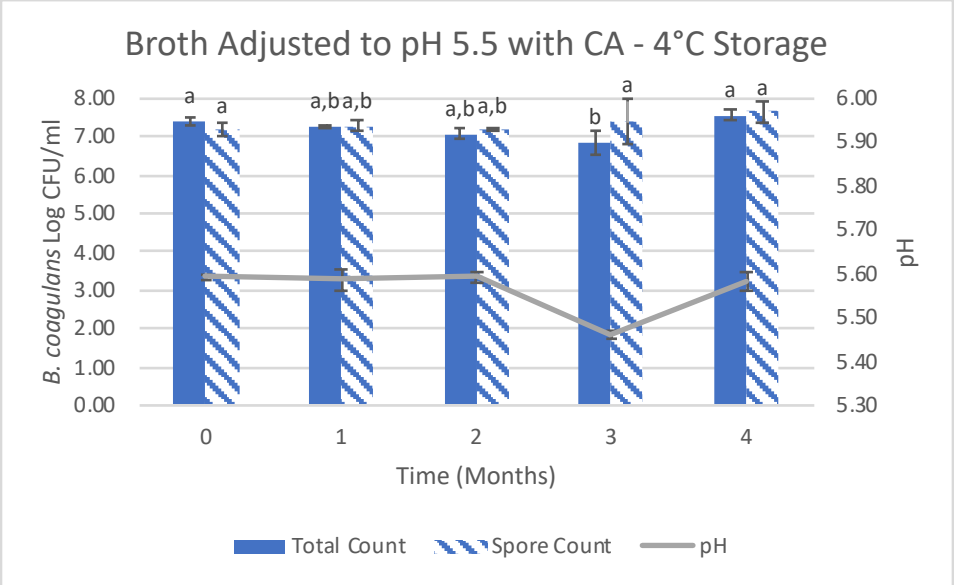

Figure 49. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 5.5 with CA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

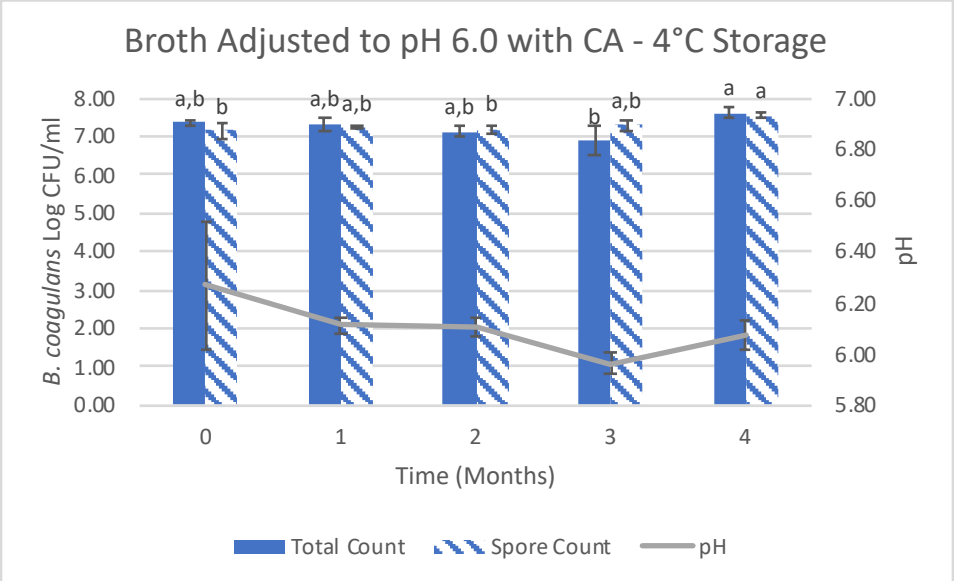

Figure 50. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 6.0 with CA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

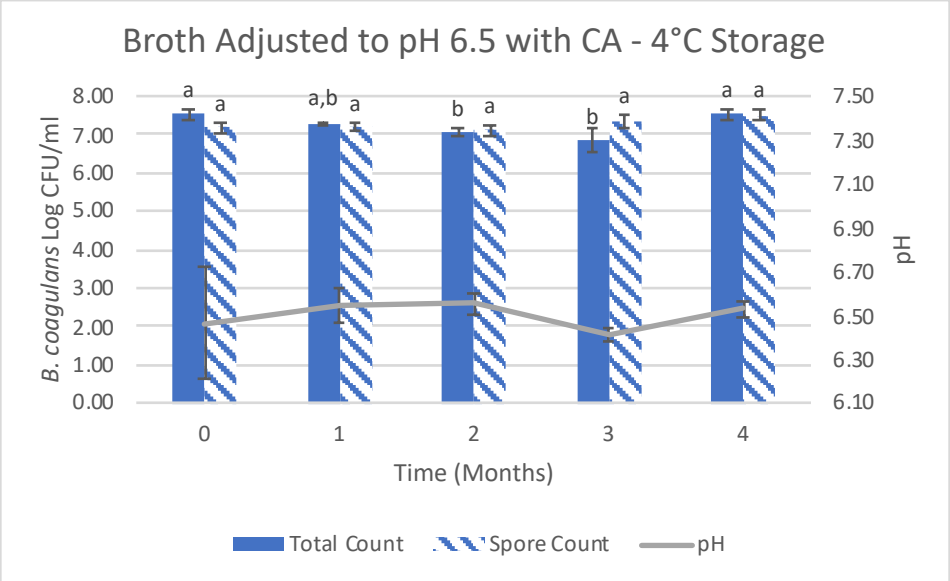

Figure 51. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 6.5 with CA as denoted by total and spore counts. Stability tests were done during storage at 4˚C with the pH of the broth monitored throughout 4 months of storage.

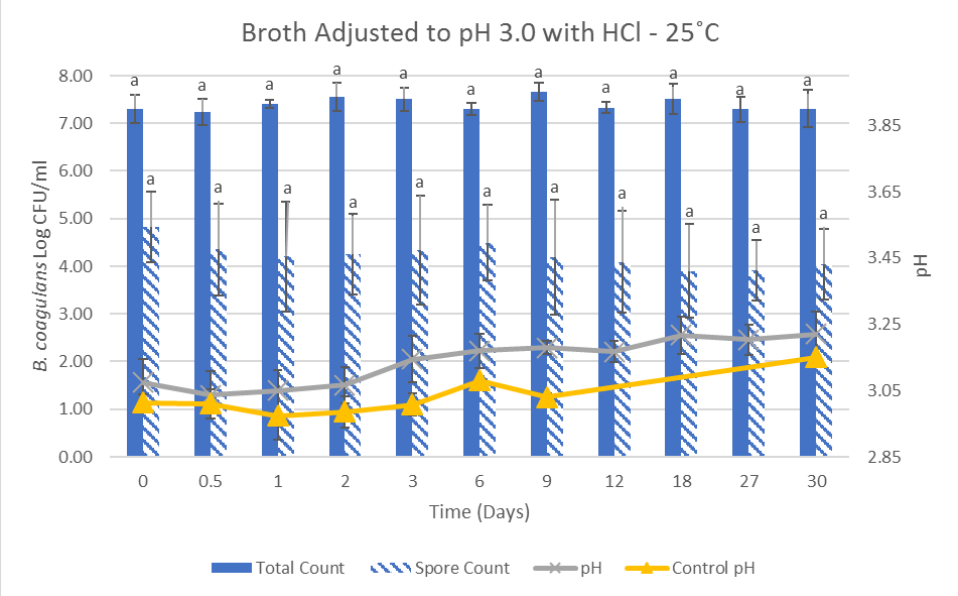

Figure 52. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 3.0 with HCl as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

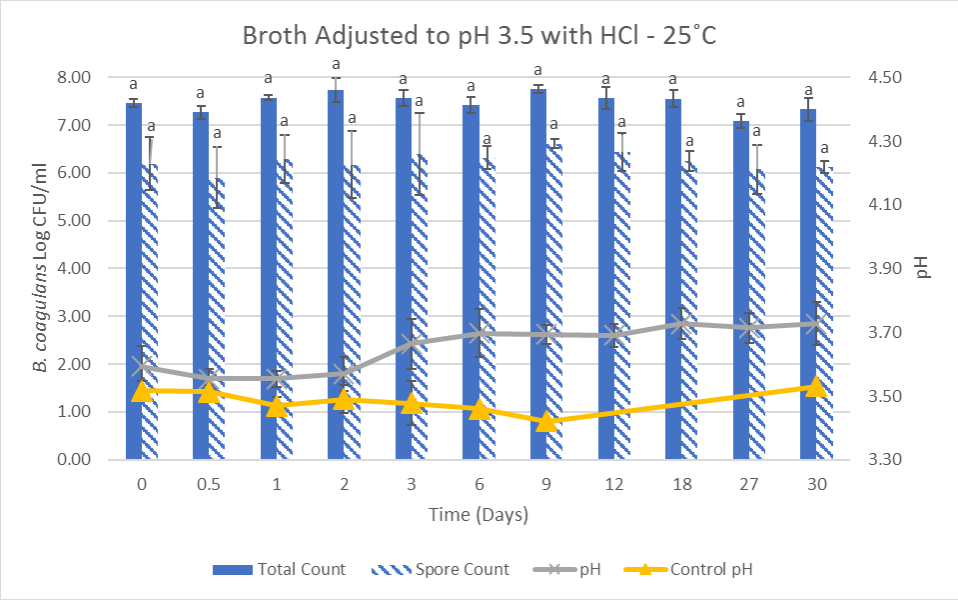

Figure 53. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 3.5 with HCl as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

