ABSTRACT

CAULEY, SARAH MARIE. Survival of Commercially Available Lyophilized *Lactobacillus plantarum* and *Pediococcus acidilactici* Probiotic Cultures in Acidified, Refrigerated Cucumbers (Under the direction of Dr. Ilenys Pérez-Díaz and Dr. Suzanne Johanningsmeier).

The majority of probiotic food products on the market are dairy products. Some consumers are unable to digest lactose or choose for other reasons not to consume dairy products. Addition of probiotics to acidified cucumber pickles would offer these consumers an alternative that is also low-calorie. The aim of this study was to develop processes for the addition of commercially available, lyophilized probiotic cultures into refrigerated, acidified cucumber products that enable a high survival rate. The challenges to achieving a high survival rate in this system include low pH (3.7), presence of acetic acid (~120 mM) and sodium benzoate, low storage temperature, low nutrient content of cucumbers relative to dairy products, low osmolality of the cover brine, and desired shelf-life of 4 months. Methods tested for the incorporation of probiotic cultures into acidified, refrigerated cucumbers were 1) direct inoculation of the probiotics into the cover brine before addition to the cucumbers; 2) rehydration of cultures in cucumber juice (CJ) prior to inoculation of the acidified cucumber product; 3) elevating the temperature of the inoculated acidified cucumbers for a short period of time prior to refrigeration; 4) supplementing the brine with the cryoprotectant, trehalose; and 5) supplementing the brine with amino acids. Direct inoculation of 7 commercially available, lyophilized *L. plantarum* and *P. acidilactici* into the cover brine to achieve an initial cell count of 8.28 ± 0.65 $\log_{10}$ CFU mL^{-1} in the equilibrated, acidified cucumber product resulted in poor survival, 4.96 ± 1.28 $\log_{10}$ CFU mL^{-1} (average of the 7 strains) after 4 months. The probiotic strain, *L. plantarum* LP-115 was chosen for all further survival studies in acidified cucumbers or acidified cucumber juice (model system)

and was inoculated to 8.4 $\log_{10}$ CFU mL^{-1} unless otherwise stated. LP-115 was chosen because its efficacy as a probiotic has been the most studied. Rehydration of *L. plantarum* LP-115 in CJ incubated at 4 or 21 ºC improved survival of *L. plantarum* LP-115 in acidified cucumber compared to direct inoculation after 1 and 2 months of storage at 4 ºC ($p<0.05$). Elevating the temperature of the probiotic acidified cucumbers immediately after inoculation (8.4 $\log_{10}$ CFU mL^{-1}) for 1 day prior to refrigeration for 128 d resulted in survival of approximately 6.0 $\log_{10}$ CFU mL^{-1} compared to just over 2 $\log_{10}$ CFU mL^{-1} when directly inoculated into the brine. Incubation of *L. plantarum* LP-115 in the acidified cucumber juice model system at 14 ºC resulted in survival of 7.35 ± 0.11 $\log_{10}$ CFU mL^{-1} after 2 months, which was significantly higher than survival (2.00 $\log_{10}$ CFU mL^{-1}) in the samples incubated at 4 ºC ($p<0.05$). Trehalose supplementation (0.75%) showed no significant improvement in survival of LP-115, but supplementation of arginine (25 mM), a free amino acid mixture, and casamino acids into the acidified cucumber juice model system improved survival at 4ºC or 21ºC for 24 h prior to 4ºC. Supplementation of arginine (25 mM) in the acidified cucumbers had a greater positive impact on the survival of LP-115 when only incubated at 4ºC compared to those that were allowed to incubate at a growth-permitting temperature prior to refrigeration. The mechanism by which arginine improves survival was investigated by visualizing the surface of the cucumbers using scanning electron microscopy and looking for the presence of the arginine deiminase enzyme. The results of this study indicate that probiotics should be rehydrated in CJ prior to inoculation of the acidified cucumber product and the product could either be incubated at a growth-permitting temperature prior to refrigeration or supplemented with arginine to improve survival during refrigerated storage.

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Survival of Lyophilized Probiotic *Lactobacillus plantarum* and *Pediococcus acidilactici* in Acidified Cucumbers

by
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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Food Science

Raleigh, North Carolina
2017

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DEDICATION

I dedicate this thesis to my parents, Tom and Robin Cauley, for their never-ending and unconditional support. They are always there to listen and comfort me when I am down and there is no one else's advice that I trust more than theirs.

BIOGRAPHY

Sarah Cauley was born to Thomas and Robin Cauley on November 9, 1991 in Lutherville, MD and has a wonderfull sister named Katie. Sarah graduated from Dulaney High School where she was on the soccer, track, and lacrosse team. She then received her B.S. degree from the University of Maryland College Park in Food Science. While at UMD, Sarah worked as a lab assistant at the International Food Safety Training Laboratory. Upon graduation, she began an internship at McCormick and Company, Inc. until attending graduate school the following year at in the Department of Food, Bioprocessing and Nutrition Sciences at NCSU.

ACKNOWLEDGMENTS

I thank Dr. Pérez-Díaz and Dr. Johanningsmeier for co-advising me and giving me the opportunity to contribute to the probiotic pickle project. I have appreciated having two different perspectives throughout the process of designing experiments, performing experiments, and writing. I am also very thankful for the insight they have offered on where my strengths and weaknesses lie.

I am also thankful to Chicago Pickle Company for partially funding this project and to Mount Olive Pickle Company, especially Janet Turner and Caitlin Alberts, for providing me with the raw materials to perform my experiments.

I am so grateful to Janet Hayes for being a great friend, for teaching me much of the lab technique that I have today, and for always being there to brainstorm with me. I am also very grateful to Robert Price and Summer Conley-Payton for their technical support and friendship.

Thank you also to all of the people that were by my side throughout this process including my sister, Katie, my best friend, Hannah Strasser, Lauren Connelly, Andy Lee, Erin McMurtrie, Kat Daughtry, Kelsey Kanyuk, Dr. Kartheek Anekella, Kat Kay, and Benjamin Bowman.

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CHAPTER 1

Characteristics and Requirements of Probiotics Relevant to Survival in Refrigerated, Acidified Cucumber Pickles

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1.1 Production of a Probiotic Pickled Cucumber

1.1.1 Why a probiotic pickle?

Each year 60 to 70 million people are affected by digestive disease, increasing the demand for gastrointestinal health supplements. The increase in consumers' awareness of the interconnection between diet and overall health and disease prevention (Daliri and others, 2015), is fueling the growth in the functional ingredients market, including probiotics products (Granato and others, 2010). For example, the US market for spoonable, probiotic yogurt doubled between 2001 and 2006 reaching a market value of $294 million and probiotic/prebiotic supplements quadrupled between 2007 and 2012 (Freitas and others, 2014; Clarke and others, 2015). Globally, the probiotic market experienced an increase in value of $1 billion between 2007 and 2008 reaching $15.9 billion (Di Cerbo and others, 2015), growing to $34 billion in value in 2015 (BCC Research, 2016), and is predicted to reach $48 billion by 2017 (Johnson and others, 2014). The market value of probiotic foods and supplements is evidence to the fact that consumers are prioritizing functional foods. Consistent with market trends, a consumers' trends survey conducted in 2011 by the International Food Information Council (IFIC) showed that 96% of the 1,000 consumers surveyed in the USA, stated that food and nutrition plays a great or limited role in maintaining and improving overall health. Among these consumers, 81% of the population was aware that consumption of probiotic cultures supports a healthy digestive system (Edge and others, 2011). The evidence for therapeutic effects of probiotic cultures has prompted the need for increased scientific and regulatory efforts in order to understand their mechanism(s)

of action and to truthfully advertise products to the public. As documented by a search engine for scientific publications, PubMed, the number of publications listed when a search for "probiotic" increased over time with 2.5, 38 and 59.5% of the total number of scientific publications probiotics being published in the 90's, the first decade of the millennium, and the last 6 years, respectively. Probiotic dairy products have dominated the probiotic food market since the early 1900's when a product was introduced in Paris, based on Metchnikoff's formulation for yogurt, which exploited *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* (Molin, 2001). To date most of the probiotic products on the market are dairy products such as yogurt and kefir where strains of *L. paracasei* and *L. rhamnosus* are commonly found (Molin, 2001). Additional food products that have entered the probiotic market include cheese, ice cream, nutrition bars, chocolate, breakfast cereal, and infant formula (Granato and others, 2010; McFarland, 2015). Non-dairy food products that contain live lactic acid bacteria (LAB) include fermented vegetables such as cucumbers and cabbage; cereal based fermented products, and fermented meat products. Fermented plant products are commonly associated with *L. plantarum* (Molin, 2001). One example of a non-dairy probiotic was developed in Sweden in 1994 (Molin, 2001). This product consists of a fermented oatmeal gruel mixed into a fruit drink to deliver 10^9 colony forming units (CFUs) of *L. plantarum* 299v per 100 mL product for up to a 1-month shelf life. Yakult was first sold in Europe in 1996 and "Go" from Nestle and Actimel from Danone were introduced in 1999 (Di Cerbo and others, 2015).

Dairy products, mostly kefir and yogurt, still dominate the probiotic food market (Granato and others, 2010), which exclude consumers that have lactose intolerance and/or reservations in the consumption of dairy products for religious or personal reasons. The production of a probiotic vegetable could offer consumers a lower calorie, natural, non-dairy probiotic food product alternative. Similar to fermented dairy products, vegetable fermentations are mostly performed by lactic acid bacteria, the most relevant microbial group when discussing probiotic food products (Holzapfel and others, 2001). Although macronutrient content is lower than dairy products, vegetables contain vitamin C and B complex, provitamin A, phytosterols, dietary fiber, minerals, and phytochemicals, which can support health of both the probiotic cultures and the consumer (Swain and others, 2014).

1.1.2 Where do probiotic cultures come from?

In the early 1900s, Elie Metchnikoff observed that human ingestion of a strain of *Vibrio cholera* resulted in symptoms of infection in select patients (Bibel, 1988). In a study where animals were infected with *V. cholera*, he observed that a co-infection with *Bacilli* cultures eradicated the expected symptoms (Bibel, 1988), leading him to theorize that the natural intestinal microbiota affects the ability of a pathogen to grow and cause an infection. This new hypothesis was in line with Metchnikoff's previous research suggesting that symptoms of old age could be lessened or delayed by consumption of sour milk due to its *Lactobacilli* spp. content, which were already known to inhibit putrefactive microorganisms through lactic acid production. Since then, the term probiotic, meaning "for life" (Kechagia

and others, 2013), has been used to identify microorganisms ingested in foods or pharmaceutical supplements to maintain or improve health.

Strains of the genera *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, *Enterococcus*, *Streptococcus, Pediococcus, Leuconostoc, Bacillus,* and *Escherichia* have been most commonly studied for their health benefits (Holzapfel and others, 2001). The bacterial strains most commonly used as probiotic cultures are *Lactobacillus rhamnosus* GG, *Bifidobacterium animalis ssp* lactic, and *Lactobacillus reuteri*. Most microorganisms studied for probiotic properties were isolated from animals or humans, but there has also been research on microorganisms isolated from dairy products, dry sausages, fruits and vegetables, cereals and fish (Argyri and others, 2016). There are commercially available probiotic cultures for which studies have been performed. *Lactobacillus rhamnosus* (LGG®) (Chr. Hansen, Hoersholm, Danmark), isolated from the human intestine, has been used as a food and supplement ingredient since 1990 and has been included in over 260 clinical studies (Chr. Hansen, 2015). Danisco has performed research on their commercially available, lyophilized *Lactobacillus plantarum* LP-115 probiotic, including resistance to low pH and bile at levels relevant to survival in the gastrointestinal tract, adhesion to Caco-2 cells, inhibition of pathogens, and immunomodulatory effects in a human clinical study (Danisco, 2008). UAS Labs has also performed clinical studies on their probiotic products. For example, they showed that their Children's Formulation product reduces atopic dermatitis in preschool children in a randomized, double-blind, placebo-controlled clinical trial (Gerasimov and others, 2010).

1.1.3 Guidelines for the Production of Probiotic Food Products

The US Food and Drug Administration (FDA) has not defined the term probiotic (Hoffman and others, 2012) and there are currently no regulations for making the claim of "probiotic" on a food product in the USA (Sanders, 2008). However, experts have defined probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO and WHO, 2006). A report on the opinion of stakeholders and experts in the field on the regulation of probiotics was published in 2012 (Hoffman and others, 2012). Labeling of probiotics and communication of health benefits of probiotics to various audiences was discussed at the "Probiotic Foods and Supplements: The Science and Regulations of Labeling" Symposium in 2010 (Sanders and Levy, 2011). Currently, many probiotic products in the US are labeled with structure/function claims such as "helps naturally regulate the digestive tract" (Saldanha, 2008). Within a FAO Food and Nutrition Paper, "Probiotics in food, Health and nutritional properties and guidelines for evaluation", reports on the opinions of experts on the functional and safety aspects of food as well as guidelines developed by experts for the evaluation of probiotics in foods was published (FAO and WHO, 2006).

Many of the lactic acid bacteria (LAB) are considered "generally recognized as safe" (GRAS) and have the Qualified Presumption Safety (QPS) status due to the history of safe consumption (Stolaki and others, 2011). Thus, selected LAB can be added to food products without the FDA approval in the USA. Although a LAB may be GRAS, each probiotic culture should be investigated for its safety and efficacy as a probiotic before it is added to a

food or supplement for labeling as a probiotic product. The strain should be characterized phenotypically and genotypically (Sorokulova, 2008; FAO and WHO, 2006). Safety assessments should include an evaluation of its history of consumption and determination of antibiotic resistance and any pathogenic properties (Sorokulova, 2008; FAO and WHO, 2006). A detailed list of attributes pertaining to safety on which to evaluate potential probiotics was provided by the Joint FAO/WHO in 2002 (FAO and WHO, 2006, pg. 43), including looking for antibiotic resistance patters, undesirable metabolic activities, and evidence of side-effects in humans. The evaluation of a therapeutic effect should start with *in vitro* and animal studies, followed by human clinical studies. The FAO Food and Nutrition Paper (FAO and WHO, 2006) outlines the steps that should be taken to develop a probiotic food, including labeling requirements. *In vitro* studies commonly used to screen strains for probiotic affects include adhesion to intestinal cells, acid- and bile-resistance, and antimicrobial production (FAO and WHO, 2006). It is also important that clinical studies recruit enough animals or people for statistical significance (FAO and WHO, 2006). Enough subjects should be included in order to see a statistically significant effect if the null hypothesis is actually false. The ability to confer a health benefit at a particular cell concentration is specific to a strain and cannot be extrapolated to another strain of the same species (Sanders, 2008; McFarland, 2015). The FAO/WHO expert consultation suggests that a dairy product should not be considered probiotic if the dose is less than 10^8 viable CFU per day (Reid). However, the mechanism by which and the dose required for a strain to exert probiotic effects should be determined (FAO and WHO, 2006). The report published by

FAO and WHO (2006) contained examples of mechanisms by which a probiotic may benefit its host including competitive exclusion of pathogen binding and modulation of the immune system.

Because regulations specifically for probiotics are inexistent to date, they are regulated in a manner consistent with the type of product to which they are added (Hoffman and others, 2012). This could be a food, food additive, cosmetic, dietary supplement, or drug. The label of a probiotic product should identify the probiotic down to the strain, the number of viable bacteria at the end of shelf-life, the serving size required to confer the beneficial effect, the proper storage conditions, and the corporate contact details (FAO and WHO, 2006). Based on the opinion of experts, a probiotic that is well characterized and has been included in safety evaluation and clinical studies should be chosen for the development of a probiotic food product.

1.1.4 R*elevant physiological characteristics of probiotic LAB*

For the development of probiotic food products, it is important to understand the physiology of the probiotic strains and the state that the culture is in prior to addition to a product. Most commonly consumed and studied probiotics are LAB, which are a group of gram positive, non-spore-forming, microaerophilic, mesophilic microorganisms that are able to tolerate acid and traditionally are categorized as Lactobacillales (Felis and others, 2015; Sun and others, 2014). These microorganisms are commonly referred to as fastidious because they require preformed amino acids, purine and pyrimidine bases and B vitamins for growth (Li, 2005). Microorganisms in the LAB group do not have a complete electron

transport chain, nor do they have a citric acid cycle (Li, 2005). Those microorganisms that are considered LAB could be homofermentative, heterofementative, or facultative heterofermentative, depending on the strain. The sugars that can be used and the resulting end products will vary based on the strain and habitat of evolution.

LAB are a group of microorganisms that are relatively tolerant to organic acids and relatively low pH and therefore are able to dominate in cucumber fermentations by producing lactic acid and out-competing other bacteria that are not acid-tolerant. In a study by Pérez-Díaz et al (2011), commercial acidified cucumber products were adjusted to pH ~5.2 and inoculated to 5 $\log_{10}$ CFU mL^{-1} with *L. plantarum*, incubated at ambient temperature for 3-4 days until maximum cell density was achieved, and then stored at 4°C. The *L. plantarum* survived at levels higher than 7 $\log_{10}$ CFU mL^{-1} for over 4 months. Although this product contained the same level of acetic acid as a refrigerated, acidified (non-fermented) product, it differs in that the initial pH was 5.2 as opposed to ~3.8 and the product was incubated at an elevated temperature for 4 days before refrigeration. The elevation of pH and temperature promoted growth and metabolic activity. Although, the low pH and temperature of an acidified refrigerated pickle product does not promote metabolic activity in a refrigerated acidified pickle, their survival post fermentation indicates their capacity to survive long-term in the presence of high concentrations of organic acids and low pH (~3.8).

1.1.5 *Cucumber pickle production*

Pickles are defined as fermented or acidified vegetables covered with a solution (cover brine) that contains vinegar (acetic acid) as the major acidifying agent (Pérez-Díaz and others, 2013). Vegetables have been preserved by fermentation since before recorded history (Fleming and others, 1995). It is believed that cucumbers were first fermented in 2000 BC in the Middle East (Breidt and others, 2013). Fermentation allows for the preservation of vegetables without use of refrigeration via the production of acid by bacteria. Today, the pickled cucumber is the leading pickled vegetable in the United States, being purchased by the majority of households (Lucier and others, 2007). Non-fermented, acidified, refrigerated, and pasteurized products dominate the $2 billion US pickled cucumber market (Breidt and others, 2013). About 880, 530 tons of cucumbers were produced in the US in 2010, from which half a million were dedicated to the commercial production of pickles (Miller, 2013).

The products that fall into the category of pickled cucumber products in the US vary based on processing and flavor. Pickled cucumbers are either acidified or fermented and then pasteurized or refrigerated (Pérez-Díaz and others, 2013). In addition to acetic acid and sodium chloride, ingredients in finished products may include preservatives, sodium benzoate or potassium sorbate, calcium chloride, yellow no. 5, turmeric, and/or herbs and spices for flavor.

Methods of preserving cucumbers, other than fermentation, such as pasteurization and refrigeration emerged in the 19th century. Therefore, lactic acid fermentations as a

mechanism of preservation became less prevalent in the 20th century in industrialized countries (Molin, 2001). Pasteurization of fresh cucumber pickles began in the US in the 1940's, given that such a process allowed for more consistent quality and a milder acid flavor (Fleming and others, 1995). Refrigerated pickles were introduced into the US in the 1960's and are preserved by acetic acid, a chemical preservative, and refrigeration (Fleming and others, 1995). Refrigerated cucumbers are characterized as having a fresh cucumber aroma/flavor, due to the compounds, (E,Z)-2,6 nonadienal and (E)-2,6-nonanal (Palma-Harris and others, 2002). Although consumption of pickled cucumbers has been declining since 1976, when it reached a peak, consumption of refrigerated pickles is on the rise (Lucier and others, 2007). A USDA report suggests that this trend may be present because refrigerated pickles are crispy and crunchy and are minimally processed which may be perceived by consumers as indicators of a fresh product (Lucier and others, 2007). The succession of popularity among the various types of pickled cucumbers reflects changes in technology and consumer perspectives.