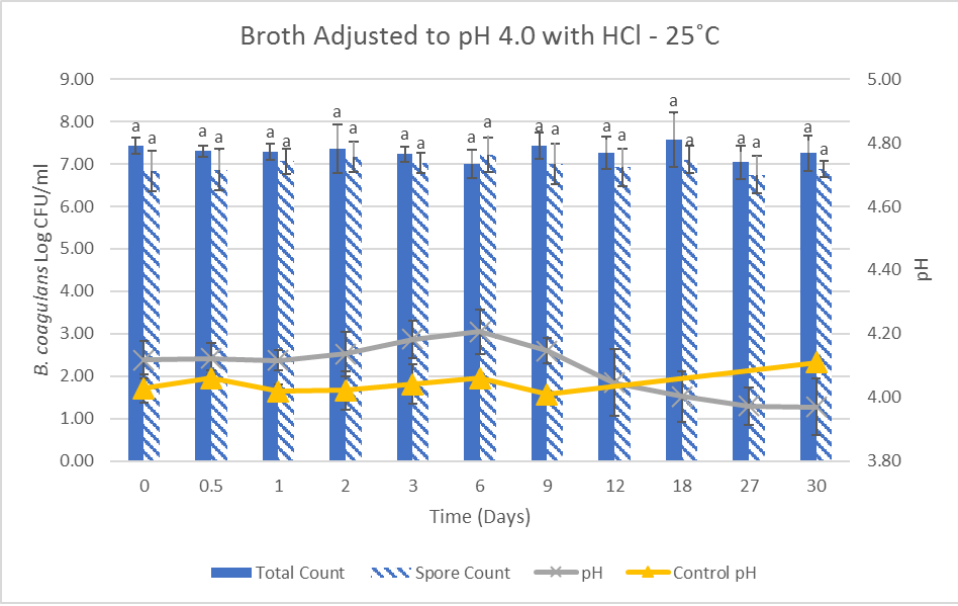

Figure 54. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 4.0 with HCl as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

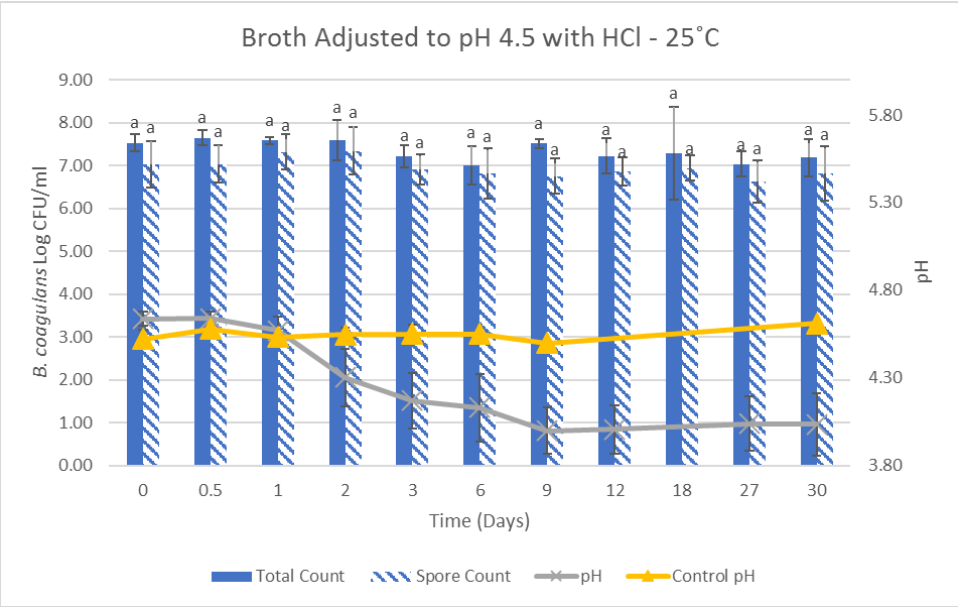

Figure 55. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 4.5 with HCl as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

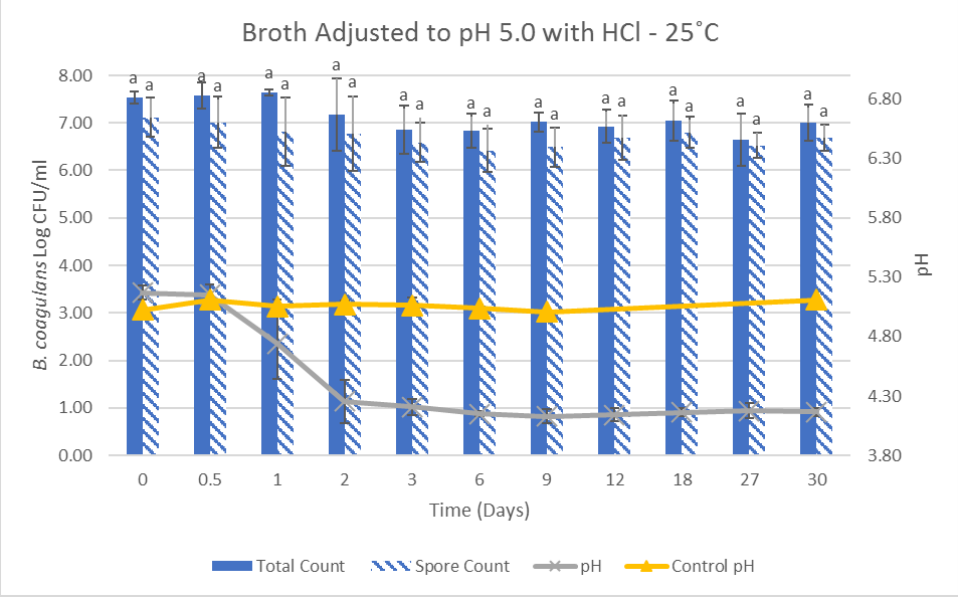

Figure 56. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 5.0 with HCl as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

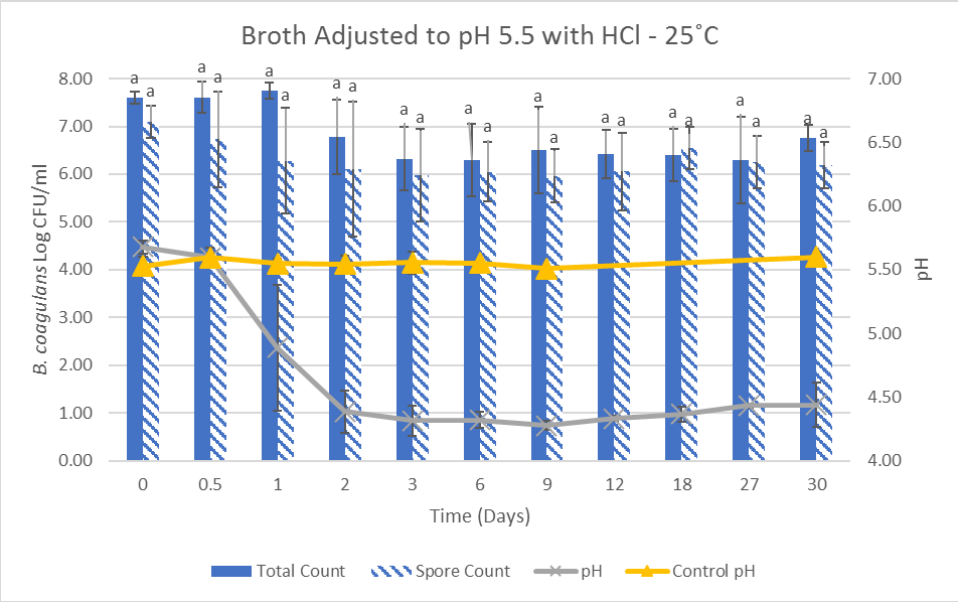

Figure 57. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 5.5 with HCl as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

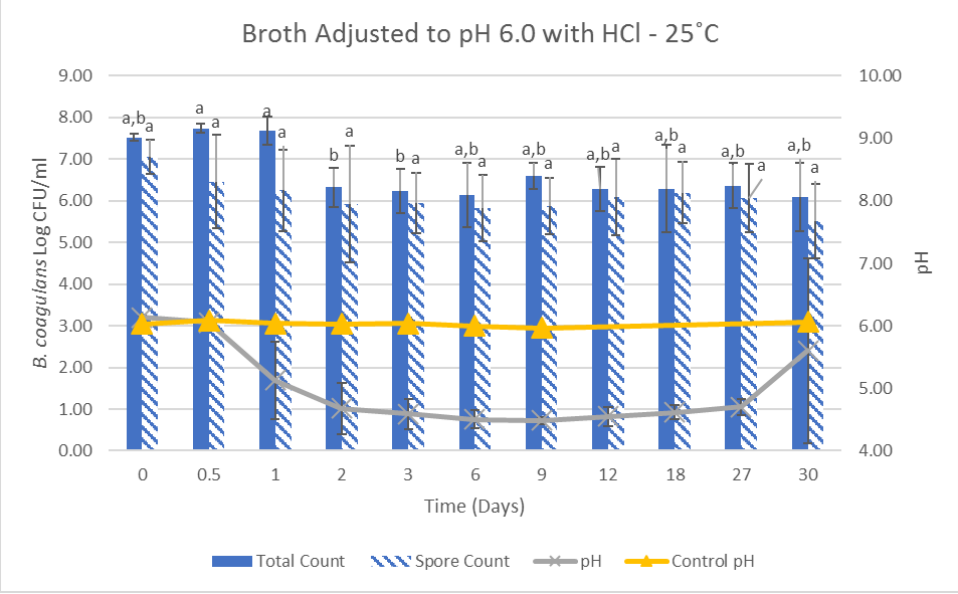

Figure 58. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 6.0 with HCl as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

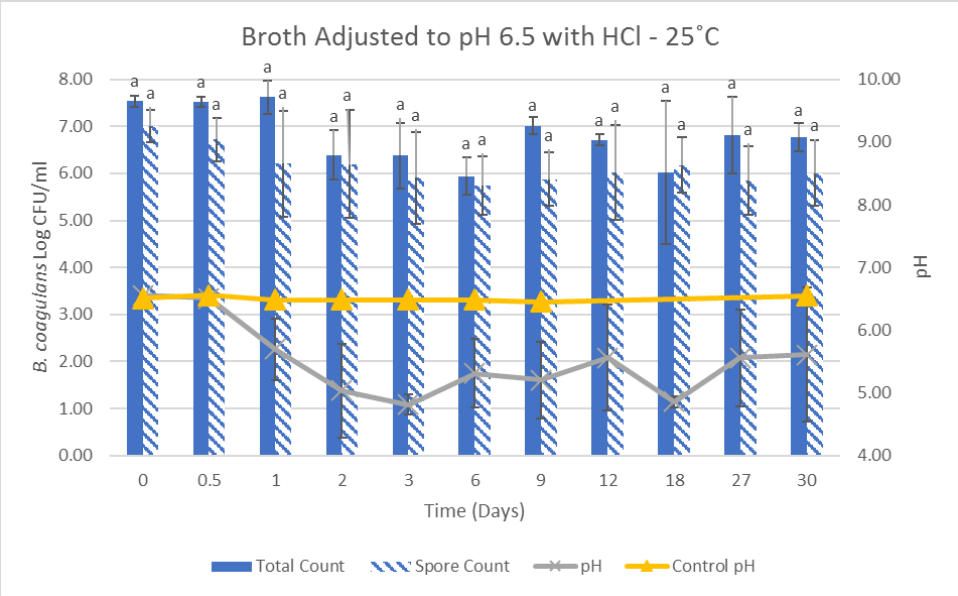

Figure 59. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 6.5 with HCl as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

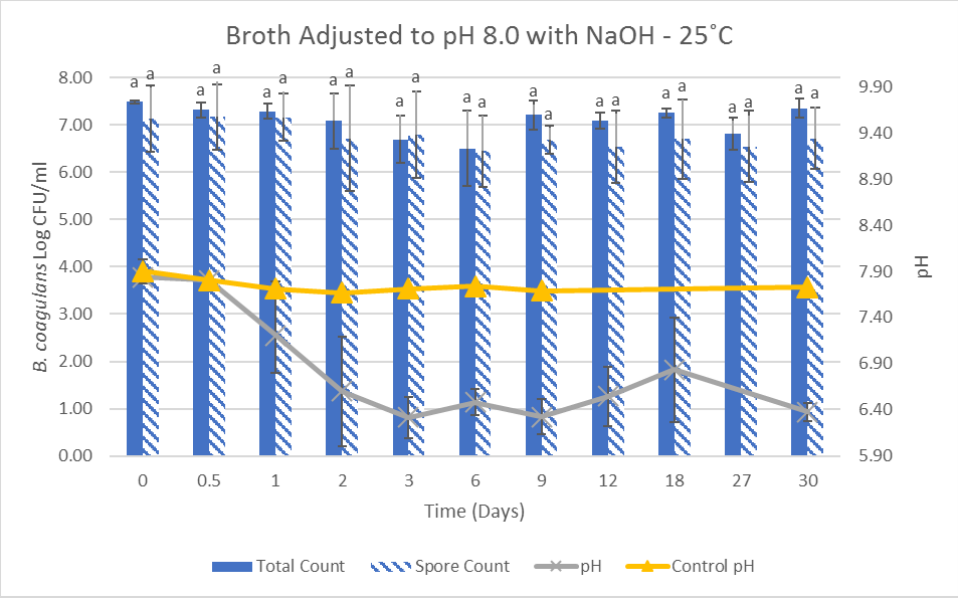

Figure 60. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 8.0 with NaOH as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

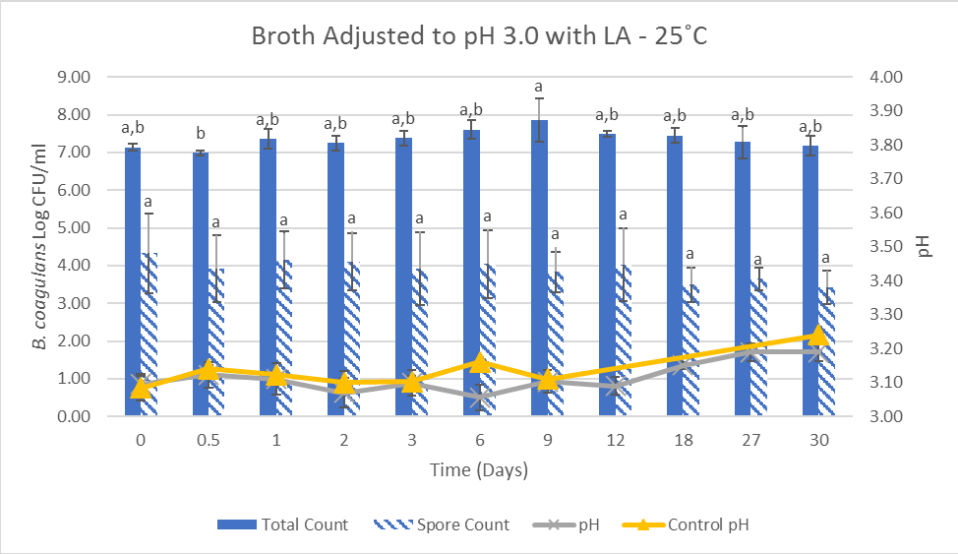

Figure 61. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 3.0 with LA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