1.1.6 *Probiotic Candidates for Probiotic Pickles*

Development of a probiotic refrigerated, acidified cucumber product requires the probiotic cultures to survive at 4 °C at pH values between 3.7 and 4.0 in combination with organic acids, primarily acetic acid and sodium chloride salt. Survival of LAB during fermentation may be indicative of acid resistance, which is needed for long term survival in a probiotic pickle product. The species of bacteria found dominating cucumber fermentations are *L. plantarum*, *Lactobacillus brevis, Pediococcus cerevisiae, Lactobacillus pentosus,* and

Lactobacillus casei (Etchells and others, 1968; Zielinska and others, 2015). Also, *Pediococcus acidilactici* has been isolated from vegetables, fruits, and cereals and grow during spontaneous fermentations of foods including cucumbers, olives, and sauerkraut (Huys and others, 2011). Since *L. plantarum* and *P. acidilactici* are both commercially available as probiotics, these species have been identified as potential candidates for probiotic pickles.

Lactobacillus plantarum is a rod shaped, GRAS (Brinques and others, 2011), facultative heterofermentative, facultative anaerobe that is pseudocatalase-positive and can grow at temperatures of 7 to 40 °C (Pot and others, 2014; Russel and others, 1995) with optimum growth occurring at 30-35 °C (Sebranek, 2005). *Lactobacillus* species can grow in the pH range between 3 and 8 (Sun and others, 2014). *L. plantarum* can colonize a variety of matrices including dairy, meat, vegetables, and the human gastrointestinal tract (Kleerebezem and others, 2003; de Vries and others, 2006). This species has also been implicated in the spoilage of meat, wine, and orange juice (de Vries and others, 2006). It can grow in these environments by out-competing other microorganisms because of its unique ability to grow at a pH of as low as 3.1 and in the presence of up to 8% NaCl (Russel and others, 1995). The diversity of environments in which this species can thrive in is a reflection of its large genome and high number of genes, which encode for sugar metabolism and extracellular activities (Angelis and others, 2016; Kleerebezem and others, 2003). A portion of the genome of *L. plantarum* WCFS1 is known as the “lifestyle adaptation region” because it encodes genes related to adaptation that it might have been acquired from other

organisms (Kleerebezem and others, 2013). This diversity of catabolism genes that have been acquired through exposure to different environments and microorganisms may improve this species' ability to utilize the various nutrients present and to survive long term in a pickled cucumber product

Facultative heterofermentative bacteria such as *L. plantarum* can utilize sugar via the EMP or phosphoketolase pathway (Kleerebezem and others, 2003). From pyruvate, *L. plantarum* has the capability to produce a number of different products including lactate, formate, acetate, ethanol, acetoin, and 2,3-butanediol (Kleerebezem and others, 2003). The effect of these byproducts on the sensory properties of a potential probiotic food product should be considered.

Strains of *L. plantarum* isolated from various origins have demonstrated probiotic qualities. A strain of *L. plantarum*, LAP6, isolated from pickled cabbage demonstrated an antagonistic affect against *Salmonella enterica* serovar Typhimurium in mice, as well as resistance to acid and bile salts and the ability to bind to mouse intestinal epithelial cells (Chiu and others, 2007). Subjects consuming *L. plantarum* LP-115, which was isolated from plant material, showed increased IgG levels following cholera vaccination (Paineau and others, 2008). In a study of 42 healthy volunteers in Sweden, *L. plantarum* was found in 56% of the participants' rectal and/or oral mucosa (Ahrne and others, 1998). Two-thirds of these *L. plantarum* strains were able to adhere to HT-29 cells in a mannose-sensitive manner.

Pediococcus acidilactici is a cocci shaped, GRAS, homofermentative, facultative anaerobe or microaerophilic LAB that is commonly isolated from plants, vegetable

fermentations, fermented sausages, fermented beverages, meat, dairy products, animals, and humans (Franz and others, 2014; Tanasupawat el al., 1993). The phenotypic qualities of *Pediococcus* spp. indicate a common ancestry with *Lactobacillus* spp. (Franz and others, 2014). Growth can occur down to a pH of 3.6, at 20 to 50 °C with an optimum of 40 °C, and in the presence of up to 10 % NaCl (Fitzsimons and others, 1992; Tanasupawat and others, 1993; Franz and others, 2014). When grown in MRS broth, the final pH falls in the range of 3.5 to 3.8 (Franz and others, 2014). Some strains can hydrolyze arginine and all require calcium pantothenate, niacin, and biotin (Tanasupawat and others, 1993). This species is able to grow in spontaneous cucumber fermentations and coexist with other LAB (Huys and others, 2011).

P. acidilactici has been studied most frequently for its probiotic effect in animals including piglets, hens, tilapia, and chickens (Ghareeb and others, 2012; Mikulski and others, 2012; Standen and others, 2013; Brousseau and others, 2015). It has also been used for the fermentation of a variety of foods including vegetables, meats, and dairy products (Simpson and others, 2002). According to Barreau and others (2012), *Pediococcus acidilactici* MA8/5M is used in swine, poultry, and aquaculture feeds as well as in human dietary supplements. However, studies have been performed with human subjects as well. *P. acidilactici* has been shown to adhere *in vitro* to Caco-2 cells and survive gastrointestinal conditions, as well as survive *in vivo* in the human gastrointestinal tract for up to 2 hours after consumption (Balgir and others, 2013; Guerra and others, 2007). Another human study observed that *P. acidilactici* LN23 administered to humans reduced symptoms after

conventionally treated bacterial vaginosis and vulvovaginal candidiasis delivered by vaginal capsules (Ehrström and others, 2010).

LABs have evolved in nutritionally rich habitats, probably leading to their fastidious nature, and therefore have many transporters to uptake nutrients from the environment (Makarova and others, 2006). Since the divergence from the *Bacilli* ancestor, *L. plantarum* has lost 137 genes and gained 182 (Makarova and others, 2006). Their niches include plants, dairy, and the GI tract of animals with a single species being found naturally in more than one niche (Makarova and others, 2006). O'sullivan and others (2009) demonstrated that species of LAB, although may show high 16S *rRNA* sequence similarity (98%), occupy different niches, which is reflected in their genome. A portion of the genome of *L. plantarum* close to the origin of replication, referred to as the lifestyle-adaptation region, contains a high number of genes encoding sugar transport, metabolism, and regulation (Kleerebezem and others, 2002). The relatively low GC content of this region indicates acquisition of these genes from a foreign organism and so adaptation capabilities seem to have been enhanced over time by horizontal gene transfer and therefore, environment of evolution (Kleerebezem and others, 2002). This would also explain the occurrence of a single strain in different types of environments. Their ability to adapt to and function in a variety of environments, ability to convert sugar to lactic acid, relatively high acid resistance, GRAS status, and ability to colonize the gastrointestinal tract has resulted in their exploitation as probiotics in food products and supplements.

1.2 Challenges to the production of probiotic pickle products

Probiotics added to a pickled cucumber product at 4ºC are unlikely to grow or be capable of metabolic activity due to the growth inhibitory temperature as well as the low pH and composition of the cover brine. However, the majority of probiotic products on the market are refrigerated products and the majority of studies that investigate the long term survival of probiotics in food products, including fruit juices, do so at 4°C (Sheehan and others, 2007; Filannino and others, 2014; Charalampopoulos and others, 2010; Shilin and others, 2015; Mousavi and others, 2011). On the other hand, storage of the product at growth-promoting temperatures, which includes room temperature for LABs, would result in the natural succession of growth phases, ending with death phase and low cell counts during the typical product shelf life.

A survey of commercially available refrigerated, acidified cucumber spears shows that on average, acetic acid concentration ranges from 58 to 110 mM, benzoate from 522 to 1005 ppm, and NaCl from 2.33 to 3.31% (Pérez-Díaz and others, 2013). The cucumbers contain fructose, glucose, and malic acid, which probiotic cultures can use as energy. However, nutrient content of a cucumber is much lower than that of dairy products, where probiotics are commonly incorporated (USDA Nutrient Database, Granato and others, 2010). Additionally, probiotics are usually added to food products as lyophilized cultures because this allows for long-term and low-maintenance storage of the culture prior to inoculation of the product (Govender and others, 2014). However, incorporation of a lyophilized culture presents a challenge because lyophilized cultures are in a metabolically inactive and damaged

state. Also, a lack of knowledge on the treatment of the culture prior to lyophilization introduces an unknown factor in designing conditions for the survival of probiotics in food matrices. Another characteristic of cucumber pickle products is the low osmolarity of the brine. Rehydration of lyophilized cells in a low osmolarity medium can result in osmotic shock in that biomolecules could be lost through the damaged cell membrane (Champagne and others, 1991). Lastly, the variability of cucumber composition as well as the variability associated with biological systems demands the need for a robust technology that will overcome this inherent variation and yield a product that consistently contains sufficient levels of probiotics. Each of these challenges and the mechanisms by which probiotic cultures can overcome these challenges will be discussed in more detail in the following section.

1.3 Strategies by which probiotic LAB may achieve long term survival in a probiotic pickle product

As stated, growth of probiotics in refrigerated acidified pickles is not plausible, especially without adaptation, and so the cells will likely divert their energy towards maintenance and stress-defense/adaptation to the harsh environment (Nyström, 2004). In general, a bacterial cell already responding to a stress in a general and/or specific manner will survive better upon a change in environment than one not expressing stress response genes (Ryall and others, 2012; Beales and others, 2004)). Additionally, survival of the lyophilized probiotics in a new/stressful environment such as an acidified pickle product may be enhanced by rehydration in a more favorable environment and pre-adapting the culture prior

to inoculation in the harsher pickled cucumber environment. It is also known that bacteria in the stationary phase of growth express a general stress response due to the depletion of nutrients and accumulation of byproducts (Poddar and others, 2013). Additionally, slow growing cultures experience a general stress response not seen in fast growing cultures (Ryall and others, 2012). The proteins probiotics may produce in response to a stressful environment include chaperones, proteases, transport systems for osmolarity regulation, catalases, superoxide dismutases, proton pumps, and decarboxylases (Mills and others, 2011). To better equip the probiotics for survival in a harsh environment, they could be manipulated in such a way so that they are experiencing a stress response prior to inoculation.

1.3.1 *Potential Effects of Lyophilization on Subsequent Long-term Survival in Pickles*

Although the use of lyophilized cultures simplifies maintenance and storage for commercial scale food production, lyophilization is a stressful process involving freezing and dehydrating the cells. This can result in damage to the cell membrane, cell wall, and DNA, a decrease in membrane fluidity, increases in DNA strand breakage, transition of RNA and DNA into stable secondary structures, altered ribosome function, protein folding, and enzyme activity (Mills and others, 2011; Carvalho and others, 2004). The impact of lyophilization, on the physiological state of cells compromises the rehydrated culture's performance. For example, strains of lyophilized *Lactobacillus* upon rehydration were shown to have reduced ability to produce lactic acid, bacteriocins, and H_2O_2, and reduced adherence to vaginal epithelial cells (Zárate and others, 2006).

The viability of the cultures after freeze-drying depends on the drying medium, including the cryoprotectants used, rehydration medium, and growth stage and cell density of the culture when lyophilized (Prakash and others., 2013). Cellular damage due to lyophilization can often be reduced in the drying stage by the addition of cryoprotectants to the drying medium (Jalali and others, 2012; Champagne and others, 1991). Sugars and polymers increase the viscosity of the pre-lyophilization medium, which lowers the rate of degradation reactions, and replace water molecules in hydrogen bonds with biomolecules, preserving the cells (Fonseca and others, 2015). For example, cryoprotectants are thought to interact with the polar head groups of lipid membranes via hydrogen bonding, thus stabilizing the membrane (FAO, 2012). The nature of the rehydration medium is very important because the cultures will need to repair damages caused by the lyophilization process (Costa and others, 2000). Rehydration in a medium with inadequate osmolarity in combination with cell membrane damage could result in a loss of proteins and plasmids upon rehydration (Champagne and others, 1991). This is relevant for lactic acid bacteria because they are known to have evolved through horizontal gene transfer, acquiring plasmids that are important for growth (Makarova and others, 2006). Rehydration of the lyophilized cultures in the same medium and same volume as the drying medium is known to provide for the best recovery (Hutkins, 2006; Carvalho and others, 2004; Costa and others, 2000). Nutrient rich media should be utilized for the rehydration of cells, to promote a desired performance, if knowledge of the drying medium composition is lacking (Costa and others, 2000; Font De Valdez, 1985). This will provide the nutrients required for the cells to repair damages and

the osmolarity required to prevent the flux of solutes through the potentially damaged membrane. Thus, factors that should be manipulated when rehydrating probiotics with the goal of obtaining high long-term survival include nutrient content, osmolarity, and volume of the rehydration medium.

1.3.2 *Response of LAB Cells to Cold Temperature*

The temperature of storage of an acidified refrigerated cucumber pickle product falls outside the range of temperatures in which most LAB can grow. Although the growth characteristics of LAB vary by species and strain, depending on the environment, the optimal growth temperature can fall in the range of 20 to 45 °C (Wheater, 1998). Although growth at a suboptimal temperature is possible, the lower limit is dependent on the substrates (Russel and others, 2002). For example, on raw cucumbers, acid-forming bacteria did not grow at 16 °C after 6 days storage in up to 85% humidity, but did at 90-95% humidity (Etchells and others, 1973). This concept is similar to the hurdle technology, which is used in food products to prevent the growth of pathogens.

The effects that cold temperature has on a cell overlap with those caused by lyophilization. Cold temperature can cause irregular solute diffusion rates, decreased fluidity of the membrane, reductions in enzyme activity, protein denaturation and misfolding, macromolecule interactions, changes in DNA supercoiling, acceleration of the transition of DNA and RNA to stable secondary structures, and reduced transcription and translation rates (Maayer and others, 2014; Derzelle and others, 2000; Barria and others, 2013; D'Amico and others, 2006). The transition of DNA and RNA into stable secondary structures results in

decreased replication, transcription, degradation efficiency, and protein synthesis (Derzelle and others, 2000; Barria and others, 2013). A decreased ability to transcribe and translate means a decreased ability to adapt to an environment. A decrease in degradation efficiency means unnecessary machinery cannot be repurposed. "Modulation of gene expression has a central role in cellular adaptation to short-or long-term environmental changes, with extensive regulation occurring at both the transcriptional and post-transcriptional level" (Lopez-Maury and others, 2008). Even if metabolism at a temperature is possible, the transport of substrates could be inhibited due to reduced fluidity of the membrane, which negatively affects the functionality of membrane-bound proteins (Beales and others, 2004). Cells can compensate by increasing the portion of unsaturated fatty acids, decreasing the length of the fatty acids, and/or increasing the number of branched fatty acids in the membrane (Beales and others, 2004).

In general, cucumber fermentations are performed at temperatures between 15 and 32 °C (Harris, 1998). Li (2005) included a table of results from a study by Chong-Rak and others (1989) which investigated the microbial profile of kimchi fermented at 5°C, 15°C, and 25°C. In kimchi fermented at 5 °C, *L. plantarum* and *P. acidilactici* were both completely absent from the product. At 15 °C, *L. plantarum* and *P. acidilactici* comprised 15% and 1%, respectively, of the microorganisms found. At 25°C, *L. plantarum* and *P. acidilactici* comprised 36.5% and 0%, respectively. Although a cold temperature will prevent growth, a high survival rate may be achieved by allowing the cell culture to incubate at an elevated temperature prior to 4 °C incubation. The study by Pérez-Díaz and others (2011) showed

that *L. plantarum* can survive at a concentration of at least 7 log CFU mL^{-1} for 4 months at 4 °C in a refrigerated, acidified cucumber product after allowing growth of the cells from ~5 $\log_{10}$ CFU mL^{-1} to maximum cell density prior to refrigeration. Filannino and others (2014) also studied the survival of *L. plantarum* at 4 °C by subjecting *L. plantarum* strains isolated from a variety of environments to fermentation at 30°C for 24 h in fruit and vegetable juices and media followed by storage at 4 °C for 21 days. In this study, most of the strains also showed high survival rates with less than 1 $\log_{10}$ CFU mL^{-1} reduction after 21 days of storage. Based on studies discussed later in this review, this phenomenon could be due to the production of cold shock proteins as the cells are experiencing the temperature change and/or allowing adaptation to the environment at the elevated temperature, which cannot occur at a low, non-growth promoting temperature. Other studies that achieve a probiotic level of survival of LAB in a food product also do so by incubating the product at an elevated temperature prior to storage at 4°C (Charalampopolous and others, 2010; Shilin and others, 2015).

Microorganisms transferred from growth temperature to a lower, non-growth promoting temperature, experience a cold shock. This results in a temporary cease of growth and protein synthesis, with the exception of cold shock proteins (CSPs) (Barria and others, 2013). Once acclimated to this lower temperature, the cells may start to grow, but at a slower rate (Barria and others, 2013). CSPs can function as chaperones by binding to single stranded DNA and RNA to prevent transition to secondary structures (Derzelle and others, 2000; Wouters and others, 1999). CSPs can also recycle structured RNA (Barria and others,

2013). It has been demonstrated that proteins for transcription and translation are produced at higher rates upon cold shock (Russel and others, 2002). Disabling ribosomes using chloramphenicol or tetracycline results in increased levels of CSPs (Russel and others, 2002). Therefore, it could be theorized that trigger for CSP production is inadequate levels of transcription and translation, which occurs in response to cold temperature.

CSP mRNA is not only expressed at high levels upon cold shock, but also during particular stages of growth. A study by Derzelle and others (2000) examined this phenomenon in *L. plantarum*. The mRNA of *cspC* and *cspP* of *L. plantarum* is present at relatively high levels in early stages of growth, reducing ten-fold when entering into stationary phase (Derzelle and others, 2000). Levels of *cspL* mRNA are highest during exponential phase and slowly decline thereafter (Derzelle and others, 2000). In response to cold shock, an exponential phase *L. plantarum* culture synthesizes a greater concentration of *cspL* than does a stationary phase *L. plantarum* culture (Derzelle and others, 2000). Stationary phase cells are inherently more stress resistant than exponential phase cells regardless of the environment (Ryall and others, 2012) and therefore may not need to synthesize CSPs in order to resist cold temperature. In a study of the cold shock of exponentially growing *L. plantarum*, stability and/or expression of CSP mRNA was increased whereas 4 non-CSP mRNA molecules that were monitored declined in concentration after cold shock (Derzelle and others, 2000). *E. coli* and *B. subtilis* also have multiple CSPs that are expressed differentially during both normal growth and upon cold shock (Derzelle and others, 2000). The CSP mRNA of *E. coli*, *B. subtilis*, and *L. plantarum*

all have a 5'UTR that is unique to the CSP and when it is expressed, indicating that this may play a role in regulation of the mRNA stability (Derzelle and others, 2000). Knowledge of the mechanism by which bacteria cope with cold temperature will aid in the development of a process that will allow for high rates of survival of probiotics in a refrigerated pickled cucumber product.