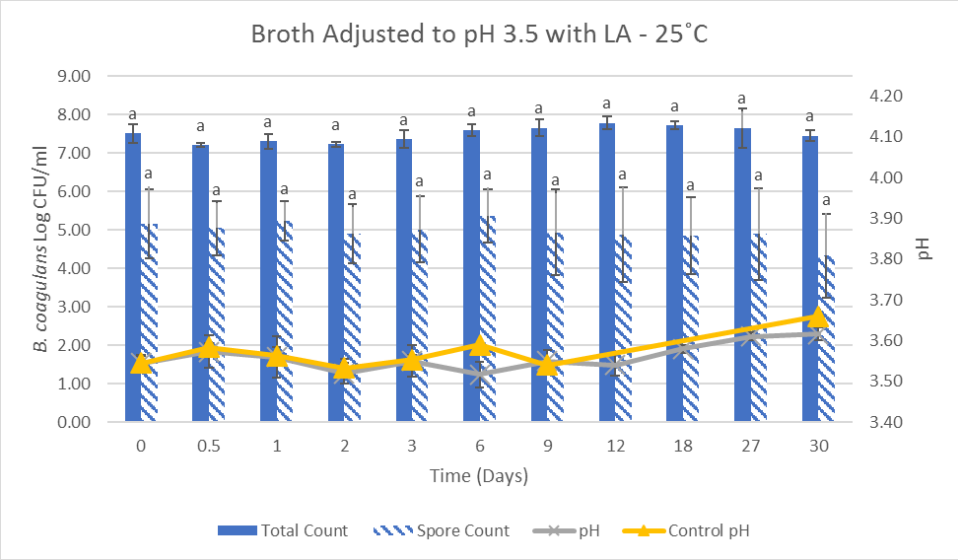

Figure 62. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 3.5 with LA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

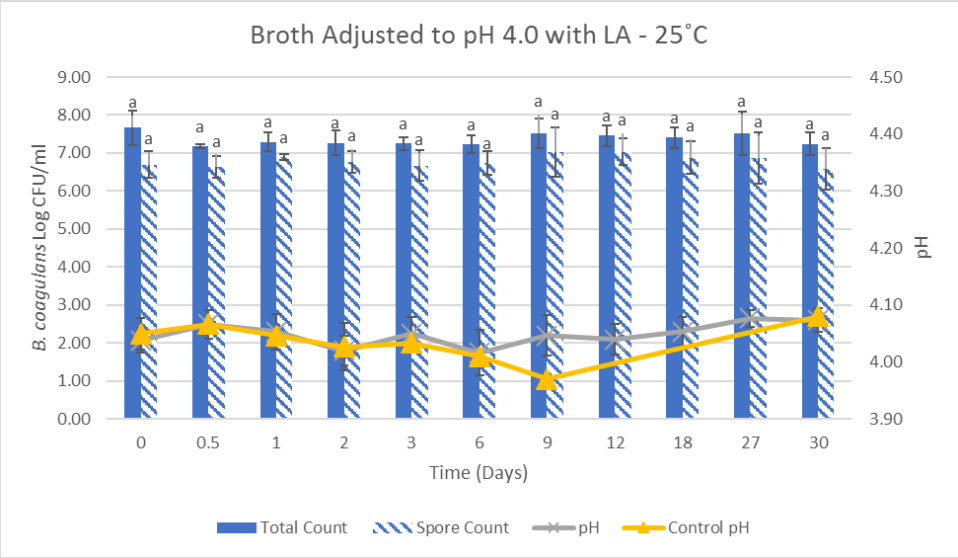

Figure 63. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 4.0 with LA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

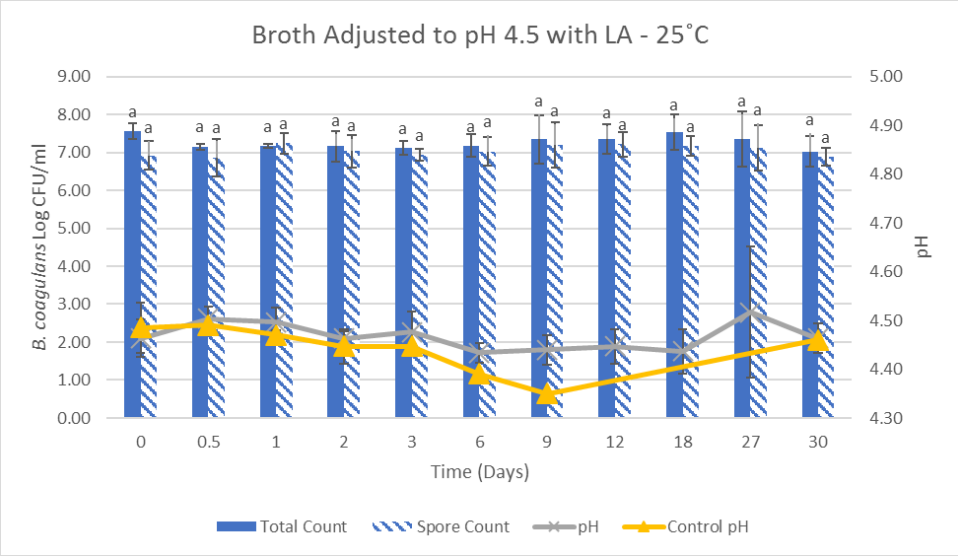

Figure 64. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 4.5 with LA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

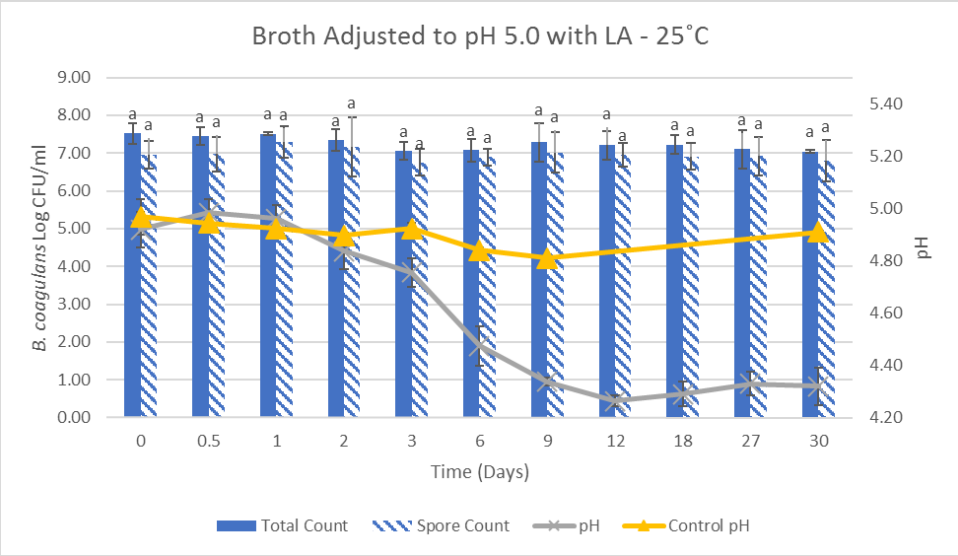

Figure 65. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 5.0 with LA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

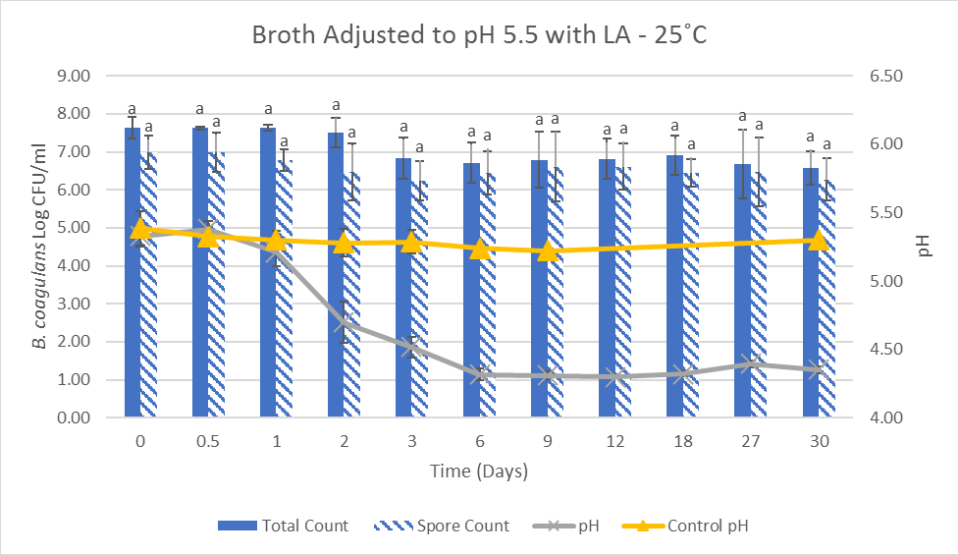

Figure 66. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 5.5 with LA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

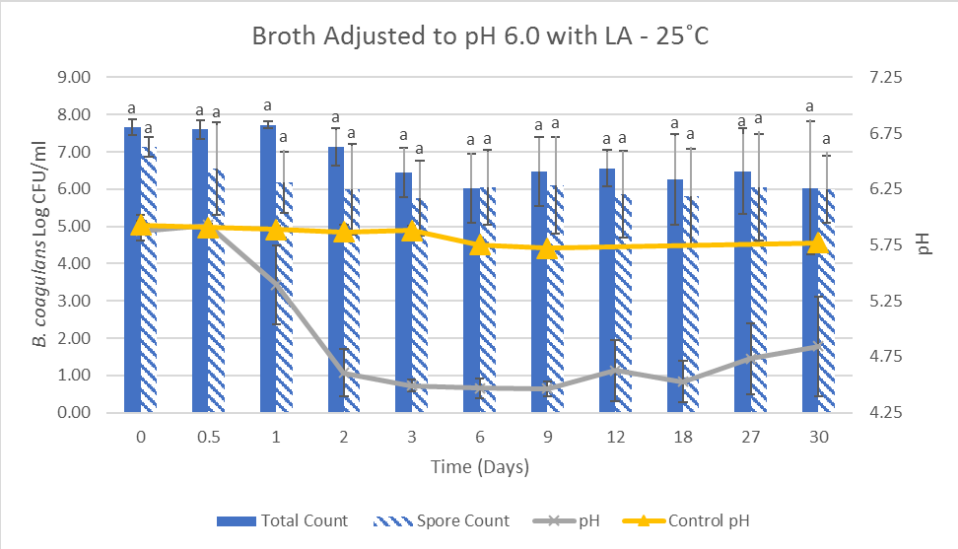

Figure 67. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 6.0 with LA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

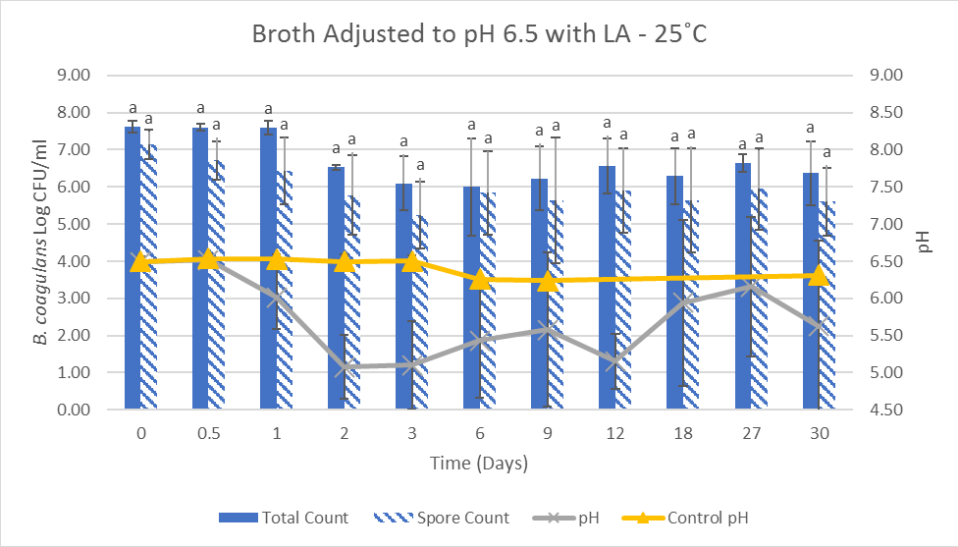

Figure 68. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 6.5 with LA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

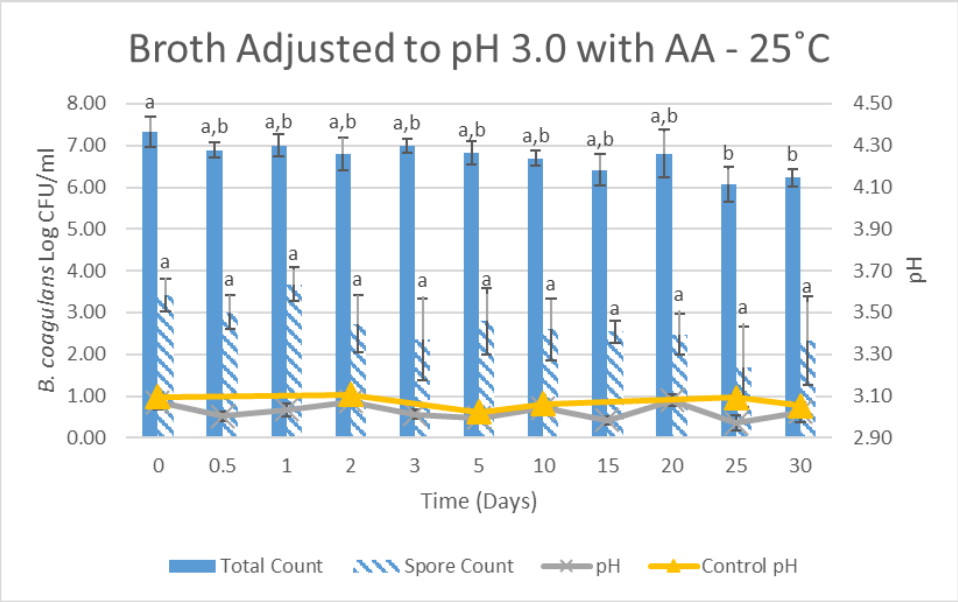

Figure 69. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 3.0 with AA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

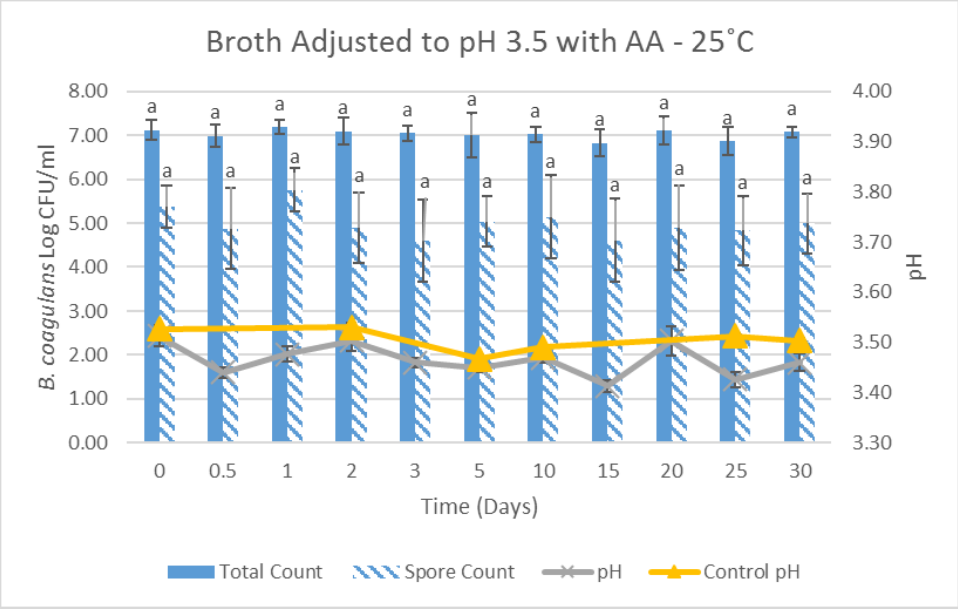

Figure 70. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 3.5 with AA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

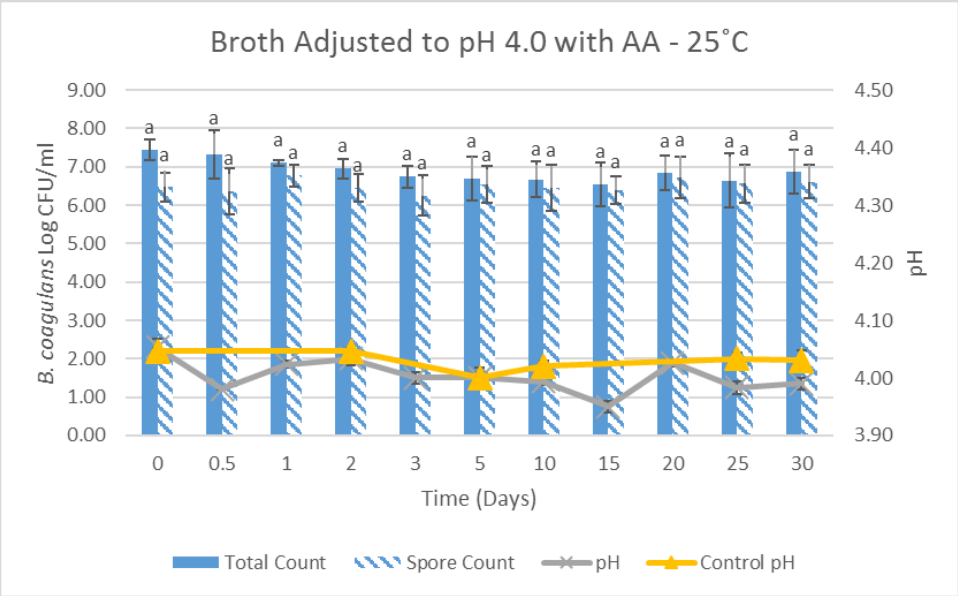

Figure 71. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 4.0 with AA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