1.3.3 *The Effect of Organic acids and low pH on Probiotic Cultures and Strategies to Compensate*

As already mentioned, on average, the pH of an acidified cucumber product is around 3.8 to 4.0 and the acetic acid concentration is in the range of 58 to 110 mM (Pérez-Díaz and others, 2013). Survival of LAB in acidic conditions varies between species and strains (Corcoran and others, 2005). The genus *Lactobacillus* can grow in the pH range of 3 to 8 (Pot and others, 2014) and *Pediococcus* between 4.5 and 7.5 (Franz and others, 2014). In a relatively low pH environment, weak acids will stay associated and are therefore able to diffuse through the bacterial membrane into the less acidic pH where they will dissociate into charged molecules that are not membrane-permeable and therefore trapped. The accumulated protons disrupt the proton motive force, disrupt pH-sensitive enzymes' activities, and can cause damage to proteins, membranes, and DNA (van de Guchte and others, 2002; Corcoran and others, 2008). For example, an acidic environment can affect transporter activity, reducing the ability of the cell to uptake nutrients (van de Guchte and others, 2002). Without the presence of a proton motive force across the cell membrane, the F_0F_1-ATPase cannot produce ATP. It can function to pump protons out of the cell, but

requires ATP to do so. The correlation between ATPase activity and acid-tolerance is supported by a study, which showed that ATPase concentration is greater in the membranes of acid-tolerant ruminal microorganisms and is active at a lower pH in acid-tolerant *Streptococcus* species than in acid-sensitive microorganisms (Cotter and others, 2003). Duary and others (2010) studied the expression of the *atpD* gene in *L. plantarum*, which encodes portions of the F1 complex of the F_1F_0-ATPase. This gene was up-regulated in 3 strains of *L. plantarum* that were inoculated in MRS broth, at pH 2.5, 3.5, and 4.5 to 10^9 CFU mL^{-1} and incubated at 37°C for 90 minutes. The degree of up-regulation seemed to correlate with the survival of the *L. plantarum* strains (Duary and others, 2010). Additional mechanisms employed for the purpose of de-acidifying the cytoplasm include amino acid decarboxylation and malolactic fermentation. Bacterial cells split their energy between maintenance and growth, using a greater portion of their energy for maintenance in a stressful environment such as that with a low pH and high concentration of organic acid. In a study by Vrancken and others (2011), *L. plantarum,* grown (30 °C) in a sourdough model system held at pH 3.5, 4.0, 4.5, 5.0, and 5.5, was analyzed for substrate utilization, gene expression, and growth. Many genes involved in carbohydrate degradation, including phosphotransferases, were expressed at lower levels in the low pH samples. On the other hand, genes for oligopeptide transport, the urea cycle, pyruvate oxidase, maltose phosphorylase, the subunits of the F0F1-ATPase, glutathione reductase, and those related to plantaricin biosynthesis were overexpressed. At low pH, there was an increase in poly(glycerol-phosphate)-alpha-glucosyltransferase, indicating an increase in cell wall

changes to incorporate more teichoic acid. It is unknown whether these same changes occur at 4°C. However, this study may be relevant to the metabolism of lactic acid bacteria that are incubated in media at a growth-promoting temperature prior to refrigeration such as those in the studies by Pérez-Díaz and others (2011) and Filannino and others (2014). Perhaps the metabolic activities observed in the *L. plantarum* strains studied by Vrancken and others (2011) are being utilized by the strains studied by Pérez-Díaz and others (2011) and Filannino and others (2014) while incubating at the growth-promoting temperature and these activities are improving survival once transferred to 4°C. Additionally, in the study by Filannino and others (2014) of the survival of *L. plantarum* in fruit and vegetable juices, malic acid was used during the initial 24 h incubation at 30°C and during storage at 4°C in inoculated cherry, pineapple, and carrot juice. Fermentation of malic acid allows for deacidification of the cytoplasm and contributes to the proton motive force, which can lead to ATP production (Fugelsang and others, 2007). The authors suggested that during fermentation and storage, the cultures in the acidic juices were devoting more energy to maintenance as opposed to survival. Knowing the mechanism by which LAB survive in a low pH environment is important in the development of a process or cover brine formulation to achieve high survival of probiotics in an acidic product.

Microorganisms will adjust their physiology when exposed to a sub-lethal stress that does not inhibit activities required for adaptation. Exposure of logarithmically growing LAB to a sub-lethal pH results in an adaptive response or acid tolerance response (ATR) resulting in an increase in acid tolerance (van de Guchte and others, 2002). Due to this phenomenon,

LABs survive better when exposed to a lethal pH if the cultures were previously exposed to a sub-lethal pH (van de Guchte and others, 2002). Acid tolerance is also increased as a result of a general stress response, which is induced in stationary phase, generally regardless of external pH (van de Guchte and others, 2002; Ryall and others, 2012). Increased expression of genes encoding F_1F_o-ATPase proton pumps, membrane proteins, and DNA and protein repair enzymes, among others will be increased as part of the ATR response (Bruno-Barcena and others, 2010). The ATR response has been shown to cross-protect the cell from other stresses including elevated temperature, oxidation and osmotic pressure (van de Guchte and others, 2002). For example, *Listeria monocytogenes* that has been acid adapted shows increased resistance to thermal stress, osmotic stress, crystal violet, and ethanol (O'Driscoll and others, 1996). Without acid-adaptation, stationary phase *Listeria monocytogenes* is resistant to pH 3.5, showing close to 100% survival after 60 minutes exposure, as compared to exponential phase cells, which survive at a rate less than 1% (O'Driscoll and others, 1996). Exponential phase *Listeria monocytogenes* that has been exposed to pH 5.5 for 1 h prior to pH 3.5 shows improved survival compared to a non-acid adapted culture (O'Driscoll and others, 1996). Ma and others (1997) showed that adaptation of *Streptococcus mutans* GS-5 to low pH not only requires exposure to a mildly low pH but such exposure has to happen in the presence of carbohydrates. *S. mutans* GS-5 did not increase acid resistance after incubation in phosphate buffer adjusted to pH 5.0, but did after incubation in growth media adjusted to pH 5.0 (Ma and others 1997). One explanation for these results is that metabolism must occur for adaptation to occur. Consistently, O'Driscoll and others (1996)

determined that acid adaptation requires protein synthesis since chloramphenicol, an antibiotic that inhibits proteins synthesis (Sigma, 1999), prevents acid adaptation. Further, when experiencing low pH, *Lactobacillus plantarum* IMDO 130201 showed increased expression of peptide and amino acid conversion genes (Vrancken and others, 2011), which may suggest that external proteins are required for adaptation to low pH.

Growing LAB generally ferment sugars to produce ATP, resulting in lactic acid and acetic acid, CO_2, and ethanol if they are heterofermenters. However, in response to acid stress, the metabolism will be shifted to produce metabolites that will improve acid tolerance rather than increase acid stress. For example, acid adaptation of *L. bulgaricus* involves altering pyruvate metabolism from acid production to pathways resulting in fatty acid biosynthesis, likely affecting membrane fluidity to improve acid tolerance (Fernandez and others, 2008). Additional pyruvate metabolism pathways that may be up-regulated rather than the lactate dehydrogenase pathway include those that lead to the synthesis of basic compounds, exopolysaccharides, and glycogen (De Angelis and others, 2016). In response to acid stress, LAB may also use citrate and malate as alternative carbon sources (De Angelis and others, 2016). LAB are capable of surviving in acidic environments if provided with the right nutrients and environmental conditions to promote adaptation.

1.3.4 *Impact of the Nutrient Content of Cucumbers and Potential Benefits of Nutrient Supplementation*

LAB are considered fastidious microbes in terms of the demands for nutrients that permit growth. LAB require nutrients to adapt to new environments and for long-term

survival. The nutrient density of a cucumber is low relative to dairy products where probiotic cultures are commonly incorporated. A comparison of nutrient content of a few yogurt types to raw cucumbers obtained from the USDA Nutrient Database shows that the energy content of yogurt is at least 4 times higher than that of a cucumber, protein content is at least 6 times higher, lipid content, in a nonfat yogurt, is higher by a factor of 10, and sugar content is higher in yogurt but varies widely. Although the nutrient content may be low compared to other foods, Stadnik (2015) suggests that raw and fermented vegetables are a good matrix for delivery of probiotics because of the availability of nutrients that enable microbial growth including prebiotics. Also, microorganisms can penetrate cucumber cell walls through tissue injury in order to access nutrients (Stadnik, 2015). An acidic environment containing a moderate amount of nutrients, such as pickles, may enable improved survival of probiotics in the gut, also harboring limited amounts of energy sources.

The nutrient content of cucumbers will vary based on the environmental conditions and agricultural practices, as well as the size of the cucumber (Lu and others, 2002). The glucose, fructose, and malic acid content of a cucumber can range from 0.8 to 1.16% (44.4 to 64.4 mM), 0.95 to 1.25% (52.7 to 69.4 mM), and 0.21 to 0.28% (15.7 to 20.9 mM), respectively (Lu and others, 2002). The microbiota of a plant or soil can affect the chemical composition of the fruit. For example, spraying a matrix of growth-promoting bacteria on tomatoes and cucumbers resulted in an increased content of physiologically important metals (Dursun and others, 2010). Use of nitrogen fertilizers for fruits and vegetables can result in

decreased levels of vitamin C and increased levels of carotene, vitamin B1, and NO_3 (Mozafar, 1993).

It is commonly theorized that LAB are fastidious in terms of nutrient requirements due to their evolution in nutrient rich environments including plants, dairy, and the animal GI tract. However, this exposure to diverse environments over time has resulted in their ability to metabolize a wide range of sugars. Kleerebezem and others (2002) reported that *L. plantarum* WCFS1 is capable of synthesizing most amino acids with the exception of the branched chain amino acids. However, the nutrient requirements vary between species and strains (Hayek & Ibrahim, 2013). Makarova and others (2006, table 8) compared the amino acid, purine and pyrimidine, fatty acid, and other biosynthetic capabilities of *Lactobacillales.* Capabilities vary with *L. plantarum*, *Leuconostoc mesenteroides, Lactococcus lactis* ssp. cremoris and lactis, and *Streptococcus thermophilus* having greater than 20 of the biosynthetic capabilities studied, with *L. brevis, L. johnsonii, L. gasseri* having less than 10, and with a few other species having intermediate levels of biosynthetic capabilities. Therefore, long term survival of LABs in food products may be enhanced by supplementation with nutrients. The protein content of a cucumber is very low compared to that found in yogurt. A comparison of the concentration of amino acids in raw cucumber with peel (11205) and 1% milk (01083) in the USDA Nutrient Database shows that amino acid content of milk is on average almost 10-fold higher than amino acid content of cucumber (Table 1.1). Amino acids are important for LAB to control intracellular pH, produce metabolic energy or redox power, and to resist stress (Fernandez and others, 2006).

Under low pH conditions, expression of genes related to peptide and amino acid metabolism is increased in *L. plantarum* IMDO 130201 (Vrancken and others, 2011). LAB have different systems for accumulating oligopeptides and for accumulating individual amino acids. In some circumstances, it is favorable to import oligopeptides as opposed to amino acids because of the energetics of transport (Konings, 2002). Proteolytic systems include cell wall-bound proteinases, which degrade extracellular protein into oligopeptides, peptide transporters that take up the oligopeptides, and intracellular peptidases that degrade peptides into shorter peptides and amino acids (Hayek and others, 2013). An advantage to transporting in oligopeptides is that any amino acids in excess after hydrolysis of the peptide can be pumped out of the cell through proton symport transporters, generating a PMF or can be converted to limiting amino acids or to metabolic intermediates. During growth in milk, *L. lactis* hydrolyzes proteins to oligopeptides using proteinase PrtP and preferentially transports oligopeptides, via the ABC transporter Opp, as a source of amino acids rather than free amino acids (Konings, 2002). Therefore, survival of probiotic cultures in a pickle product may be improved by supplementation with amino acids and/or peptides.

Supplementation of food products with amino acids is limited by 21CFR172.320. There are specifications for quality as well as qualifications for when they may be added, which are related to improving the protein content of the product. Arginine is approved for one specific use that does not fall into this category, which is as a component of a solution used as a processing aid for meat and poultry (GRAS Notice 317). Also, the effect of amino acid supplementation on the sensory attributes of the pickle would need to be investigated.

1.3.5 *Metabolism of Arginine, Glutamine, and Glutamate*

Catabolism of certain amino acids results in deacidification of the cytoplasm as well as restoration of the proton motive force. The pH of an acidified refrigerated cucumber product falls in the range of 3.7 to 4.0 (Pérez-Díaz and others, 2013). At this pH, the acetic acid will passively diffuse through the cell membrane and dissociate within the cytoplasm, lowering the cytoplasmic pH. This will limit the production of ATP by glycolysis due to the pH-dependent glycolysis enzymes (Cotter and others, 2003). It is therefore relevant to discuss mechanisms by which LAB deacidify their cytoplasm and produce ATP without further acidification as is the result of glycolysis. Catabolism of glutamine, glutamate, and arginine can deacidify the cytoplasm and that of arginine results in ATP production. The capability to utilize these pathways is strain specific, likely due to their niche of evolution. In a study of *L. plantarum* growth in sourdough at pH in the range of 3.5 to 5.5, growth in the lower pH environments utilized amino acid catabolism for survival rather than for processes characteristic of growth (Vrancken and others, 2011).

The arginine deiminase (ADI) pathway is found, strain-specifically in many LABs including *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Streptococcus*, and *Weisella* (Kowalczyk and others, 2016; Stadnik and others, 2015). The ADI pathway consists of 3 enzymes, arginine deiminase (ADI) or dihydrolase, ornithine transcarbamoylase (OTC), and carbamate kinase (CK). Also important is the transport protein, which imports, powered by the concentration gradient, arginine in exchange for ornithine, a byproduct of this pathway. Angelis and others (2002) summarized the purpose

of the ADI pathway in 3 activities, providing ATP when carbohydrates are in low concentration, providing carbamoyl phosphate for citrulline or pyrimidine biosynthesis, and protecting the cell against acid stress or starvation. The ADI pathway produces ATP and increases the pH of the cytoplasm/environment by consuming intracellular protons and producing ammonia (Arena and others, 1999). In line with its various purposes, it has been observed that the ADI pathway is regulated by catabolite repression in *Lactobacillus sakei*, possibly via the PTS-CcpA signal transduction system (Zúniga and others, 1998). Production of ATP from arginine catabolism does not result in formation of substrates that lower the pH. Therefore, arginine metabolism is an alternative to energy production, which could be used to power the F_0F_1-ATPase in a low pH environment. Under conditions when glycolysis is inhibited, if possible in the strain, the ADI pathway will be upregulated until the intracelullar pH is increased to a level that promotes activity of the glycolytic enzymes (Endo and others, 2014).

Liu and others (1998) observed the ADI pathway in heterofermentative lactobacilli from wine, but not homofermentative lactobacilli from this environment. Angelis and others (2002) isolated 70 strains of LAB from sourdough and concluded that only the heterofermenters displayed activity of all 3 enzymes. There was one of nine *L. plantarum* strains that showed ADI and OTC, but not CK activity. However, 2 strains of *L. plantarum* isolated from orange peel were found to utilize either arginine or citrulline via the ADI pathway (Arena and others, 1999). Also, regardless of a CK capability, ornithine is still formed, which results in production of ammonia, alkalizing the cytoplasm.

Homofermentative LAB found in other environments hydrolyzed arginine, including a *L. plantarum* strain isolated from fish and a *P. acidilactici* from fermented sausages (Liu and others, 1998). *L. brevis* isolated from fermented vegetables was also able to hydrolyze arginine (Fleming and others, 1985). The ability to utilize the ADI pathway is likely strain-specific and a result of the niche in which the strain evolved. Regulation of arginine can vary within the LABs. Liu and others (1998) reviewed various studies that show in some LAB, arginine is catabolized along with glucose, but not until after fructose was depleted. In another study, arginine catabolism of lactococci did not occur until glucose depletion, but did occur concurrently with metabolism of galactose (Liu and others, 1998). ADI pathway enzyme synthesis seems to depend on the sugars that are present in the media (Liu and others, 1998).

Glutamine catabolism results in deacidification of the cytoplasm through a process called glutamine deamidation. This involves import of glutamine, which is then converted to glutamate by a glutaminase, producing ammonium. Glutamate decarboxylase converts glutamate to GABA consuming one proton when the extra- and intra- cellular pH is less than 4.0 (Teixeira and others, 2014). Glutamine or glutamic acid is transported intracellularly in exchange for gamma-aminobutyrate (GABA), creating an electrogenic potential (Teixeira and others, 2014). This successive conversion to molecules that consume a proton upon formation and are then pumped out of the cell contributes to the proton motive force across the membrane and increases the pH of the cytoplasm.

Su and others, (2011) found that having glutamine deamidation and glutamate decarboxylation capability increases the competitiveness of *L. reuteri* in sourdough. Amino acid catabolism in the type of sourdoughs studied occurs after microbial growth has lowered the sourdough pH to 3.2-3.6 (Su and others, 2011). Glutamine is converted to glutamate via glutaminase or glutamine synthetase (Vermeulen and others, 2007). Glutamine synthetase requires ATP and is found in the genome of *L. plantarum* WCFS1 (Vermeulen and others, 2007). Glutaminase, however, does not require ATP (Vermeulen and others, 2007). Both enzymes are reversible in their conversion of glutamate and glutamine (Jeon and others, 2009). Sanchez and others (2007) observed that *Bifidobacterium longum* Biotype Longum overproduced glutamine synthetase at a low pH.

L. reuteri 100-23 has three glutaminase genes with one, *gls3*, being on an operon with *gadB*, which encodes glutamate decarboxylase, along with the putative genes for the glutamate/GABA antiporter. *L. reuteri* was harvested in early stationary phase and challenged at 37 ˚C in lactate buffer (pH 3.5, 24 h) or phosphate buffer (pH 2.5, 10 h) supplemented with 25 mM of arginine, glutamine, or glutamate. Glutamate and glutamine improved survival in the phosphate buffer (pH 2.5) and arginine improved survival in the lactate buffer (pH 3.5). This study shows that the ability to utilize these amino acids for survival depends on the environment and they are not all redundant.

Not only are there FDA regulations on amino acid supplementation restricting supplementation to certain scenarios, but also decarboxylation of certain amino acids results in the production of biogenic amines. Biogenic amines are organic, basic, nitrogenous

compounds that can cause food-borne illness (Spano and others, 2010). Although regulations limiting the concentration of biogenic amines in foods do not exist, it is known that accumulation in food products should be avoided (Stadnik and others, 2015; Spano and others, 2010). Conditions required for the accumulation of biogenic amines are the presence of precursors and decarboxylase-positive microorganisms and conditions that promote bacterial growth, decarboxylase synthesis, and decarboxylase activity (Stadnik and others, 2015). LAB can decarboxylate arginine, producing the biogenic amine, agmatine, which can then be catabolized further to form putrescine (Wunderlichová and others, 2014). Agmatine, among other biogenic amines, is a health concern when consumed in certain amounts. Ornithine, an intermediate of the ADI pathway, can be decarboxylated to the psychoactive amine, putrescine (Wunderlichová and others, 2014; Stadnik and others, 2015).

Supplementation of a pickled cucumber with arginine, glutamine, and glutamate might improve survival of probiotics. However, their addition to this product is currently not permitted and there may be sensorial changes as well as safety concerns involving production of biogenic amines.

1.4 Potential processes for the manufacture of probiotic pickles

Based on this literature review, changes to the typical process and/or formulation of refrigerated pickles should be assessed to determine the effect on long term survival of probiotic cultures in acidified cucumber pickles.

Potentially effective changes to the process include:

- Elevating the temperature of the product storage for a brief period prior to 4°C storage to allow metabolism to occur.
- Rehydrating the lyophilized probiotic cultures in a medium with a higher osmotic pressure and nutrient content than that of the brine, prior to transferring the cells to the brine

Changes to the formulation that may enhance survival include:

- Supplementation with amino acids or peptides
- Supplementation with cryoprotectants
- Addition of organic acid or strong acids to lower the pH of the rehydration media in order to acid-adapt probiotic cultures.

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Table 1.1 – Amino acid content of raw cucumber with peel and 1% milk according to the USDA Nutrient Database.

	Raw Cucumber	1% Milk	
	11205	1083	
	Concentration (g/100g)		Ratio of Value for 1% Milk / Value for Raw Cucumber
Tryptophan	0.005	0.049	9.80
Threonine	0.019	0.157	8.26
Isoleucine	0.021	0.211	10.0
Leucine	0.029	0.341	11.8
Lysine	0.029	0.276	9.52
Methionine	0.006	0.087	14.5
Cystine	0.004	0.032	8.00
Phenylalanine	0.019	0.168	8.84
Tyrosine	0.011	0.168	15.3
Valine	0.022	0.233	10.6
Arginine	0.044	0.126	2.86
Histidine	0.01	0.094	9.40
Alanine	0.024	0.12	5.00
Aspartic acid	0.041	0.264	6.44
Glutamic acid	0.196	0.729	3.72
Glycine	0.024	0.074	3.08
Proline	0.015	0.337	22.5
Serine	0.02	0.189	9.45
		Average	9.39
		Standard deviation	4.76

CHAPTER 2

Effect of Sodium Benzoate, Storage Temperature and Rehydration Matrix on the Survival of Lyophilized Probiotic *Lactobacillus plantarum* in Acidified, Refrigerated Cucumbers

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Short Title: Development of probiotic pickles

2.1 Abstract

Currently, dairy products dominate the probiotic food market. Addition of probiotic lactic acid bacteria to refrigerated acidified cucumbers would offer consumers a non-dairy, low calorie, health-promoting alternative. In this study, processes were developed to achieve a long-term, high rate of survival of commercially available, lyophilized, probiotic *Lactobacillus plantarum* and *Pediococcus acidilactici* cultures. Studies performed were based on the characteristics of this system that are likely to prevent survival such as the low temperature of incubation (4ºC), the presence of acetic acid and sodium benzoate (a preservative), and the relatively low osmolality of the brine. The effect of sodium benzoate concentration in the cover brine formulation on the survival of probiotics was investigated. The methods tested to improve survival of the probiotics included directly inoculating the lyophilized probiotics into the cover brine prior to pouring over the cucumbers, rehydration of lyophilized probiotic cultures in cucumber juice prior to inoculation of the pickled cucumbers, incubating the probiotic pickles at growth-permitting temperatures prior to refrigerated storage, and trehalose supplementation. Sodium benzoate (up to 9mM) had no negative effect on survival of the probiotics tested when incubated in the full strength brine for 2.5 h at 4ºC. However, direct addition of 7 lyophilized probiotic cultures into the cover brine resulted in cell death averaging 3.5 ± 1.2 $\log_{10}$ CFU mL^{-1} after 1 month at 4ºC. Rehydrating *L. plantarum* LP-115 in CJ prior to inoculation into the acidified cucumbers improved survival for 1 month with a cell count of ~4.5 $\log_{10}$ CFU mL^{-1} compared to just above 2 $\log_{10}$ CFU mL^{-1} when the lyophilized cells were added directly into the product.