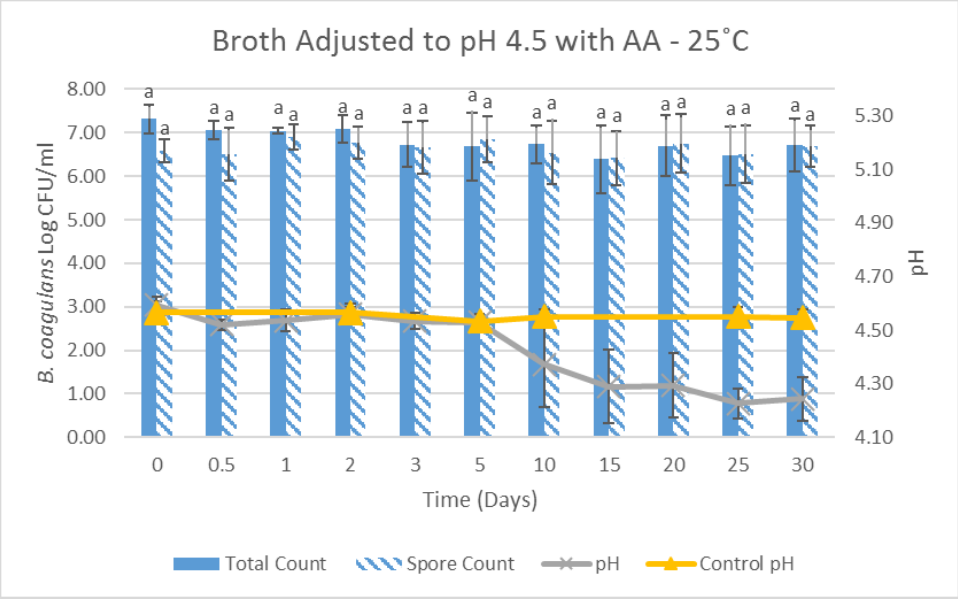

Figure 72. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 4.5 with AA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

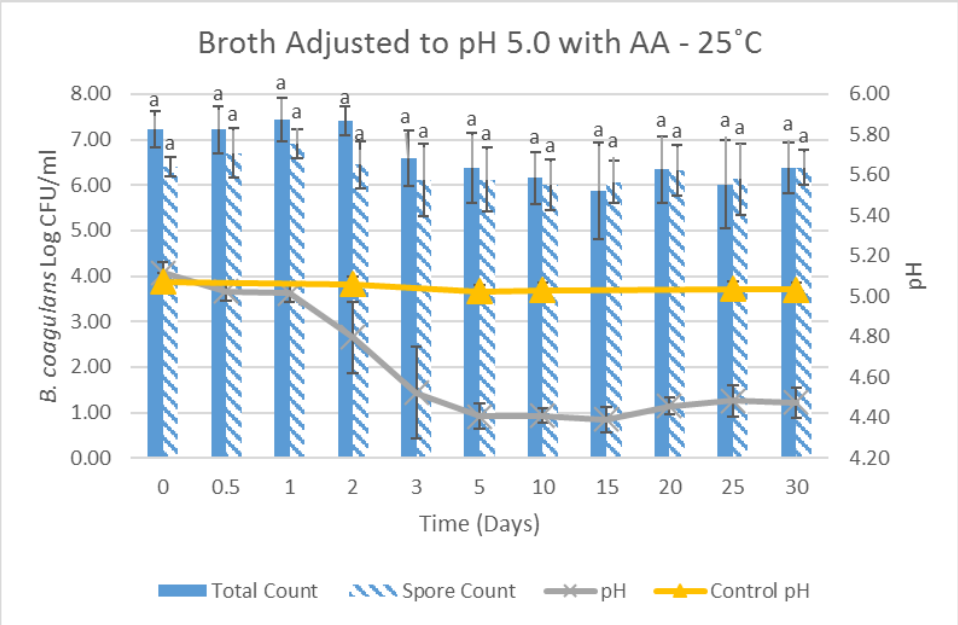

Figure 73. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 5.0 with AA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

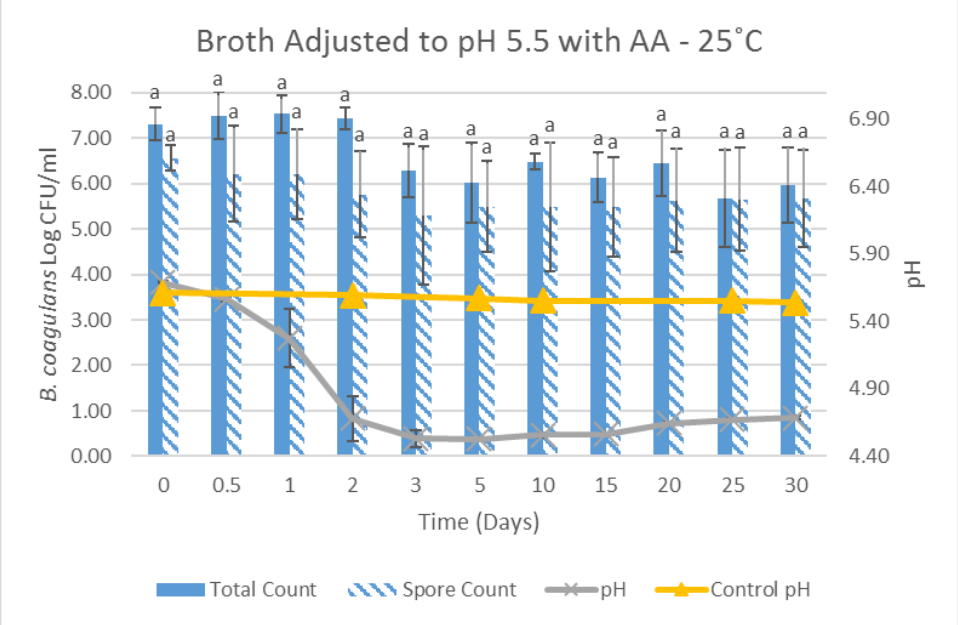

Figure 74. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 5.5 with AA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

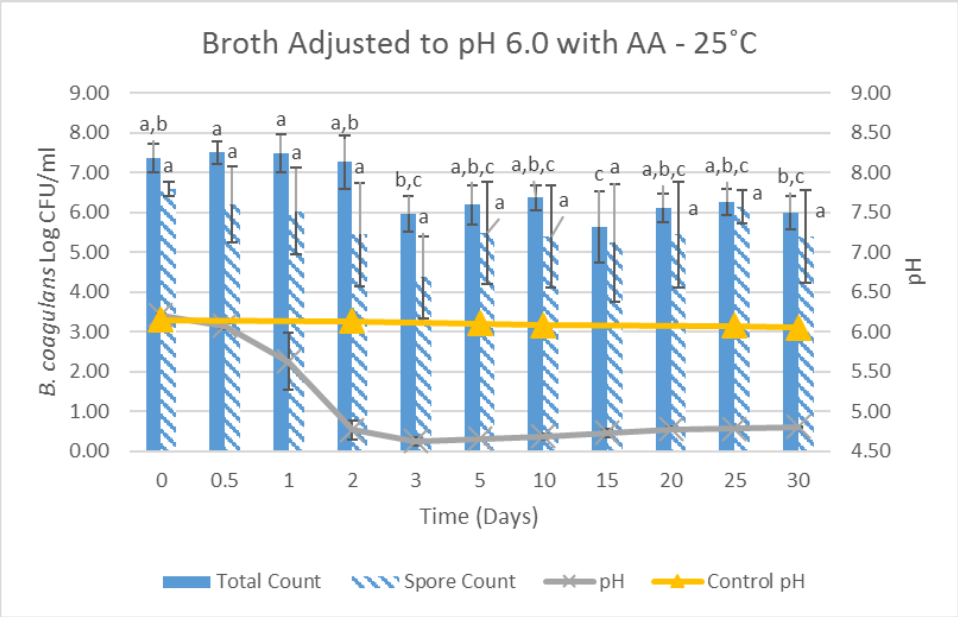

Figure 75. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 6.0 with AA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

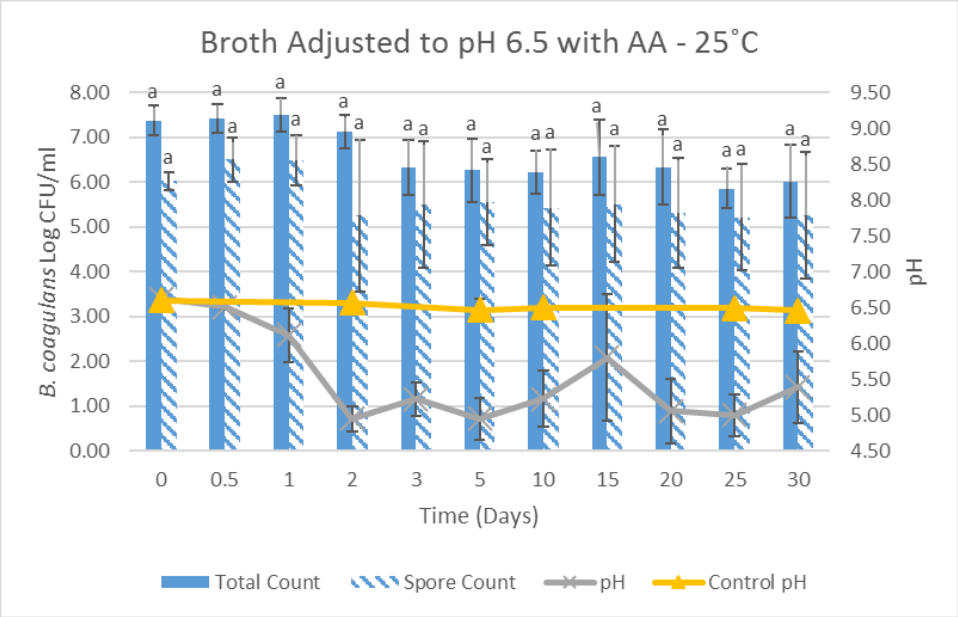

Figure 76. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 6.5 with AA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

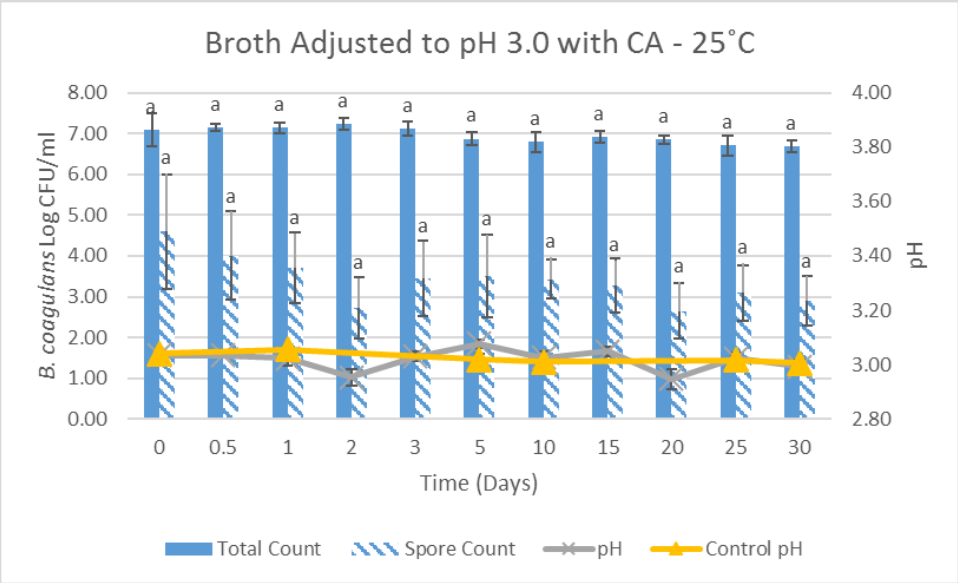

Figure 77. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 3.0 with CA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

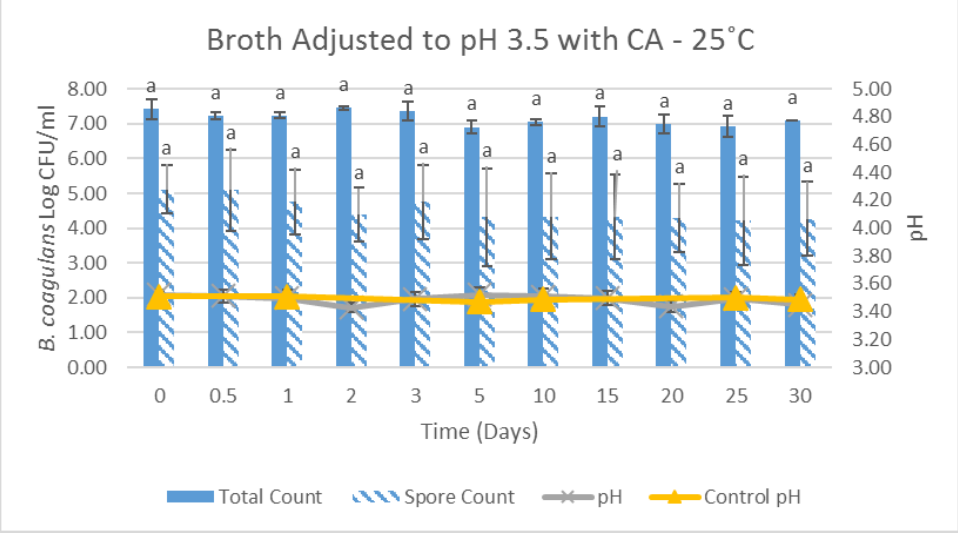

Figure 78. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 3.5 with CA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

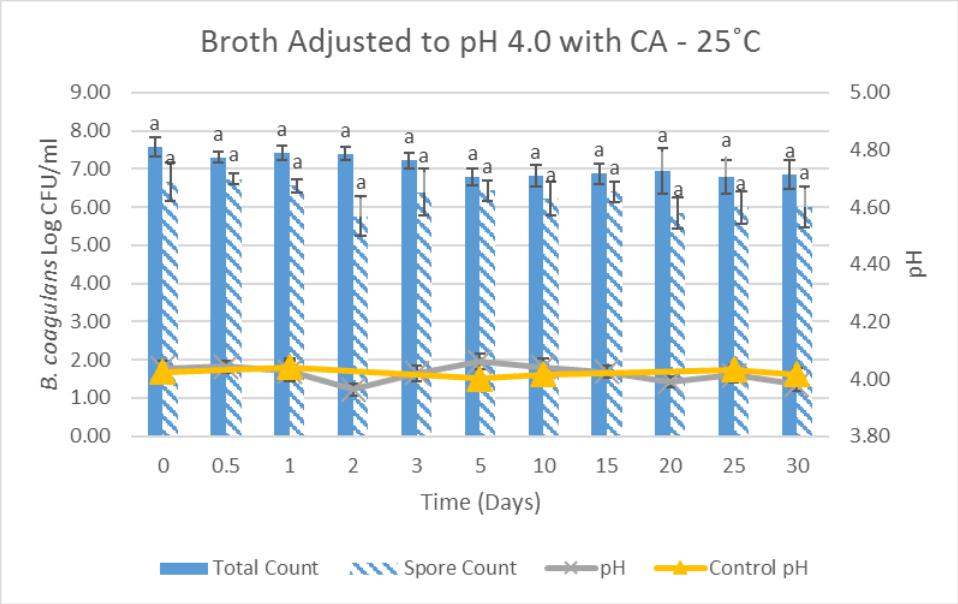

Figure 79. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 4.0 with CA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

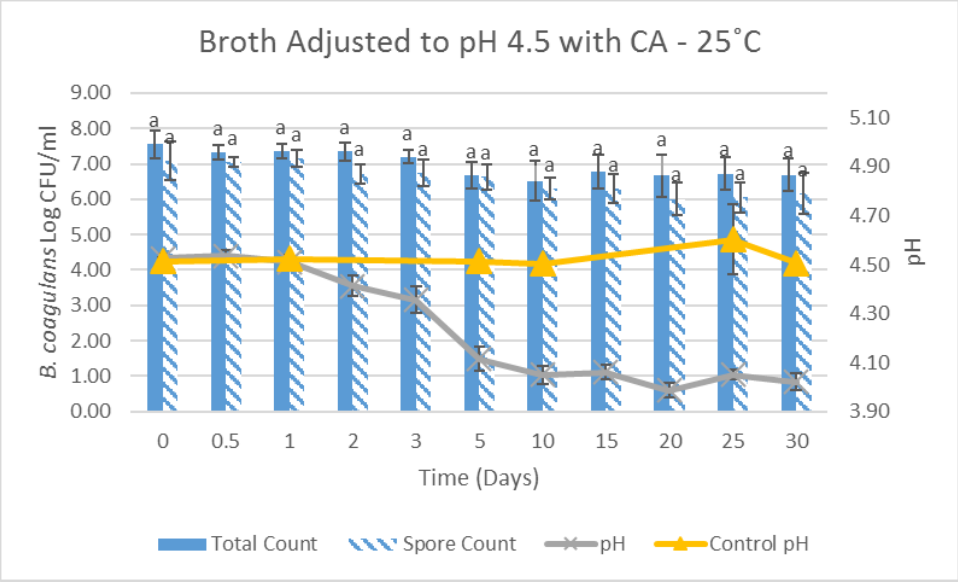

Figure 80. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 4.5 with CA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