Elevating the temperature (21°C) of the pickle product for at least 1 day prior to refrigerated storage improved the survival of *L. plantarum* LP-115 to about 6.0 $\log_{10}$ CFU mL^{-1}. The results of this study indicate that probiotic cultures should be incubated in CJ prior to inoculation into the acidified cucumber and could be incubated at a growth-promoting temperature for 1 day prior to refrigeration to further improve survival.

2.2 Introduction

Recently, consumers have shown interest in minimally processed, health promoting foods. The functional food market in North America and globally is on the rise. This includes probiotic cultures, which are commonly consumed via dairy products and as supplements. Probiotic cultures are defined as "live microorganisms which when administered in adequate amounts, confer a health benefit on the host" (Morelli and others, 2012). The market value of spoonable, probiotic yogurt in North America increased over two-fold between 2001 and 2006 (Freitas and others, 2014) and globally, this product accounted for 34.5% of total sales ($21.3 billion) of probiotic foods in the US in 2012. Consumption of probiotic cultures or prebiotics as supplements is also increasing in popularity in the United States with consumption increasing four-fold between 2007 and 2012 (Clarke and others, 2015). Factors contributing to the development of the probiotic market include the high rate of digestive issues in North America (Research and Markets, 2015), the aging population, the high cost of health care, and the growing understanding of the link between diet and well-being (Granato and others, 2010; Malla and others, 2013). Studies on consumer preference of probiotic delivery systems found that consumers prefer

delivery of probiotic cultures via food as opposed to as a supplement (Jacobs and others, 2014; Wan, 2015). These studies are supported by the fact that probiotic foods accounted for 92.4% of the $23.1 billion in probiotic (ingredients, supplements, and foods) sales globally (Hung and others, 2015). The global probiotic food market is dominated by dairy-based products, which are mostly sold in Europe (Saxelin, 2008). Incorporation of probiotic cultures into pickled cucumbers would expand the probiotic food market, by offering consumers a non-dairy, low calorie food delivery system.

Consumer preference for fresh, minimally processed food products has affected the pickled cucumber market. Although consumption of pickled cucumbers has been on the decline since its peak in 1985, refrigerated cucumbers, which are more crispy and perceived as fresher, are increasing in popularity (Lucier and others, 2007). Both fermented and acidified cucumber pickles can be found on the market with the former containing lactic acid that is produced by LAB (LAB) and the latter containing acetic acid, which is added to the product. Species of LAB that perform the fermentation include *Lactobacillus plantarum* and *Pediococcus* spp. (Etchells and others, 1968; Huys and others, 2011). These species can also be found in the human gut, raw plants, meat products, and as commercial probiotic cultures (Giraffa, 2014).

The aim of this study was to develop a probiotic, refrigerated cucumber pickle with minimal changes to the product formulation, sensory profile, and process flow. Therefore, the work in this study involved developing and testing methods that would allow for survival of commercially available, lyophilized, probiotic strains of *L. plantarum* and *P. acidilactici*

in an acidified, refrigerated, cucumber product. The challenges for a probiotic culture to survive in a refrigerated acidified cucumber product were the primary considerations in this study. Potential hurdles in the targeted product include a storage temperature of 4°C, use of sodium benzoate as a preservative, product acidification with acetic acid in the form of vinegar, acidic pH, and the low osmolalilty of the cover brine. On average, acidified, refrigerated (4°C) cucumber products have a pH in the range of 3.7 to 4.0 with an acetic acid, benzoic acid and NaCl concentrations ranging from 58 to 100 mM, 4 to 8 mM, and 2.33 to 3.31% (400 to 566 mM), respectively (Pérez-Díaz and others, 2013).

Pérez-Díaz and coworkers (2011) published a method that achieved survival of a *L. plantarum* starter culture (LA0445) to 7 $\log_{10}$ CFU mL^{-1} for 4 months at 4°C in a refrigerated, fermented cucumber product. This strain was isolated from a commercial fermented cucumber product at pH 3.3 (McDonald, 1993). The method described requires elevating the acidified cucumber product pH to 5.2 followed by inoculation to ~5 $\log_{10}$ CFU mL^{-1} and incubation at a growth-promoting temperature for 4 days prior to refrigerated incubation. However, there are consequences of this process that must be considered. This process would likely result in a product with a different sensory profile than an acidified, refrigerated product, the product must initially have a pH of 4.6, and its implementation would require modifications to processing facilities that only manufacture acidified, refrigerated cucumber products. Therefore, other potential processes are investigated in this study.

Lactic Acid Bacteria (LAB) are commonly studied for their probiotic potential *in vitro* and *in vivo*. In general, LAB grow at temperatures between 7 and 45°C, depending on the strain and environment (Pot and others, 2014; Wheater, 1955). Significant deviations from the optimal growth temperatures for *L. plantarum* (30-37 ˚C) results in decreased functionality, including alteration of membrane fluidity, reduced enzyme activity, protein denaturation and misfolding, macromolecule interactions, changes in DNA supercoiling, transition of DNA and RNA to stable secondary structures, and therefore, reduced transcription and translation rates (De Maayer and others, 2014; Derzelle and others, 2000; Barria and others, 2013; D'Amico and others, 2006). Therefore, the manufacturing and storage temperature of refrigerated pickled cucumber products was expected to have a significant impact on the long-term survival of the lyophilized probiotic culture.

Bacteria have a variety of mechanisms to cope with cold temperature including production of cold shock proteins (CSPs) and synthesis or accumulation of trehalose. Activating these mechanisms will require regulation and metabolic activity of the cells, which is unlikely to occur at the growth-inhibiting temperature of 4°C. In *L. plantarum*, CSPs are produced during growth and upon a transition from incubation at a growth-promoting temperature to a growth-inhibitting temperature (Barria and others, 2013; Derzelle and others, 2000). Trehalose acts as a cryoprotectant for *L. plantarum* improving survival during storage after freeze-drying and freezing (Gisela and others, 2014; Carvalho and others, 2002; Giulio and others, 2005). *Lactobacillus acidophilus* survival is also enhanced by trehalose if cells are exposed to freeze-thaw cycles (Duong and others, 2006). Survival of

mid-log phase (37 ˚C) *E. coli* MC4100 cells is greater upon cold shock if cells are incubated at 16 ˚C prior to cold shock because trehalose is produced during this 16°C incubation(Kandror and others, 2002). Although *L. plantarum* cannot synthesize trehalose, it is able to transport and regulate this compound (Duong and others, 2006).

Sodium benzoate is added to pickled cucumber products to prevent the growth of spoilage microorganisms and stabilize the product during transit (Pérez-Díaz and others, 2010). A concentration of 9 mM or higher of sodium benzoate inhibits the growth of LAB in acidified (pH 3.5) cucumber products (Pérez-Díaz and others, 2008). However, concentrations of 6 mM sodium benzoate or lower are permissive of a complete fermentation. Although, the legal limit for sodium benzoate in food products is 0.1 % or about 7 mM (21CFR184.1733), the exposure of the probiotics to the cover brine, which may contain up to 2-fold more sodium benzoate prior to equilibration with the cucumbers, may impact the survival of the probiotic cultures immediately after addition to the product.

Being a weak acid with a pka of 4.75, acetic acid will be mostly undissociated in an acidified cucumber product, which ranges in pH from 3.7 to 4.0 (Pérez-Díaz and others, 2013). In the undissociated form, the acid can diffuse through the cell membranes. The cellular internal pH required for metabolic activity is often higher than the product pH, enabling acetic acid to dissociate into ions that are not membrane-permeable. The accumulation of protons in the cytoplasm disrupts the proton motive force (PMF), enzyme activity, and protein, membrane, and DNA functionality (van de Guchte and others, 2002; Corcoran and others, 2008). Therefore, this study attempted to evaluate the effect of acid

adaptation prior to inoculation into the acidified cucumber product on long term survival of probiotics in the acidified cucumber product.

This study developed and assessed processes to manufacture an acidified, refrigerated cucumber product that would allow for a high rate of survival of commercially availably lyophilized probiotic cultures without the need for a full fermentation prior to refrigeration. Regulatory agencies and clinical studies indicate that many probiotic strains studied can confer therapeutic effects at 9 $\log_{10}$ CFU mL^{-1} per serving (Hill and others, 2014). A common serving size of a pickled cucumber product is 28 g. Based on these parameters, the concentration of probiotic cultures required in the cucumbers for the duration of the product shelf life is 7.5 $\log_{10}$ CFU mL^{-1}. Experiments performed include incubation of probiotics in cover brine containing various levels of the preservative, sodium benzoate, direct inoculation of lyophilized cultures into the cover brine, rehydration of the lyophilized probiotic cultures in acidified cucumber juice (~220 mM acetic acid) prior to inoculation of the acidified cucumber pickles, and incubation of the product at 21°C for 24 h prior to 4 °C long-term storage with and without trehalose supplementation. Details of the experiments performed in this study are summarized in Table 2.2.

2.3 Materials and Methods

2.3.1 Probiotic cultures and media preparation

Lyophilized probiotic cultures of *Lactobacillus plantarum* and *Pediococcus acidilactici* were acquired as samples from companies selling them commercially and were coded for reference throughout the document (Table 2.1). The concentration of the

lyophilized probiotic cultures was determined by plating on Lactobacilli MRS media (Fisher Scientific, Pittsburgh, PA, USA, 288130) using an Eddy Jet 2 automated spiral plater (IUL, Barcelona, Spain), after serially diluting in sterile saline solution (0.85%).

The cucumber juice (CJ) used for experimentation was prepared from size 2A (2.7 cm to 3.2 cm diameter; 15.9 cm long) pickling cucumbers received from a local processor, which were homogenized with a Warring 700s blender and frozen at -20°C in plastic bottles. The cucumber slurry was thawed and centrifuged at 9000 rpm (25°C) using an Eppendorf Centrifuge 5810R for 20 minutes in 50 mL aliquots to remove particulate. The supernatant was filter-sterilized using a 0.2 µm polyethersulfone filter (Sigma-Aldrich, St. Louis, MO, U.S.A.) and stored at -20 °C until use.

The cover brine was prepared to contain approximately 270 mM acetic acid added as vinegar, 1.1 M or 6.4 % NaCl (Fisher Scientific), 21 mM anhydrous $CaCl_2$ (received from a local pickled cucumber processer), and 9 mM sodium benzoate (Fisher Scientific). After equilibration of the cover brine with the cucumbers, solutes were diluted to approximately 122, 493, 9.5 and 4.1 mM acetic acid, NaCl, $CaCl_2$ and sodium benzoate, respectively. The cover brines used were filter-sterilized through 0.2 µm polyethersulfone filters (Fisher Scientific) prior to refrigeration.

Acidified cucumbers were prepared in 8 oz. jars by combining fresh cucumber slices and filter-sterilized cover brine using a 55:45 (w:v) pack out ratio (cucumbers to cover brine). The concentration of solutes in the product is therefore 2.2 times lower than the concentration in the cover brine. Cucumbers were washed with water and sliced to 1/8”

using an automated slicer (Hobart, Ohio, United States). Jars were closed with commercial metal lug caps, which were heated in boiling water for 15 s to soften the sealing compound and then immediately applied to the filled jars. The acidified CJ (model system) was prepared to equilibrate at a pH of 3.75 ± 0.1 by combining CJ and cover brine and supplementing with nominal amounts of 1 M HCl or 5N NaOH, as required to achieve the targeted pH.

2.3.2 Assessment of Survival as a Function of Time

Survival of probiotic cultures was assessed by plating samples from the cover brine or cucumber slices using an Eddy Jet 2 spiral plater (IUL, Barcelona, Spain) on Lactobacilli MRS media (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) supplemented with 10% cycloheximide (MRS-cy) after serial dilutions in 0.85% saline solution. MRS-cy plates were incubated anaerobically for 48 h at 30 °C. Colonies growing on MRS-cy plates were counted with a Flash & Go Automatic Colony Counter (IUL, Barcelona, Spain). The cucumbers were prepared for microbial analysis by blending into a slurry using Waring 700s blender (Waring, Stamford, CT, U.S.A.), followed by homogenization for 2 min at maximum speed using a stomacher (Seward^{TM}, Bohemia, NY) and Lab Blender Filter Bags (Seward). The filtrate was serially diluted in saline solution (0.85%) and plated on Lactobacilli MRS media (Benton, Dickinson and Company). The lower limit of detection is calculated by averaging 0 and the lowest concentration that could be measured, which is 2 $\log_{10}$ CFU mL^{-1}.

2.3.3 Measurement of fermentation metabolites by HPLC

Fermentation cover brine samples were collected aseptically and spun at 8,000 rpm for 10 min. in an Eppendorf Benchtop Refrigerated Centrifuge 5810R (Hamburg, Germany) to remove residual particulate matter. Cover brine supernatants were transferred to HPLC vials. Quantification of organic acid and sugar concentrations were measured by an HPLC method modified from that published by McFeeters and Barish (2003) using an Aminex HPX-87H resin column (300 x 7.8 mm, Bio-Rad Laboratories, Hercules, CA) at 65 ºC with 0.01N sulfuric acid at a flow rate of 0.9 mL/min. A Shimadzu Prominence HPLC UV-Vis detector (SPD-20A, Columbia, MD, USA) set to collect data at a wavelength of 210 nm was used to detect malic, lactic, succinic, propionic, and butyric acids. A Shimadzu Refractive index detector (RI-10A) was used to measure glucose, fructose, and acetic acid. External standardization of the detectors was done using at least 5 concentrations of the standard compounds.

2.3.4 Direct Inoculation of Probiotic Cultures in Jars of Acidified Cucumber Chips

Probiotic cultures were inoculated into cover brine and mixed by shaking and inverting immediately before pouring into 8 oz. jars already packed with the correct mass of cucumber chips. The cover brine in this experiment did not contain NaCl. Day 0 counts were calculated based on the concentration of the stock lyophilized culture (Table 2.1) and the amount of probiotic lyophilized cultures in grams added to the cover brine. Two jars per strain (technical replicates) for each time point as well as 2 uninoculated jars for each time point were prepared. Jars were sacrificed for microbial analysis on days 4, 8, 32, and 128.

Cucumbers were processed according to section 2.3.2 and the slurry and cover brine samples were frozen at -20°C. Cell counts were measured from both the brine and cucumber samples of each jar.

2.3.5 Effect of Sodium Benzoate on Survival of Probiotic Cultures in the Cover Brine

The equilibration rate of sodium benzoate in acidified cucumbers was determined by measuring its concentration in aliquots of cover brine as a function of time for up to 6 h. The lids of the jars were equipped with a rubber septum in the center, so that cover brine samples could be collected with a syringe and needle assembly (BD, New Jersey, USA).

Three probiotic cultures, two *L. plantarum* strains (LP-A and LP-C) and one *P. acidilactici* (PA-B) were prepared as inocula in filter-sterilized (PES filter) CJ to approximately 11.7 (strain LP-A), 11.8 (strain PA-B), and 10.3 (strain LP-C) $\log_{10}$ CFU mL^{-1}, respectively. The CJ inocula were incubated at 4°C for 1 h prior to inoculation. Aliquots of the CJ inocula were transferred to cover brine in order to assess the effect on survival of the exposure to the cover brine that may be experienced prior to equilibration with cucumbers. There were 5 levels of the sodium benzoate treatment, four different cover brines containing 0, 3, 6, or 9 mM sodium benzoate and a 0.85% saline control. The cover brines and saline solution were filter-sterilized with a 0.2 µm PES membrane Corning™ Disposable Sterile Bottle-Top Filter (Fisher Scientific). Aliquots of the refrigerated CJ inocula were transferred to the cover brines to 9.0 (LP-A), 9.1 (PA-B), and 8.3 (LP-C) $\log_{10}$ CFU mL^{-1}, respectively, followed by incubation at 4°C for 2.5 h. Survival of the probiotic cultures was assessed by enumeration of CFUs on Lactobacilli MRS medium.

The experiment was run in duplicate and the data was analyzed using JMP. The data was modeled using ANOVA with cell count (log_{10} CFU mL^{-1}) after the 2.5 h incubation as the response variable and probiotic strain, treatment (4 levels of sodium benzoate and the saline control), and the interaction of strain and treatment as the effects. Significant differences between treatments were determined using the LSMeans Differences Tukey HSD test.

2.3.6 Effect of Cucumber Juice Adaptation on Survival of the Probiotic Cultures in Acidified Cucumbers

L. plantarum was inoculated to 10.8 ± 0.08 log_{10} CFU mL^{-1} into CJ or CJ adjusted to pH 4.0 using vinegar (20% acetic acid) and incubated for either 2 or 18 hours at 4 °C (4 treatment combinations) prior to inoculation of acidified cucumbers. The CJ inocula was added to jars at a volume needed to achieve an initial cell count of approximately 8.4 log_{10} CFU mL^{-1} in acidified cucumber jars. Uninoculated CJ was used to inoculate placebo jars for every treatment. Jars were then stored at 4°C and sampled on day 32 for microbial analysis. This experiment was performed again with a different lot of cucumbers and the CJ rehydration media was supplemented with 0.06% sorbic acid to proactively address food safety concerns that may arise from the implementation of the process at the commercial scale. For the purpose of statistical analysis these two experiments were treated as replicates based on the assumption that sorbic acid has no effect on lactic acid bacteria survival at that level (Costilow and others, 1955; Zoecklein and others, 1995). Within each replicate, all treatments were performed in duplicate.

Data was analyzed in JMP using the ANOVA model with sorbic acid (Sorbic), acetic acid (Acetic), time of incubation in the cucumber juice (Time), Sorbic*Acetic, Sorbic*Time, and Acetic*Time] as effects and cell count in $\log_{10}$ CFU ml^{-1} as the response variable. The data point for each replicate is an average of the technical replicates.

2.3.7 Effect of Temperature Modification and Trehalose Supplementation on Survival of the Probiotic Culture in a Cucumber Juice Model System

The effect of trehalose supplementation and temperature modification on survival of the probiotic cultures was assessed in a model system, which consists of cover brine and filter-sterilized CJ as opposed to cover brine and sliced cucumbers. Trehalose dehydrate (US Biological) was dissolved in the cover brine at 0.75% prior to mixing with the CJ at a ratio of 55:45 (CJ to cover brine, v/v). The probiotic cultures were pre-adapted in CJ for 18 h at 4 °C and then aliquots of this CJ inoculum were transferred to the CJ model system to 8.4 $\log_{10}$ CFU mL^{-1}. The refrigerated CJ model system samples with and without trehalose were subjected to 5 temperature profile treatments (table 2.3) for a total of 10 treatment combinations. An uninoculated control was run for every inoculated sample. Jars of the experimental and control treatments were sacrificed on every time point. Samples were obtained on days 16, 32, and 64. Cell counts were enumerated on Lactobacilli MRS-cy plates as described above and samples frozen at -20 °C for HPLC analysis of organic acids and sugars at a later time. There were three independent replicates for treatments 21-4°C, 21-14-4°C, 14°C, and 4°C on days 16 and 32 and 2 independent replicates on day 64. Data was analyzed in JMP using the ANOVA model. The effects in the model were Trehalose,

Temperature profile, Incubation time, Trehalose*Temperature profile, Temperature*Incubation time, Trehalose*Incubation time, and Replicate and the cell concentration on day 16, 32, and 64 in $\log_{10}$ CFU mL^{-1} was used as the response. The details of the significant effects were analyzed using an LSMeans Differences Tukey HSD test.

2.3.8 Effect of Temperature Modification, and CJ Rehydration on Long-term Survival in Acidified Cucumbers

The treatments chosen for this experiment are based on experimental results indicating probiotic rehydration in CJ prior to inoculation (Figure 2.4) and incubation of the probiotic at an elevated temperature (Figure 2.5) improve long-term survival of LP-A. The objective is to compare survival among potential processes to determine the most effective methods. The direct inoculation method, described above, was performed as a control, representing an unacceptable survival rate. Combinations of the incubation temperature (4°C or 21°C) of rehydration in acidified CJ (~220 mM acetic acid), three temperature profiles in the acidified cucumbers, and two inoculation levels (5.8 or 8.4 $\log_{10}$ CFU mL^{-1}) of the acidified cucumber on long term survival of probiotic cultures in acidified cucumbers were tested (Table 2.5). Temperature profiles of acidified cucumber jars include 4°C, 21°C for 24 h followed by 4°C, and 21°C for 5 d followed by 4C. All treatments are inoculated to 8.4 $\log_{10}$ CFU mL^{-1} (high inoculation level) and adjusted to pH 3.75 (high pH) other than one of the 2 treatments incubated for 5 d at 21°C prior to 4°C, which was inoculated to 5.8 $\log_{10}$ CFU mL^{-1} (low inoculation level) and adjusted to pH 4.6 (high pH) with 10 N NaOH. This

5-day incubation at 21°C prior to 4°C incubation and adjustment of pH to 4.6 is based off a study published by Pérez-Díaz and others (2011).

The lids of the jars were equipped with rubber septa in order to allow sampling of the cover brines without opening the jars. Samples of cover brine were taken at 24 h for all treatments and on day 5 for treatments incubated at 21°C for 5 d prior to 4°C. These samples were enumerated on Lactobacilli MRS-cy media and frozen at -20 ˚C for chemical analysis at a future date. Cover brine samples were also obtained from all treatments on day 16 for enumeration of LAB and chemical analyses. Jars were sacrificed to obtain lactobacilli cell concentrations within the cucumber chips and cover brine separately on days 32, 64, and 128. Samples of the cucumber slurry and cover brine were frozen at -20 °C for further analyses.

Three replicates of this experiment were performed using a different lot of 2A (2.7 cm to 3.2 cm diameter; 15.9 cm long) cucumbers for each replicate. Only 2 replicates will be analyzed and reported because they were modified from the first replicate. However, HPLC analysis of fermentation substrates and products was performed on the CJ inocula from the first replicate. In the first replicate, the CJ inocula was not acidified for every treatment, unlike the second two replicates. Between all replicates, there were 3 CJ adaptation treatments; CJ1) pH unmodified, 18 h, 4°C; CJ2) pH 4.0 with acetic acid, 18 h, 4°C; CJ3) pH 4.0 with acetic acid, 24 h, 21°C. The acidified CJ adaptations were supplemented with 122 mM acetic acid and adjusted to pH 4.0 with nominal volumes of HCl or NaOH as needed.

Survival of probiotic cultures in this system was modeled using ANOVA in JMP using cell counts from samples taken on day 32, 64, and 128. The dataset included all data

from replicates 2 and 3 and treatments H/L/21/4 and H/L/None/4 (Table 2.5) from replicate 1. Survival in $\log_{10}$ CFU mL^{-1} was modeled by the full factorial of Treatment, Component (cucumber or brine), and Date of analysis.

2.4 Results

The direct inoculation of commercially available probiotic cultures in cover brine prior to pouring over cucumbers resulted in the reduction of cell densities to less than 7 $\log_{10}$ of CFU g^{-1} of cucumber after 4 days of 4°C storage (Figure 2.1). Probiotic cell count reduction was variable between cultures. The proportion of the cell concentration of cucumber to brine was also observed to be variable between the strains (data not shown). By day 4, cell counts had declined by at least 2 $\log_{10}$ CFU mL^{-1} and up to greater than 5 $\log_{10}$ CFU mL^{-1} (Figure 2.1). For probiotic cultures PA-B, LP-C, LP-D, and LP-G, cell counts were relatively stable from day 4 until day 128, whereas viable cell counts continued to decline for probiotic cultures LP-A, LP- E, and PA-F. Survival rates were estimated at between 0.01 and 0.1% for PA-B, LP-C, and LP-D and <0.001 for LP-A, LP-E, PA-F, and LP-G after 128 days.

Overall, there was no effect of increasing sodium benzoate concentration in the cover brine on survival of LP-A, PA-B, and LP-C after incubation for 2.5 h at 4°C. In the ANOVA which models the cell counts of all strains together using the effects of Treatment (Sodium benzoate) ($p=0.0006$), Strain ($p=0.0091$), Treatment*Strain ($p=0.0051$), and Initial cell count ($p=0.9277$), all effects are significant other than initial cell count. In the reduced model (Figure A.1) only including Treatment ($p=0.0003$), Strain ($p<0.0001$), and Treatment*Strain

(p=0.0031), survival of strain C in the cover brines is significantly lower than in the saline control. There were no significant differences between the cell counts of all other treatment and strain combinations. However, in this model, the Residual by Predicted Plot (Figure A.1C) indicates the assumption of homogeneity of variance is violated. Consistently, it appears that the error associated with the cell counts between replicates is greater for strain LP-C than the other strains.

Therefore, the survival was also modeled separately for each strain (Figure 2.3 and A.2). In the ANOVA of LP-A (R^2=0.88), LP-B (R^2=0.89), and LP-C (R^2=0.84), the effect of sodium benzoate is significant with p-values of 0.0176, 0.0124, and 0.0306, respectively. Significant differences between sodium benzoate concentrations and the control were determined by performing a LSMeans Differences Tukey HSD in JMP (Figure 2.3). The LSMeans Differences Tukey HSD test of Treatment for LP-A shows that there is no significant difference between the saline control, and cover brines with 3 and 6 mM sodium benzoate. However, the cover brines with 0 and 9 mM resulted in significantly lower survival than the saline control. The LSMeans Differences Tukey HSD test for LP-B indicates that survival in the cover brine treatments with 3, 6, or 9 mM sodium benzoate are not significantly different from that in the saline control. However, survival in the cover brine with 0 mM sodium benzoate is significantly lower. The LSMeans Differences Tukey HSD test for LP-C indicates that survival in the cover brines with 0 and 3 mM sodium benzoate resulted in significantly lower survival rates of LP-A than the saline control. There

is no evidence that increasing the concentration of sodium benzoate affects survival of the probiotics tested.

This study demonstrates that the probiotic cultures will not experience any significant cell count reduction due to the sodium benzoate when they are inoculated into the cover brine and before the sodium benzoate equilibrates between the cucumber and cover brine. Although there is no effect of sodium benzoate, it is obvious that strain LP-C is less resistant to the cover brine. Also, the survival of LP-A and LP-B in the cover brine with 0 mM sodium benzoate was statistically significantly lower than survival in the saline control.

The effect of rehydrating the lyophilized probiotic LP-A in cucumber juice with or without acetic acid incubated for 2 or 18 h on the survival of the cells in acidified cucumber pickles after a 1-month 4°C incubation was assessed. There was no statistically significant effect of Sorbic acid, Acetic acid, Time of incubation (2 or 18 h), or any of the two-way interactions on the survival of the probiotic cultures after incubation in the acidified cucumbers for 1 month (Figure A.5). Reducing the model by eliminating the Time*pH and Sorbic*Time interaction terms results in an increase in significance of Sorbic ($p=0.0540$) and Sorbic*pH ($p=0.0563$) effects with an R^2 of 0.89 (Figure A.4). On average, after 32 days of incubation in the acidified cucumbers, the probiotic counts were reduced from 8.4 $\log_{10}$ CFU mL^{-1} to 4.9 ± 0.5 $\log_{10}$ CFU mL^{-1} or a survival rate of 0.0316%. Survival rate of LP-A as a result of direct inoculation in the full-strength brine prior to mixing with the cucumber chips was 0.000279% on day 32.

In the study on the effect of temperature modification and trehalose supplementation on survival of the probiotic cultures in a CJ model system, the null hypothesis that trehalose has no effect on survival of LP-A during long-term storage could not be disproved and that incubation of the acidified CJ model system at an elevated temperature prior to refrigeration statistically significantly enhances long-term survival (Figure 2.5).

In the ANOVA model (Figure A.5) that included the full factorial of the effects, Storage time, Temperature Profile, and Trehalose, the effect of Storage time ($p<0.0001$), Temperature Profile ($P<0.0001$) and Storage time*Temperature Profile ($p<0.0001$) were significant. A LSMeans Differences Tukey HSD test was run on the Storage time*Temperature interaction term (Table 2.3). The cell count as a result of the 21-14°C and 14°C temperature profile did not significantly decrease compared to day 0 (8.41 $\log_{10}$ CFU mL^{-1}) until day 64 (7.04 $\log_{10}$ CFU mL^{-1}) and 32 (7.59 $\log_{10}$ CFU mL^{-1}), respectively. Samples subjected to the 21-14-4°C and 21- 4°C temperature profiles resulted in significantly lower cell counts of 7.45 $\log_{10}$ CFU mL^{-1} and 7.08 $\log_{10}$ CFU mL^{-1}, repectively, by day 32 compared to day 0. By day 64, cell counts in samples incubated at 14°C for all or most of the shelf life (21-14°C and 14°C) were significantly higher than in treatments that were incubated at 4°C after incubation at a growth-permitting temperature (21-14-4°C and 21-4°C). The samples only incubated at 4°C resulted in cell counts significantly lower than at time 0 by day 16 and the counts in samples incubated at this temperature were significantly lower than any other treatment at any time point (other than 0).

The data was also analyzed in SAS using code that can be found in Appendix A, section *Comparison of two different models in SAS*. In addition to analyzing the data as an ANOVA in the same way as was done in JMP, the survival was modeled allowing dependence of cell count on time to vary across temperature. This was done by comparing slopes of the cell count as a function of time for each temperature profile (R^2=0.93). Based on this analysis, all temperature profile levels are significantly different from one another ($p<0.007$) except treatments 14°C and 21-14°C.

HPLC analysis was performed on day 64 to assess metabolic activity based on treatment (Table 2.4). It appears that the probiotic cultures that are allowed to incubate temporarily at an elevated temperature prior to refrigeration (21-14-4°C and 21-4°C) metabolized a little over half of the malic acid to produce lactic acid. The probiotic cultures that were incubated at 14°C utilized almost all malic acid and some glucose and fructose and produced lactic acid. HPLC analysis of the 4°C treatments showed no reduction in glucose, fructose, or malic acid and no lactic acid production.

The study of the effect of temperature profile in the acidified cucumbers and CJ rehydration on long-term survival of the probiotic LP-A in acidified cucumbers demonstrated that survival is significantly affected by rehydration of the lyophilized probiotics in CJ and temperature profile (temperature of the CJ rehydration and incubation temperature of the acidified cucumbers) (Figure A.6). By day 32, cell counts in the cucumber portion of the treatment where the probiotic cells are not rehydrated in CJ (treatment H/L/None/4) are significantly lower than in any other treatment (Table 2.5). The treatment where probiotic

cells are rehydrated in CJ and incubated in acidified cucumbers at 4 ºC only (treatment H/L/4/4) shows significantly lower survival than probiotics in the samples incubated in the acidified cucumber product at 21ºC for 1 day or in the samples inoculated to 5.8 $\log_{10}$ CFU mL^{-1}, adjusted to pH 4.6, and incubated in the acidified cucumber product at 21ºC for 5 days prior to refrigeration. These last two treatments resulted in the highest cell count by day 64. The differences seen on day 64 are the same as on day 128. For at least 32 days, the treatment where the probiotics are rehydrated in the CJ at 21°C for 24 h prior to inoculation into the acidified cucumbers, which are incubated at 4°C only, (treatment H/L/21/4) results in better survival than the treatments where the probiotics are never exposed to the elevated temperature (treatments H/L/None/4 and H/L/4/4). However, the survival in treatment H/L/21/4 is not as effective as treatments where cultures are incubated at 21°C in the acidified cucumber product (treatments H/L/4/21(1)-4 and L/H/4/21(5)-4). In summary, steps that improve the survival of probiotics, in order of ascending efficacy are rehydration in 4°C CJ, rehydration in 21°C CJ, incubation in the acidified cucumbers at 21°C, and incubation in the acidified cucumbers at 4°C.

HPLC analysis of the CJ rehydrations was performed (Figure 2.8). CJ inoculum 1 was utilized in a replicate that was not repeated and so was not discussed in the survival results. The LP-A rehydrated in CJ1 (unmodified CJ, 18 h, 4°C) utilized glucose, fructose, and malic acid and produced lactic acid. When rehydrated in CJ2 (pH 4.0, 18 h, 4°C), the probiotic appears to be using the fructose, but not glucose or malic acid, and producing lactic acids. LP-A rehydrated in CJ3 (pH 4.0, 24 h, 21°C) used all of the fructose and malic acid,

but again no glucose, and produced lactic acid. Acetic acid concentration in CJ2 and CJ3 did not appear to change over time (data not shown).

2.5 Discussion

The direct inoculation method represents the most practical approach to produce a probiotic pickle product. However, the data presented here indicates this process results in poor survival (Figure 2.1). The probiotic culture PA-B survived best with a cell concentration of ~6.4 $\log_{10}$ CFU mL^{-1} in the cucumber after ~1 mo. of refrigerated storage (day 32) (Figure 2.1). Based on the survival rate of LP-B, it is predicted that this process would require increasing the initial concentration by 100-fold to approximately 11 $\log_{10}$ CFU mL^{-1}, which is characterized by significant cloudiness and viscosity that would be difficult to manage in a production setting and unlikely to be acceptable to consumers. Therefore, manipulation of the formula and process were tested in further experimentation in order to improve long-term survival.

Processes developed and tested were designed based on the various stressors in the acidified cucumber system in order to achieve survival of probiotics to 10^9 CFU per serving or 7.5 $\log_{10}$ CFU g^{-1} cucumber. Such sources of stress include the preservative, sodium benzoate, and acetic acid in the cover brine, incubation of the product at a temperature that precludes microbial growth (4°C).

Although, the use of lyophilized cultures is one of the most practical ways to manipulate live cells in a commercial setting, this process intrinsically compromises the physiology of viable cells, affecting their ability to survive and grow in a new environment

(Beales and others, 2004). The growth rate and phase, stress exposure, population size and culture density, and phenotypic and genotypic heterogeneity within a bacterial population prior to lyophilizing are only some of the pre-existing stress factors that could impact the performance of a commercially prepared lyophilized probiotic culture (Ryall and others, 2012). Exposure of cells to conditions that elicit a general stress response can improve the ability of the culture to survive in a new and potentially stressful habitat, such as acidified cucumbers. In the commercial production of refrigerated pickles, it is a common practice to fill containers with cut or whole cucumbers and cover them with a concentrated brine (cover brine) containing acetic acid, NaCl, spices, and sodium benzoate immediately prior to sealing the jars such that the ingredients equilibrate with the cucumbers to the desired formulation in the finished product. Incorporation of probiotics immediately prior to sealing would expose the cells to the unequilibrated cover brine, and therefore to concentrations of the solutes that are two-fold higher than what is present in the equilibrated product. On average, full strength cover brine is composed of 150 mM acetic acid added as vinegar, 1 M or 5.7% NaCl, 22 mM anhydrous $CaCl_2$, and 13 mM sodium benzoate at a pH of 3.0 (Pérez-Díaz and others, 2013).

The ability of the commercially available probiotic cultures to cope with the sodium benzoate stress in the cover brine was assessed because growth (30°C) of *L. plantarum* in an acidified pickle product containing 150 mM acetic acid at pH 3.5 is delayed at least 5-fold by 9 mM and higher sodium benzoate (Pérez-Díaz and others, 2008). Based on the Henerson-Hasselback equation, under these conditions it is expected that 83 and 95% of the sodium benzoate and acetic acid, respectively, exist undissociated. The pH of the unequilibrated

cover brine, 3.0, is lower than that of the equilibrated product resulting in a greater proportion of undissociated to dissociated acid, which would be 94 and 98% of the benzoic and acetic acids, respectively. The concentration of undissociated acids should be considered when assessing the antimicrobial effect of organic acids because this is the form in which they can passively diffuse through the membrane.

The effect of supplementing cover brines with sodium benzoate at concentrations as high as 9 mM on the survival of LP-A and PA-B during the time in which they are exposed to the cover brine was negligible. The data was analyzed in such a way that the lack of homogeneity of variance wouldn't affect the analysis of the effect of sodium benzoate within strain. However, it should be noted that it is possible that the apparently large error associated with survival of LP-C could be due to an outlier in one of the replicates. Both probiotic cultures experienced less than 0.3 $\log_{10}$ CFU mL^{-1} cell reduction in all brine samples except for in the case of the brine supplemented with 0 mM sodium benzoate and inoculated with LP-B which resulted in a cell count significantly lower than the saline control by a little over 0.5 $\log_{10}$ CFU mL^{-1}. It is relevant to note that the pH of the cover brine was not adjusted after addition of sodium benzoate, meaning the pH of the system increased with increasing concentrations of sodium benzoate. The pH dependence of the proportion of undissociated acetic acid may explain the reduced cell count as a result of cover brine supplementation with 0 mM as compared to those containing 3, 6, or 9 mM sodium benzoate. Together these observations suggest that at the concentrations tested, there is no negative effect of sodium benzoate on survival of cultures in the full strength cover brine, which is

relevant during the time of incubation before the product is equilibrated. Additionally, strict parameters to control the pH of the cover brines should be implemented, in particular if PA-B is to be used for the production of an acidified cucumber product

Interestingly, equilibration of sodium benzoate in the acidified cucumber chips occurred in 10 minutes (Figure 2.2), suggesting that the suspended cells only encountered sodium benzoate stress for a short period of time. Therefore, although the 2.5 h incubation assessed in this experiment represents an extreme scenario for cucumber chips, it may be appropriate for whole cucumbers, expected to take longer to achieve equilibration (Potts and others, 1986).

Survival in the cover brine containing 0 mM sodium benzoate was indicative of the resistance of the culture to the other stressors in the system studied including 270 mM acetic acid added as vinegar, 1.1 M NaCl, and the cold temperature of incubation (4°C). LP-C survived at a significantly lower rate in the brine with 0 mM sodium benzoate than LP-A or LP-B in this same sample. This suggests that LP-C is less resistant to one of the other stressors or a combination of the stressors in the system than are LP-A or LP-B. It is important to note that the results of this experiment are not representative of what happened in the direct inoculation experiment. Unlike the direct inoculation experiment, in this experiment the lyophilized probiotics were incubated in CJ prior to inoculation.

Next, it was hypothesized that acid-adapted probiotic LP-A would be able to survive at a higher rate in the acidified cucumbers than non-acid-adapted lyophilized probiotics. Probiotic LP-A was chosen because although it did not show a high survival rate in the direct

inoculation study, survival of this strain was not affected by sodium benzoate and its potential to have a therapeutic effect is more documented than any other culture tested. To test this hypothesis, the lyophilized probiotic cells were rehydrated in acidified (acetic acid) CJ medium (pH 4.0) prior to inoculation of the acidified cucumbers. When exposed to sub-lethal stresses, lactic acid bacteria can express the acid tolerance response, increasing acid resistance (van de Guchte and others, 2002; O'Driscoll and others, 1996; van de Guchte and others, 2002). Rehydration in CJ, as opposed to the cover brine, allows for the cultures to be revived in a relatively high osmolality medium (due to nutrients and concentration of culture) and one that has nutrients available to support adaptation prior to experiencing the cover brine (Costa and others, 2000; Ma and others, 1997; O'Driscoll and others, 1996; Vrancken and others, 2011). In the acidified cucumber product, all of the solutes from the cucumber are diluted about two-fold and do not reach equilibration until after at least 1 day (data not shown), potentially limiting their availability to the probiotics in the brine. There was no significant difference in long-term survival of the probiotic cultures when the cells were rehydrated in CJ containing acetic acid versus no acidification.