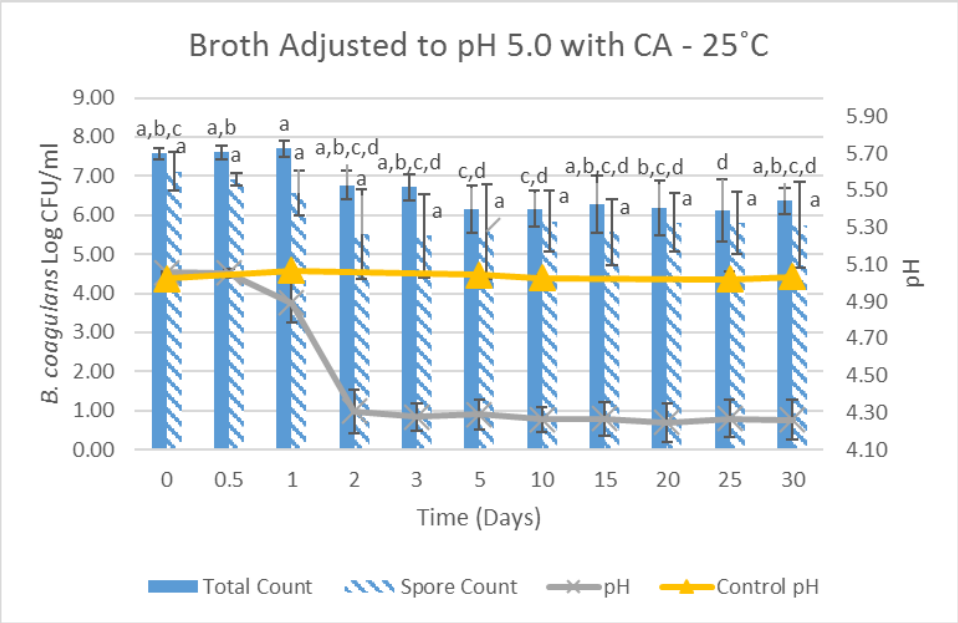

Figure 81. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 5.0 with CA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

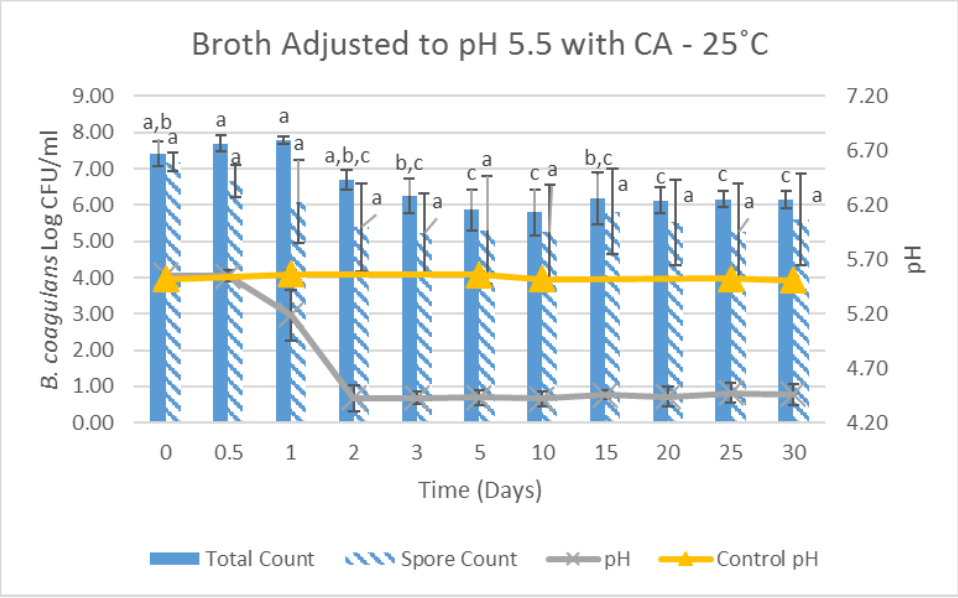

Figure 82. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 5.5 with CA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

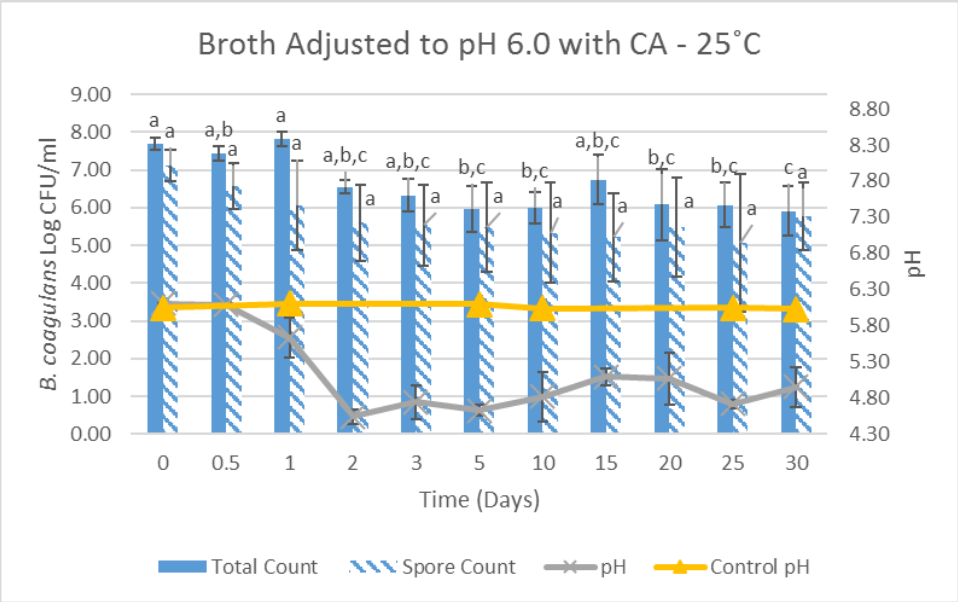

Figure 83. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 6.0 with CA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.

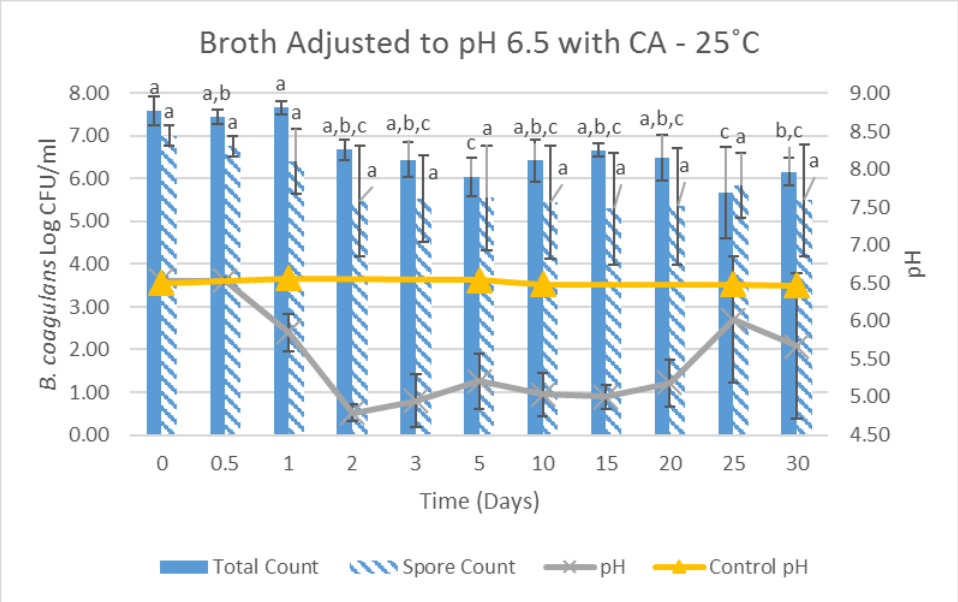

Figure 84. Stability of *Bacillus coagulans* Unique IS-2 in broth with pH adjusted to pH 6.0 with CA as denoted by total and spore counts. Stability tests were done during storage at 25˚C with the pH of the broth monitored throughout 30 days of storage.