Time of incubation in the CJ rehydration media was assessed by comparing survival after a 2 or 18 h incubation. There was also no significant effect of time of incubation in the CJ medium (2 or 18 h). On average, survival of LP-A in the acidified cucumbers after incubation in the CJ medium was 4.9 ± 0.5 $\log_{10}$ CFU mL^{-1} (Figure 2.4). Incubation time was hypothesized to have an effect because stress resistance and the production of cold shock proteins (CSPs) have been observed to be dependent on stage of growth (van de Guchte and

others, 2002; Derzelle and others, 2000). The survival of LP-A after CJ incubation was greater than 100 times that observed in the directly inoculation experiment. It is suspected that incubation in the CJ medium prior to inoculation allows the cells to recover homeostasis due to the higher nutrient content and osmolarity of this medium compared to the cover brine.

Although rehydration of the lyophilized cultures in CJ seemed to improve survival compared to rehydration directly in the cover brine, long-term survival in the acidified cucumbers was not improved by acidifying the CJ for rehydration as opposed to non-acidified CJ. This suggests that either there is no negative effect of the acetic acid and/or low pH of the system on long term survival or that the cultures were not able to adapt, possibly due to the growth-inhibiting incubation temperature of the CJ rehydration (4°C).

It was next hypothesized that after rehydration in the CJ and inoculation of the acidified cucumber product, allowing the probiotics to incubate briefly at an elevated temperature (21°C) prior to refrigeration and that supplementation of the product with trehalose, a cryoprotectant, would improve long-term survival. This was tested in the model system with probiotic culture LP-A. The assumption was that the transcription, translation, and regulation required for adaptation is not physiologically possible at the non-growth permitting temperature of 4 °C (Derzelle and others, 2000), but that adaptation to this environment could occur at a growth-permissive temperature of 21°C. It was also considered that the transition from high temperature to low temperature over a period of time would allow the culture to sense the unfavorable change in temperature and respond accordingly,

synthesizing CSPs (Derzelle and others, 2000; Barria and others, 2013; Varon and others, 1993). Supplementation with the cryoprotectant, trehalose, was tested for every temperature profile tested. The effect of incubating the model system at 21°C (24 h) followed by 14°C (24 h) followed by refrigeration was tested because Kandror and others (2002) observed that *E. coli* MC4100 produced trehalose at 16 °C after experiencing a temperature downshift from 37 °C and survived to a higher cell density when subsequently transferred and incubated at 4 °C. Trehalose has been observed to act as a cryoprotectant for *L. plantarum*, improving survival during storage after freeze-drying and freezing (Gisela and others, 2014; Carvalho and others, 2002; Giulio and others, 2005). Although *L. plantarum* cannot synthesize trehalose, it is able to transport and regulate this compound (Duong and others, 2006). This study shows that incubation of the lyophilized probiotics in the acidified CJ model system at an elevated temperature prior to refrigeration, improves the long-term survival compared to immediate incubation at 4 °C. This is in agreement with the study by Filaninno and others (2014) where fruit and vegetable juices inoculated with strains of *L. plantarum* to 7.0 $\log_{10}$ CFU mL^{-1} were incubated for 24 h at 30 °C followed by storage at 4 °C. They observed minimal cell count reductions of less than 1 $\log_{10}$ CFU mL^{-1} after 21 days at 4 °C.

Incubation of the probiotic LP-A at the higher temperatures (treatments 21-14°C and 14°C) resulted in significantly enhanced survival by day 64, suggesting that the changes induced at the higher temperatures in treatments 21-14-4°C and 21-4°C did not allow for activities essential to survival to be active during long-term incubation at 4 °C. This observation is consistent with the observed significant difference in survival between the 4°C

and 14°C treatments. However, the difference in survival of LP-A between treatments 4°C and 14°C incubation is significantly greater than the difference between 21-4°C and 21-14°C, 0.72 versus 2.73 $\log_{10}$ CFU g^{-1} cucumber, respectively (Figure A.5E).

The probiotic LP-A was capable of surviving significantly better for 2 months when stored at 14 °C or 21-14°C than at any other temperature profile. 14 °C is at the lower end of the growth temperature range for Lactobacillaceae (Wright and Axelsson, 2011) suggesting that the probiotic is able to grow very slowly at 14 ˚C, remaining metabolically active and slowly using the available nutrients and generating byproducts, essentially experiencing an extended log phase. Additionally, it has been observed that slow-growing cultures of *E. coli* experience a general stress response not experienced by fast-growing cultures (Ryall and others, 2012). The hypothesis that the probiotics are growing slowly at 14°C is supported by the results of the HPLC analysis of organic acids, showing utilization of about 20% of the glucose and fructose and the majority of the malic acid and formation of about 30 mM lactic acid after 2 months of incubation (Table 2.4). On the other hand, no lactic acid production was observed from the cultures incubated at 4 °C, suggesting a state of dormancy or lack of metabolic activity under such conditions. Malic acid was utilized in the samples incubated at 14 °C, 21-14-4 °C, and 21-4 °C temperature profiles. It is likely that the cells were decarboxylating the malic acid to lactic acid to maintain a higher intracellular pH and to replenish the proton motive force in response to acid stress (Filannino and others, 2014; De Angelis and others, 2016). Although, together these observations suggest that a 14°C storage

may provide the optimal conditions for long term survival of the probiotics, such process would be considered temperature abuse.

We finally aimed at observing the survival of the probiotic culture LP-A in jars of acidified cucumber chips, as opposed to a CJ model system, in response to variable temperature profile treatments for the rehydration in acidified CJ and storage in the acidified cucumber chips (Table 2.5 and Figure 2.7).

It was learned that to produce a probiotic pickle product with a 4-month shelf-life, the product should be inoculated by LP-A after the culture is rehydrated in acidified CJ incubated at 4°C and incubated in the acidified cucumber product for 24 h at 21 °C prior to refrigeration. However, the initial inoculation level must be increased in order to bring the cell count at 4 months to greater than 7.5 $\log_{10}$ CFU mL^{-1}. This must however be balanced with a concentration that will not create a challenge for processors and that will be acceptable to consumers. The data gathered in this study can be used to infer the initial cell count of probiotics that is required to know that a certain percentage of jars, 95% for example, contain 7.5 $\log_{10}$ CFU mL^{-1} for the duration of the shelf life. However, due to the low number of replicates, there is a low confidence that the standard deviation observed is representative of the standard deviation of the population. More replicates should be repeated and then the concentration of probiotic microorganisms required at either 2 or 4 months can be calculated based on a z-value of 1.65 (α =0.05), the survival rate, and the predicted population standard deviation. For example, in the process in which the probiotics were incubated in the acidified CJ for 18 h at 4°C and then in the acidified cucumbers at 21°C for 24 h prior to

refrigeration, the cell count on day 64 was 6.90 with a standard deviation of 0.45 $\log_{10}$ CFU g^{-1} cucumber for a survival rate of 5.3%. Here the required cell count on day 64 would be 8.24 $\log_{10}$ CFU g^{-1} cucumber (Equation A.1A) and therefore, the required initial cell count would be 9.52 $\log_{10}$ CFU g^{-1} cucumber (Equation A.1B). Other methods of inferring the required initial cell count are examined in appendix A.

Equation 1a: $\mu - (1.645)(0.45) = 7.5$

$$\mu = 8.24$$

Equation 1b: $Initial\ cell\ count = log_{10}\left(\frac{10^{8.24}}{0.053}\right) = 9.52$

Based on this data, assuming the survival rate and standard deviation would be the same in future replicates, and a z-value of 1.65 (α =0.05), the cell count required to know that 95% of the product contains 7.5 $\log_{10}$ CFU g^{-1} cucumber after 64 days is 8.24 $\log_{10}$ CFU g^{-1} cucumber (Equation A.1A). The initial cell count required can be extrapolated from this and the already observed survival rate of 5.3% (Equation A.1B).

The treatment that actually resulted in the highest survival of LP-A (L/H/4/21(5)-4) was that where the cultures were rehydrated at 4°C and then inoculated into the acidified cucumbers to 5.8 $\log_{10}$ CFU mL^{-1} followed by a 5-day incubation at 21°C before refrigeration. The cell count on day 64 was 8.3 $\log_{10}$ CFU mL^{-1}, which may guarantee a cell count of 7.5 $\log_{10}$ CFU mL^{-1} depending on the results of further replication. However, the cell count after 128 days of storage was lower than 7.5 $\log_{10}$ CFU mL^{-1}. Because this process is characterized by a full fermentation occurring prior to 4°C incubation, the highest initial cell count that can be achieved is the maximum cell density that the culture can grow to in

this system. Therefore, with further replication, this process may prove adequate for a 2-month shelf life.

2.6 Conclusion

This is the first study that investigated methods for the development of a probiotic pickled cucumber product as well as the concentration effect of sodium benzoate on survival in the cover brine at 4°C. The methods investigated include: (1) direct inoculation of the probiotic cultures in the cover brines, (2) rehydration of lyophilized probiotic cultures in acidified cucumber juice at 21 ˚C and 4 ˚C, (3) trehalose supplementation in acidified cucumber jars, (4) incubation of the acidified cucumber product at 21°C for 1 or 5 days prior to refrigeration, and (5) probiotic culture inoculation at high and low levels.

Based on observations made in this study, the process that has the most potential to yield a probiotic pickle product is that where lyophilized cultures are rehydrated in CJ and incubated at 4 ˚C for 18 h prior to addition to the acidified cucumber pickles for inoculation, followed by initial product storage at 21 °C for 24 h and then refrigeration at 4 ˚C. The initial cell count would need to be increased in order to provide buffer space for variability in survival rates. More replicates should be run in order to determine the concentration of probiotic cultures required at time 0 to consistently ensure at least 7.5 $\log_{10}$ CFU g^{-1} cucumber at the end of shelf life. In contrast to the method proposed by Pérez-Díaz and others (2011), which requires fermentation for multiple days and an initial increase in pH, this study developed a process that enables the manufacture of a probiotic pickle product with only 1 day of incubation at an elevated temperature without elevating the pH above 4.6.

2.7 References

North america probiotic (nutraceuticals) market by application (functional food, functional beverages, animal feed, dietary supplements, personal care) and by geography- trends and forecast upto 2019. 2015.

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Yonak SL and Porubcan RS, inventors; Master Supplements Inc, assignee.Enzyme and prebiotic combinations for enhancing probiotic efficacy**.**

Table 2.1. Information on probiotics that were assessed and inoculation levels into the acidified cucumbers in the direct inoculation study based on the concentration of the lyophilized cultures.

Strain	ID	Source	Reference	Lyophilized Culture Concentration [Log(CFU mL^{-1})]	Mass Added to Cover Brines (g/L of cover brine)	Estimated Initial Count in Jars [Log(CFU mL^{-1})]
L. plantarum LP-115	LP-A	Dupont Nutrition and Health	Danisco, Costa and others (2014), Daniel and others (2006), Paineau and others (2008), Yonak and others (2015), Dharmasena and others (2015)	11.6	2.6	8.79
P. acidilactici A571	PA-B	Dupont Nutrition and Health	N/A	11.8	2.3	8.79
L. plantarum 10B	LP-C	Lyoferm, Inc.	N/A	10.3	2.4	7.33
L. plantarum LM 10B	LP-D	Lyoferm, Inc.	Lyoferm (2013)	10.8	2.4	7.79
L. plantarum	LP-E	Biosource	N/A	10.6	2.4	7.67
P. acidilactici	PA-F	Biosource	Borowski and others (2009)	11.8	2.4	8.80
L. plantarum A139	LP-G	Biena, Inc.	N/A	11.6	1.9	8.78

Table 2.2. Summary of experiments conducted and specifications of the rehydration medium and brine formulation tested for enhancing the survival of probiotic cultures in pickles.

Experiments Conducted	Probiotic Culture Rehydration Matrix	Probiotic Culture Rehydration Conditions	Acidified Cucumber Incubation Temperature (°C)	Experimental Media	Sampling Schedule	Tests Performed
Direct Inoculation of Probiotics	Cover brine without NaCl	None	4	Acidified cucumber chips	Days 0, 4, 8, 32 and 128	Counts on MRS
Effect of Sodium Benzoate Prior to Equilibration	CJ	4 ˚C for 1 h	4	Acidified cucumber chips	Hourly for 6 h	Counts on MRS, and HPLC
Effect of CJ Rehydration	CJ	2h 4 ˚C	4	Acidified cucumber chips	Days 8 and 32	Counts on MRS, pH
	Acidified CJ (pH 4) with acetic acid	2 h	4			
	CJ	18 h	4			
	Acidified CJ to pH 4 with acetic acid	18 h	4			
Effect of Temperature Modification and Trehalose	CJ	18 h at 4 ˚C	21-14-4, 21-4, 21-14, 14, 4	CJ model system with and without trehalose	Days 16, 32, 64, and 128	Counts on MRS, HPLC and pH
Effect of Incubation at Elevated Temperature	Acidified CJ or CJ	18 h, 4 ˚C	21-4, 4	Acidified cucumber chips	Days 1, 6, 16, 32, 64, and 128	Counts on MRS
		24 h, 21°C	4			

Table 2.3. Survival of LP-A in the acidified cucumber juice (CJ) model system with various temperature profiles.

		Incubation Time (days)			
		0	**16**	**32**	**64**
Treatment ID	**Temperature Profile (°C)**	**Cell Count as a Function of Incubation Time[a] (log_{10} CFU mL^{-1})**			
4°C	4	8.41 ± 0.03^{A}	6.71 ± 0.35^{D}	4.93 ± 0.77^{E}	2.00 ± 0.00^{F}
21-14°C	21 (24 h)[b] -14	8.41 ± 0.03^{A}	8.02 ± 0.038^{AB}	7.71 ± 0.24^{ABC}	7.04 ± 0.066^{CD}
21-14-4°C	21 (24 h) -14 (24 h) - 4	8.41 ± 0.03^{A}	7.85 ± 0.41^{ABC}	7.45 ± 0.18^{BCD}	5.73 ± 0.49^{E}
21-4°C	21 (24 h) - 4	8.41 ± 0.03^{A}	7.93 ± 0.30^{AB}	7.08 ± 0.48^{CD}	4.96 ± 0.66^{E}
14°C	14	8.41 ± 0.03^{A}	7.78 ± 0.38^{ABC}	7.59 ± 0.30^{BC}	7.35 ± 0.11^{BCD}

[a]Data points are averages of all samples given that temperature profile regardless of trehalose supplementation. Different superscripted letters represented means that are significantly different (α=0.05). [b]Time of incubation at the temperature is in parentheses. A dash indicates a change of incubation temperature.

Table 2.4. Quantification of organic acids and sugars in samples collected from model system samples inoculated with LP-A and incubated at various temperature profiles.

	Substrates Utilized (mM)			Products (mM)
	Malic Acid	**Glucose**	**Fructose**	**Lactic Acid**
Equilibrated Cucumbers[a]	10.70 ± 0.51	25.91 ± 0.92	29.59 ± 0.20	BLD
Treatment ID				
14°C	0.62 ± 0.31	28.15 ± 1.11	24.39 ± 1.03	30.82 ± 1.20
4°C	9.46 ± 1.50	28.57 ± 2.08	31.29 ± 3.20	BLD
21-14-4°C	4.04 ± 0.02	31.43 ± 0.22	34.25 ± 0.76	22.74 ± 2.68
21-4°C	4.12 ± 0.02	31.43 ± 0.06	34.256 ± 0.99	18.86 ± 2.20

Results are shown as averages and standard deviation of 2 replicates.
[a]The data for the equilibrated cucumbers was obtained by HPLC analysis of the raw cucumber and then the concentration initially in the acidified cucumbers was calculated based on the pack out ratio of cucumbers to brine.

Table 2.5. Cell count of LP-A in pickles as a result of modification to the temperature profile of the rehydration medium and the acidified cucumbers.

Treatment ID[a]	Inoculation Level (log_{10} CFU mL^{-1})[b]	pH[c]	Temperature Profile		Component[f]	Storage Time (Days)			
			CJ Rehydration[d]	Jar Temp. Profile[e]		0	32	64	128
						Average Cell Count (log_{10} CFU mL^{-1})			
H/L/None/4	8.4	3.75 ± 0.05	None	4°C	C	6.45 ± 1.23^{BCDEFH}	2.16 ± 0.28^{LM}	2.55 ± 0.71^{KLM}	2.64 ± 0.57^{KLM}
H/L/None/4	8.4	3.75 ± 0.05	None	4°C	B	6.45 ± 1.23^{BCDEFH}	2.00 ± 0.00^{M}	2.20 ± 0.17^{KLM}	2.00 ± 0.00^{M}
H/L/4/4	8.4	3.75 ± 0.05	4°C, pH 4.0, 18 h	4°C	C	8.20 ± 0.11^{ABC}	4.36 ± 0.15^{FGHIJK}	3.11 ± 1.57^{IJKLM}	2.39 ± 0.54^{KLM}
H/L/4/4	8.4	3.75 ± 0.05	4°C, pH 4.0, 18 h	4°C	B	8.20 ± 0.11^{ABC}	4.19 ± 0.30^{GIJK}	3.10 ± 1.56^{IJLKM}	2.00 ± 0.00^{LM}
H/L/4/21(1)-4	8.4	3.75 ± 0.05	4°C, pH 4.0, 18 h	21°C (1 d) →4°C	C	8.20 ± 0.11^{ABC}	7.66 ± 0.24^{ABCD}	6.90 ± 0.45^{ABCDE}	5.96 ± 0.56^{DEFGH}
H/L/4/21(1)-4	8.4	3.75 ± 0.05	4°C, pH 4.0, 18 h	21°C (1 d) →4°C	B	8.20 ± 0.11^{ABC}	7.20 ± 0.14^{ABCD}	7.11 ± 0.28^{ABCDE}	6.11 ± ^{CDEFGH}

Table 2.5 Continued

L/H/4/21(5)-4	5.8	4.6 ± 0.05	4°C, pH 4.0, 18 h	21°C (5 d) →4°C	C	5.72 ± 0.099^{DEFGH}	8.34 ± 0.56^{AB}	7.21 ± 0.59^{ABCD}	6.65 ± 0.57^{ABCDE}
L/H/4/21(5)-4	5.8	4.6 ± 0.05	4°C, pH 4.0, 18 h	21°C (5 d) →4°C	B	5.72 ± 0.099^{DEFGH}	8.72 ± 0.065^{A}	8.21 ± 0.23^{ABC}	6.59^{ABCDEFGH}
H/L/4/21(5)-4	8.4	3.75 ± 0.05	4°C, pH 4.0, 18 h	21°C (5 d) →4°C	C	8.20 ± 0.11^{ABC}	6.37 ± 0.024^{BCDEFGH}	2.35 ± 0.50^{KLM}	2.34 ± 0.49^{KLM}
H/L/4/21(5)-4	8.4	3.75 ± 0.05	4°C, pH 4.0, 18 h	21°C (5 d) →4°C	B	8.20 ± 0.11^{ABC}	6.89 ± 0.57^{ABCDE}	2.71 ± 1.01^{JKLM}	6.59^{LM}
H/L/21/4	8.4	3.75 ± 0.05	21°C, pH 4.0, 24 h	4°C	C	8.18 ± 0.087^{ABC}	6.34 ± 0.44^{BCDEH}	4.99 ± 0.45^{EFGHI}	2.71 ± 0.63^{KLM}
H/L/21/4	8.4	3.75 ± 0.05	21°C, pH 4.0, 24 h	4°C	B	8.18 ± 0.087^{ABC}	5.92 ± 0.48^{DEFGH}	4.53 ± 0.47^{FGIJ}	2.00 ± 0.00^{M}

*Data with different letters in the superscript are significantly different (α=0.05).
[a]Treatment ID is inoculation level/pH/temperature of CJ rehydration/temperature of the acidified cucumbers. H is for high inoculation or pH (8.4 $\log_{10}$ CFU mL^{-1} of 4.6) and L is for low inoculation or pH (5.8 $\log_{10}$ CFU mL^{-1} or 3.75). [b]The inoculation level is the concentration of LP-A in the acidified cucumbers at time 0. [c]The pH is that of the equilibrated acidified cucumbers. [d]Temperature, pH, and time of incubation of LP-A in the CJ rehydration. [e]Jar temperature profile is the temperature of the acidified cucumber chips once inoculated. The → indicates transfer of samples from one temperature of incubation to another. [f]Portion of pickles from which cells where enumerated, C=cucumber, B=brine.

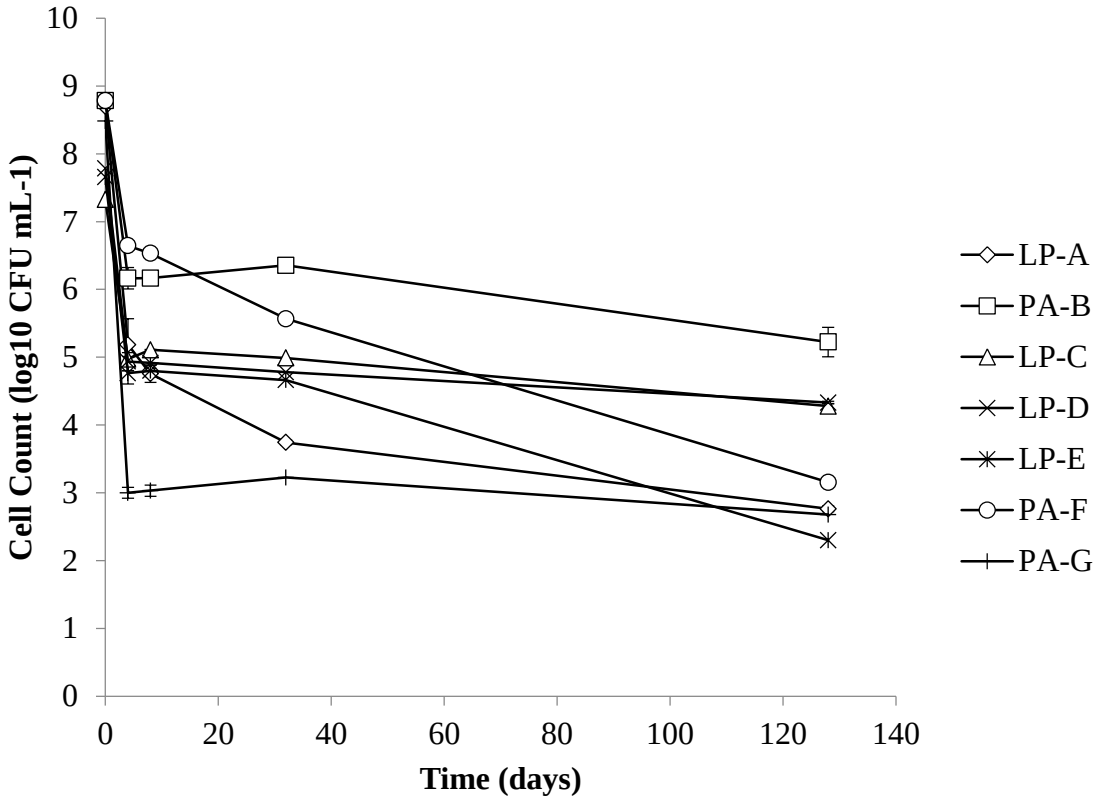


Figure 2.1. Survival of 7 probiotic strains as a function of time after direct addition to the pickling brine and incubation at 4°C.

Cell counts represent survival within the cucumber portion of the acidified cucumbers.

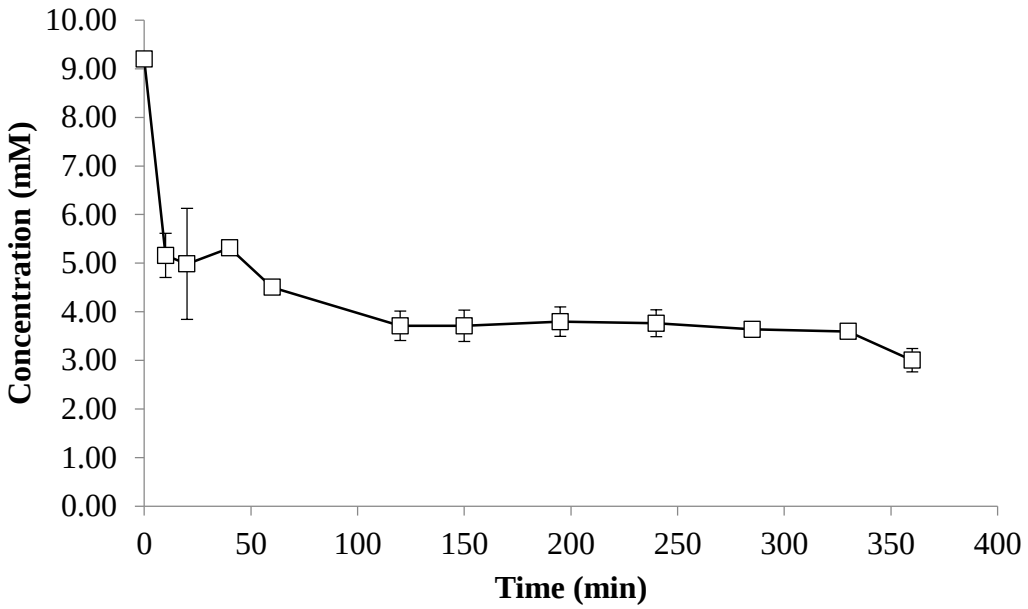


Figure 2.2. Rate of equilibration of benzoic acid in refrigerated, acidified cucumbers. Data are averages of 2 replicates and error bars are standard deviation.

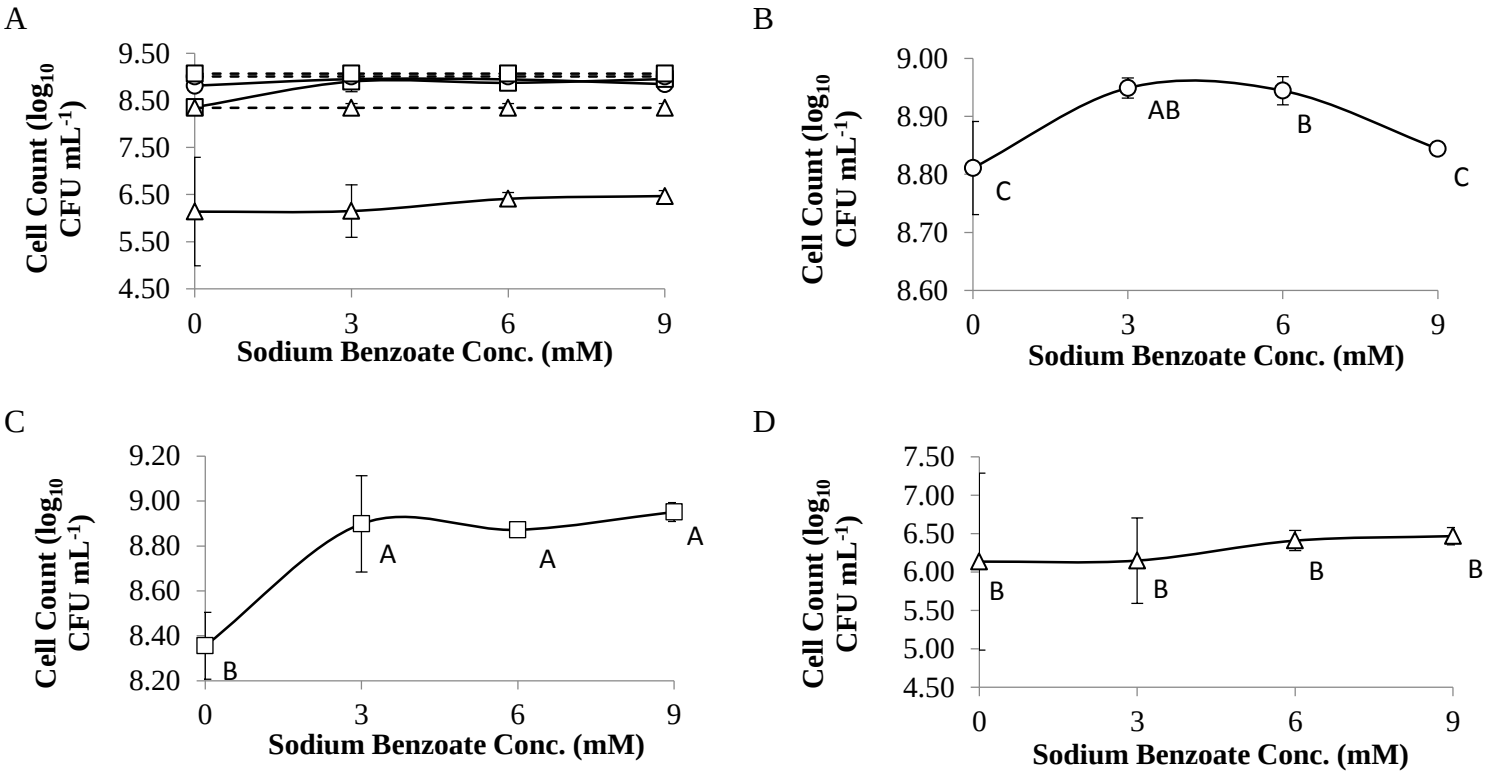


Figure 2.3. The effect of sodium benzoate concentration on survival of LP-A, LP-C, and PA-B in the pickling brine.
A) Data with non-dashed trend lines represent cell counts of LP-A (circle), LP-C (triangle), and PA-B (square) after incubating in cover brine containing 0, 3, 6, or 9 mM for 2.5 h at 4 ˚C. Data with dashed trend lines represent the cell count in the control after 2.5 h. B, C, and D) Cell count of LP-A, PA-B, and LP-C, respectively, in cover brine as a result of sodium benzoate concentration after 4 ˚C incubation for 2.5 h. Lettering indicates significant differences between treatments within each strain. Cell count in the saline control is not shown in B, C, and D graphs, but for all strains, cell count in the saline is assigned the letter A. Error bars are standard deviation of 2 replicates.

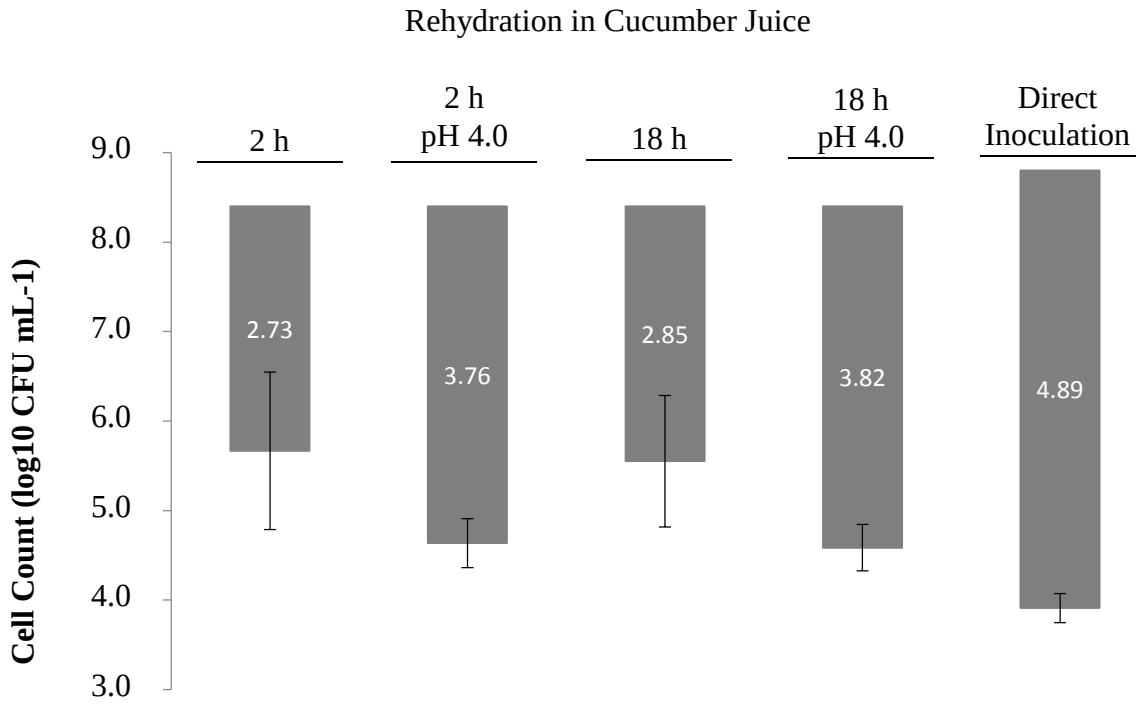


Figure 2.4. Comparison of cell count reduction of LP-A between the Cucumber Juice Adaptation study and the Direct Inoculation study.

The top of the bar represents the initial inoculation level and the bottom of the bar represents the cell count after storage at 4°C for 32 days. Number within bar indicates cell count reduction in $\log_{10}$ CFU mL^{-1}. Incubation time and pH are listed above the bars. Error bars are standard deviations of duplicates.

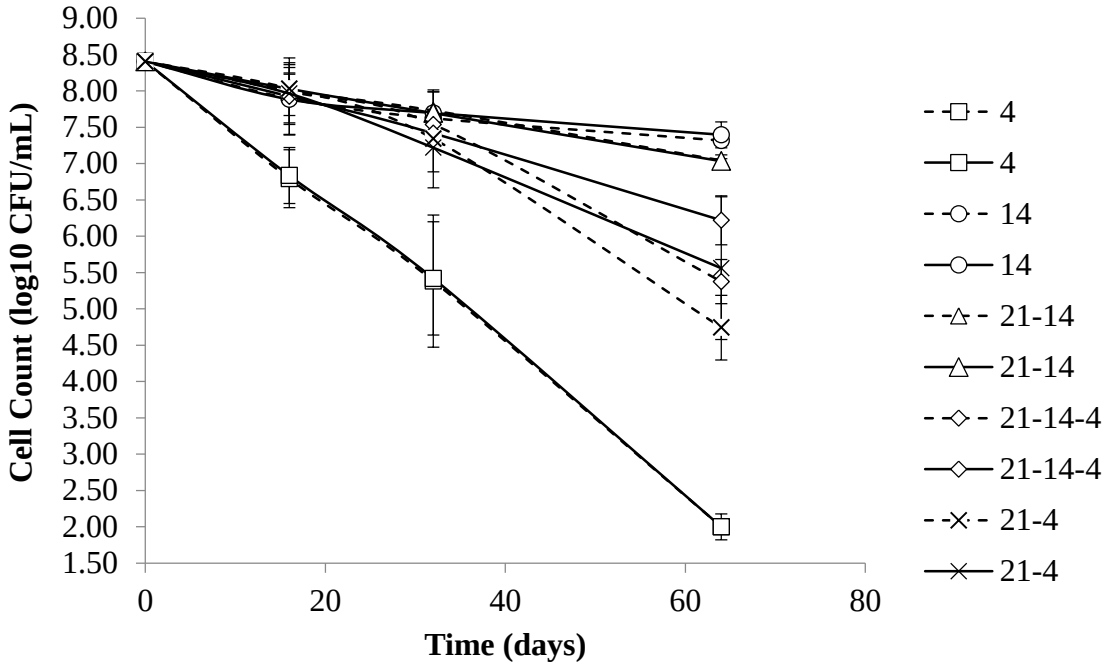
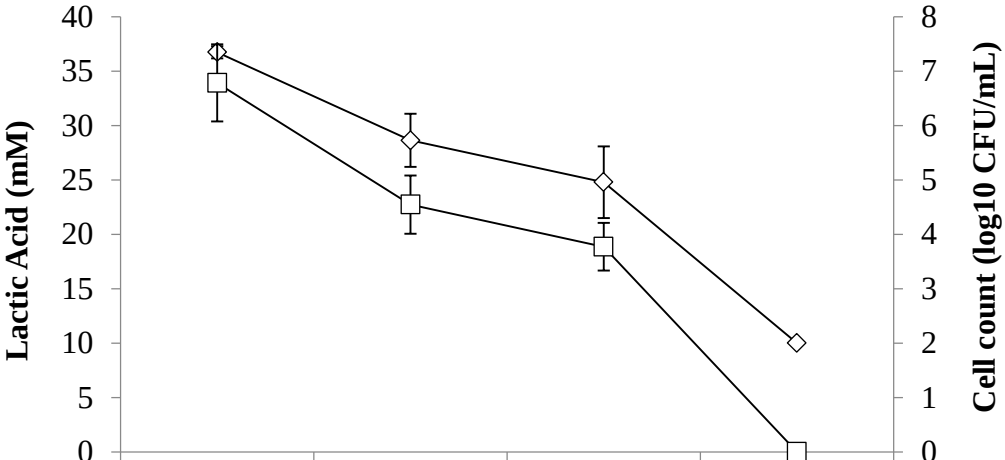


Figure 2.5. The effect of temperature profile and trehalose supplementation on survival of LP-A in the acidified CJ model system as a function of time.

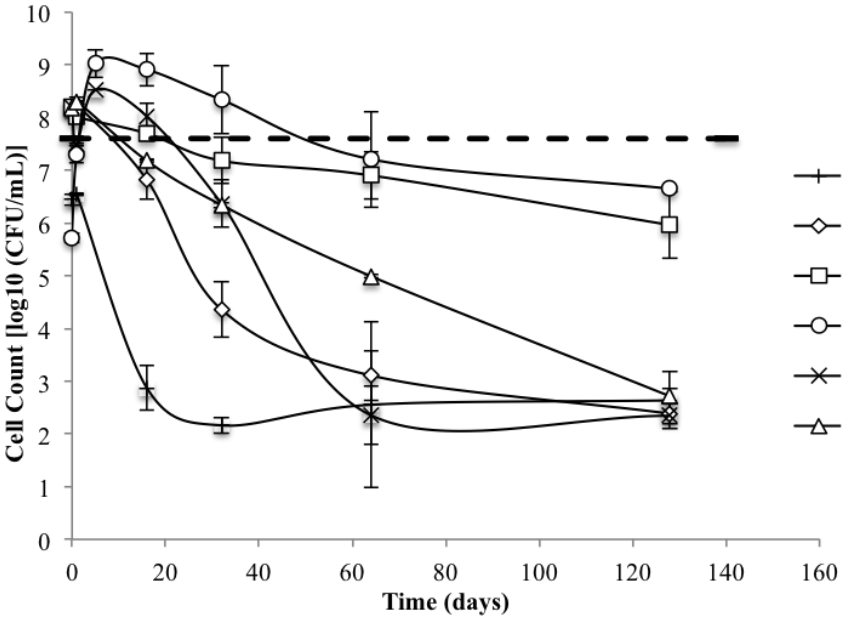
The samples that contain trehalose are represented by a dashed line and the samples that do not contain trehalose are represented by a solid line. Temperature profile treatments are listed in the legend: 4: 4 °C storage for 2 months; 14: 14°C storage for 2 months; 21-14: 21°C for 24 h followed by 14°C for 2 months; 21-14-4: 21°C for 24 h followed by 14°C for 24 h followed by 4°C for 2 months; 21-4: 21°C for 24 h followed by 4°C for 2 months.



	14	21-14-4	21-4	4
Cell Count	7.35	5.73	4.96	2
Lactic acid	33.93	22.74	18.86	0

Figure 2.6. Survival of probiotic LP-A after 64 days in acidified cucumber juice appears to be correlated with lactic acid production.

Temperature profile treatments are listed directly beneath the x-axis, 14, 21-14-4, 21-4, and 4. Error bars are standard deviation. Lactic acid data is the average of two replicates. Cell count data is the average of 4 replicates for each time point.



Trt	CJ (°C)	Acidified CJ (°C)	Inoculation Level (log_{10} CFU mL^{-1})
1	None	4	8.4
2	4	4	8.4
3	4	21 (1d) → 4	8.4
4	4	21 (5d) → 4	5.8
5	4	21 (5d) → 4	8.4
6	21	4	8.4

Figure 2.7. Effect of modification to temperature profile on survival of LP-A as a function of time.

Temperature profile is a combination of probiotic culture rehydration temperature and temperature of incubation in the acidified cucumber pickles. TRT 1: Rehydration: 4˚C Product: 4 ˚C (Control); TRT 2: Rehydration: 4˚C, Product: 4˚C; TRT 3: Rehydration: 4˚C, Product: 21˚C (24 h)-4 ˚C; TRT 4: Rehydration: 4˚C, Product: 22 ˚C (5 d)-4 ˚C, inoculation level: 5.8 log_{10} CFU mL^{-1}; TRT 5: Rehydration: 4˚C, Product: 21˚C (5 d)-4 ˚C; TRT 6: Rehydration: 21˚C, Product: 4˚C. Initial inoculation concentration is 8.4 log10 CFU mL^{-1} unless otherwise stated.

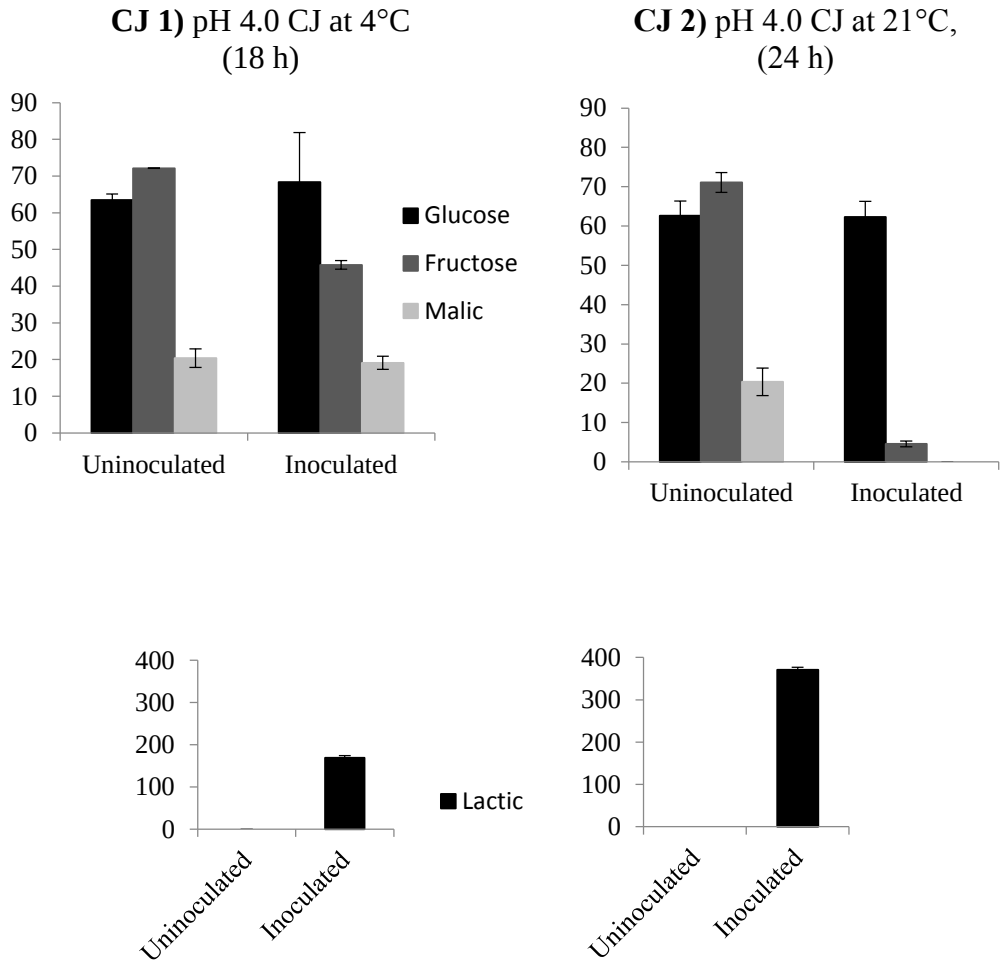


Figure 2.8. HPLC analysis of organic acids and sugars in acidified CJ inocula compared to the uninoculated CJ inocula.

CHAPTER 3

Effect of Arginine Supplementation on the Survival of Lyophilized Probiotic *Lactobacillus plantarum* in Acidified Refrigerated Pickles

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3.1 Abstract

Acidified refrigerated cucumber products have a pH and storage temperature that is suboptimal for growth and survival of probiotic cultures. Additionally, the protein content of this type of product is relatively low compared to the dairy products where probiotics are most commonly found. It was previously observed that elevating the temperature of incubation of a commercially available *L. plantarum* LP-115 probiotic either during rehydration in acidified cucumber juice or in the acidified refrigerated cucumber product improved the long term survival of the probiotics in the acidified refrigerated cucumber product (Chapter 2). In this study, the effect of amino acid supplementation on long-term survival of probiotic *Lactobacillus plantarum* LP-115 in refrigerated, acidified cucumber was investigated. Acidified cucumber juice (model system for pickles) was supplemented with citrulline, arginine, glutamine, glutamate, tryptone, casamino acids, a free amino acid mixture, or the free amino acid mixture supplemented with citrulline, arginine, glutamine, glutamate and incubated at either 4˚C or 21˚C for 24 h followed by 4˚C. The effect of arginine supplementation was further tested in acidified cucumber pickles incubated at three different temperature profiles. Supplementation with arginine, casamino acids, and the free amino acid mixtures improved survival of *L. plantarum* LP-115 compared to the control. Arginine improved survival in all temperature treatments and was especially effective at improving survival in the treatments where the acidified cucumbers were incubated at 4˚C. Preliminary experiments with scanning electron microscopy (SEM) showed that cells in the treatment supplemented with arginine appeared to be existing in biofilms at a higher rate than

treatments lacking arginine. This study demonstrated that long term survival of *Lactobacillus plantarum* LP-115 can be enhanced by either elevating the temperature of incubation of the acidified cucumbers for the first 24 h of storage or by supplementing with arginine. The mechanism by which arginine improves survival of *L. plantarum* LP-115 in this system requires further investigation.

3.2 Introduction

Consumption of probiotic dairy products and supplements, containing live microorganisms that confer a health benefit, continues to increase in the global market (Freitas and others, 2014; Clarke and others, 2015). Incorporation of probiotics into pickled cucumbers could offer consumers a non-dairy, low calorie probiotic food product. Although consumption of pickled cucumbers has been on the decline since its peak in 1985, refrigerated cucumbers, which are crispier and perceived as fresher, are increasing in popularity (Lucier and others, 2007). Refrigerated cucumbers are manufactured by acidification or fermentation by LAB, including *Lactobacillus plantarum* and *Pediococcus* spp. (Etchells and others, 1968; Huys and others, 2011).

We have developed and tested methods that allow survival of commercially available, lyophilized, probiotic strains of *L. plantarum* and *P. acidilactici* in an acidified refrigerated, cucumber product for 2 months (Chapter 2). Hurdles for the survival of probiotic *L. plantarum* and *P. acidilactici* in the targeted product that were considered in the previous study included a suboptimal growth permissive temperature (4˚C), the presence of the preservative, sodium benzoate, acidification with acetic acid in the form of vinegar, an acidic

pH of 3.7 to 4.0, and limited levels of nutrients relative to dairy products. We aimed at maintaining 7.5 $\log_{10}$ CFU/g cucumber of the probiotic throughout the shelf life of the pickle product. Processes that were tested included direct inoculation of lyophilized cultures into the acidified cucumber product, rehydration of the probiotic culture in cucumber juice (21°C or 4°C) prior to inoculation in the product, incubation of the probiotic pickles at 21°C for 24 h prior to 4°C long-term storage with and without trehalose supplementation, and incubation of the acidified cucumbers at 21°C for 1 versus 5 days prior to refrigeration. Elevation of the temperature of the acidified cucumber to 21°C for 24 h or 5 days prior to 4°C storage with cucumber juice rehydration for 18 h at 4°C resulted in the highest survival rates for the *L. plantarum* probiotic culture (LP-A).

LABs are commonly considered fastidious microorganisms. They have evolved in many different environments including plants, dairy, and the GI tract of animals and the same species can be found in multiple different environments. Since their divergence from the genus *Bacilli*, the *Lactobacillales* have lost many genes related to their further evolution in nutritionally rich environments. *Lactobacillales* have lost unnecessary genes such as those encoding biosynthetic enzymes including those for specific amino acid biosynthesis (Makarova and others, 2006). On the other hand, they have acquired, mostly through horizontal gene transfer, genes such as peptidases for utilization of a diverse pool of nutrients (Makarova and others, 2006). The protein content of cucumbers is relatively low, especially compared to dairy products, and so it is possible survival in this system will be limited by nutritional content. Especially considering that amino acids can aid LAB in acid resistance,

which is important in an acidified cucumber product with a pH of 3.7 to 4.0 and acetic acid concentrations over ~120 mM. Under these conditions, the cytoplasmic pH could drop below that which is permissible for growth of *L. plantarum*, due to the influx of protonated organic acids, which then dissociate into anions and protons within the cell (McDonald and others, 1990). McDonald and others (1990) observed *L. plantarum* WSO to cease growth when its internal pH reached 4.6 to 4.8. Vrancken and others, 2011 observed increased expression of peptide and amino acid metabolism genes rather than pathways characteristic of growth by *L. plantarum* growing in sourdough at a pH of 3.5 or 4.0 compared to pH 4.5, 5.0, or 5.5. Decarboxylation or deimination of particular amino acids results in deacidification of the cytoplasm and ATP synthesis either directly or via generation of proton motive force, increasing acid resistance (Pessione and others, 2016). Therefore, low protein content could inhibit the ability of a probiotic microorganism to cope with the environment of an acidified cucumber.

Catabolism of glutamine, glutamate, and arginine by LAB, including certain strains of *L. plantarum* (Spano and others, 2004; Siragusa and others, 2007), results in consumption of protons and production of ammonia and/or carbon dioxide (CO_2) in environments with pH levels that are suboptimal to cellular enzyme activity (Spano and others, 2010; van de Guchte and others, 2002), which is advantageous for LAB Spano and others (2004) suggested that LAB strains that are able to obtain energy from metabolism of arginine might be more competitive in wine, which exposes cells to acid stress. Glutamine can be deamidated by LABs, converting it to glutamate with the production of ammonia. Glutamate can be further

metabolized to GABA, consuming one proton when the intra- and extra- cellular pH is less than its pka of 4.2. Glutamine or glutamate can be imported in exchange for GABA. Similarly, arginine can be metabolized by some of the LAB via the arginine deiminase (ADI) pathway to produce ammonia, CO_2, and ATP. It has been observed that arginine metabolism is regulated by the presence/absence of various sugars (Liu and others, 1998) and the pH of the environment (Endo and others, 2014; Poolman and others, 1987). The increase in pH as a result of the activity of the ADI pathway restores activity of the pH-sensitive glycolytic enzymes (Endo and others, 2014). Although the catabolism of arginine can be very beneficial for a cell experiencing organic acid stress, this metabolic activity in food products is concerning given the possible formation of the biogenic amines, agmatine and putrescine (Wunderlichová and others, 2014).

Probiotics that will potentially be added to products that contain biogenic amine precursors should be screened for the capacity to perform these pathways. Ideally, this will be investigated using genetic and biochemical tests. The enzymes encoding the ADI pathway enzymes have been sequenced in *Lactobacillus sakei, Oenococcus Oeni,* and *Lactobacillus plantarum* (Zuniga and others, 1998; Tonon and others, 2001; Spano and others, 2004). It is the objective of this study to determine if nutrient content alteration in the form of amino acid supplementation could further improve the survival of a probiotic *L. plantarum* in acidified refrigerated pickles. Moreoever, it was the intent to determine if amino acid supplementation could be an alternative to temporary elevated temperature incubation to achieve long term survival of the probiotic culture in acidified refrigerated

pickles. Elimination of the need for elevated temperature storage would enable the production of probiotic refrigerated pickles in currently existing manufacturing facilities, without the requirement for changes in infrastructure.

3.3 Materials and Methods:

3.3.1 Microorganisms and cucumber juice (CJ) and cover brine preparation

Commercially available probiotic *L. plantarum* cultures LP-115 and WCFS1 (LA1196) were obtained from Dupont-Danisco as a lyophilized powder and the U.S. Department of Agriculture-Agriculture Research Service, Food Science Research Unit (Raleigh, NC, U.S.A) culture collection as a frozen stock. The survival of *L. plantarum* LP-115 was studied in Chapter 2 under the code name, LP-A. The frozen stock culture of *L. plantarum* WCFS1 was prepared in MRS broth supplemented with 10% glycerol (Sigma-Aldrich, Saint Louis, MO, U.S.A.). The probiotics were inoculated in filter-sterilized CJ at a concentration of approximately 10.8 $\log_{10}$ (CFU mL^{-1}).

Cucumbers, size 2A (2.7 cm to 3.2 cm diameter; 15.9 cm long), were received from a local processor and rinsed with tap water immediately before processing. Cucumber juice (CJ) was prepared by blending cucumbers using a Nutribullet (brand, location) followed by filtering out particulates using cheesecloth and centrifugation at 5,000 rpm for 20 minutes at 20°C. The particulate free CJ was filter-sterilized using 0.2 µm bottle top filters with a polyethersulfone membrane (Fisher Scientific, Pittsburgh, PA, USA). The sterilized CJ was frozen at -20°C until used for experimentation. As needed, the sterile CJ was thawed

overnight by incubation at 4 °C and used for the adaptation of cells or diluted with cover brine for experimentation in a model system

Cover brine was prepared in deionized water to contain sodium chloride, calcium chloride, sodium benzoate and acetic acid added as vinegar to 496 mM,9.5 mM, 4.1 mM and 122 mM, respectively, after equilibration with the cucumbers. Cover brines were filter sterilized using 0.2 µm filters with a polyethersulfone membrane (Fisher Scientific) and refrigerated until used.

3.3.2 Effect of Amino Acid Supplementation on Long-term survival of LP-A in a Cucumber Juice Model System

This experiment assessed the effect of 9 amino acid treatments and two temperature profiles. The levels, types and sources of amino acids tested are described in Tables 3.1 and 3.2. Two temperature profiles were included in this experiment, 4 ˚C long-term incubation and 21 ˚C incubation for 24 h followed by 4 ˚C for 4 months. For each treatment, there was one inoculated and one uninoculated sample for each time point. Samples were sacrificed at each time point in order to not alter the headspace of samples that would be analyzed at a later date. Samples were sacrificed on days 16, 32, and 64. Appropriate dilutions were plated on MRS media using an Eddy Jet 2 spiral plater (IUL, Barcelona, Spain) and incubated at 30 ˚C for 48 h prior to enumeration. Colonies on plates were counted using a Flash & Go Automatic Colony Counter (IUL, Barcelona, Spain). Remaining aliquots were frozen at -20 °C for further analysis including HPLC analysis of organic acids and sugars and pH measurements.

Amino acids were added to the cover brines as described in Table 3.1 and mixed. All cover brines were prepared as described above and stored at 4 °C for less than 24 h before combining with CJ. The acidified CJ model system used consisted of a mixture of filter-sterilized cover brine and CJ at a ratio of 45:55 (v/w), respectively. After an 18 h incubation at 4°C, 0.042 mL aliquots of CJ inoculum (~10.6 $\log_{10}$ CFU mL^{-1} LP-A) was transferred to 10 mL of acidified CJ for each treatment for each time point. This resulted in an initial cell count in the acidified CJ of approximately 8.2 $\log_{10}$ CFU mL^{-1}. Aliquots of 0.042 mL of uninoculated CJ were also transferred to separate samples of 10 mL of acidified CJ for each treatment for each time point as a control. Samples were stored according to their randomly assigned temperature incubation profile. This experiment was not repeated.

3.3.3 *Effect of Arginine Supplementation and Incubation Temperature on Long-term Survival of Probiotic L. plantarum in Acidified Cucumber Chips*

Cucumber chips and cover brines were packed into 8 ounce jars at a ratio of 55:45 (w:v), respectively, and then inoculated with the CJ inoculum prepared as described above. Cucumbers were sliced using a Continuous-Feed Food Processor (Hobart, Troy, Ohio, U.S.A.). The temperature profile was considered a combination of the CJ inoculum incubation temperature and the acidified cucumber jar incubation temperature. The temperature profile levels included (1) 18 h incubation at 4 °C of the CJ inocula followed by a 4°C incubation of acidified cucumber jars; (2) 24 h incubation at 21 °C of CJ inocula followed by a 4 °C incubation of acidified cucumbers; and (3) 18 h incubation at 4 °C of the

CJ inocula followed by a 24 h incubation of acidified cucumber jars at 21 °C prior to long term storage at 4 °C.

Combinations of arginine and temperature profile levels described in Table 3.3 were assessed. The cover brine and cucumbers were combined immediately before inoculating with the probiotic cultures. Jars were hermetically sealed and stored as indicated in table 3.3. The data for treatments 21-4-0, 4-21-0, and 4-4-0 (Table 3.3) were reported in the second chapter of this thesis. They are being reported again in this chapter because these same treatments were performed with the addition of 25 mM arginine to the acidified cucumber product as part of the same experiment. It is necessary to incorporate the data already reported in chapter 2 into the analysis of the arginine supplemented treatments in order to determine the effect of arginine under the various temperature profile treatments.

An uninoculated and inoculated jar was packed for every treatment and every time point. Samples of cover brine and cucumber were taken from every treatment, inoculated and uninoculated, on days 32, 64, and 128. Brine samples were obtained on day 16 by pulling brine samples through the rubber septa with a sterile syringe and needle assembly (). Microbial analysis was performed as described below and aliquots were frozen at -20°C for further analyses.

For microbiological analysis cucumber slices were aseptically collected and blended in sterilized Waring 700s glass blending cups (Waring, Stamford, CT, U.S.A). The cucumber slurry was homogenized after blending, in a stomacher using Seward^{TM} Stomacher^{TM} Lab Blender Bags (Seward Limited, Worthing, West Sussex, UK) equipped with filters. Cover

brine samples and cucumber filtrate were diluted in 0.85% sterile saline as needed and plated on Lactobacilli MRS supplemented with 1% cycloheximide (cy) (Remel, San Diego, California, USA) medium using an Eddy Jet 2 spiral plater (IUL). Plates were incubated anaerobically for 48 h at 30°C and colonies counted using a Flash & Go Automatic Colony Counter (IUL, Barcelona, Spain).

In order to determine the effect of arginine under the various temperature profiles on long-term survival of *L. plantarum* LP-115, the data collected was modeled in JMP using ANOVA. The dataset included MRS-CY counts from the brine and cucumber. The response variable in both models was the cell count as enumerated on MRS-cy plates. The effects in the model were the full factorial of Component (brine or cucumber), Arginine, TemperatureProfile (in acidified cucumbers and CJ), and date of analysis (0, 32, 64, and 128) (Table A.9A). Day 0 cell counts in the acidified cucumbers were calculated based on the cell counts of the CJ inoculums and the volume of CJ inoculums transferred to the acidified cucumbers. Insignificant effects were eliminated, starting with the most insignificant and highest interaction effects, until only significant effects were left in the model (Table A.9B). The data was also run with component as a by variable and the full factorial of the remaining effects so that the other effects would be analyzed within the brine and cucumber separately (Table A.9D and A.9E). Day 16 data was included for the brine. The Tukey HSD test was run on the three-way interaction for the brine and cucumber separately to determine significant differences between treatments (Table 3.3).

3.3.4 Visualization of the probiotic cultures on cucumber chips

Sample preparation and operation of the SEM for visualization of probiotics on cucumbers was performed by Valerie Lapham at the Center for Electron Microscopy at North Carolina State University. Slices were cut to a surface area of 5 mm^2 and a thickness of 1 mm. Slices were fixed with 3% glutaraldehyde in 0.05 M potassium phosphate buffer, dried by critical point drying with CO_2 (Tousimis Research Corp., Rockville, Md), and coated with a gold-palladium alloy (80:20) in a Hummer 6.2 (Anatech, Union City, CA, U.S.A.). Samples were visualized at 15kV with a JEOL JSM-5900LV SEM (Peabody, MA, U.S.A.) operating at high vacuum in secondary electron mode.

Three cucumber chips were taken from treatments 21,4,0 and 21,4,20 (table 3.3) where probiotic LP-A was incubated in CJ for 24 h at 21ºC prior to inoculation into the acidified cucumbers, with and without 25 mM arginine, and incubated at 4ºC. Two to three slices were taken each from different parts of the mesocarp and skin portion of each of the three cucumber chips. Slices from the skin of each treatment were processed together and slices from the mesocarp of each treatment were processed together. A total of four to five of the slices from each vial were visualized using the SEM. It was attempted to obtain pictures that were representative of each treatment as well as pictures that showed attributes of each treatment that were unique or not seen on the slices of other treatments.

The cucumber chips were prepped for SEM analysis after the incubation for 64 days. The cell count in the treatment without arginine was approximately 5.0 $\log_{10}$ CFU mL^{-1} and in the treatment with arginine was 5.8 $\log_{10}$ CFU mL^{-1}.

3.3.5 Assessment of the presence of the ADI pathway associated genes in L. plantarum WCFS1

Total genomic DNA was extracted from *L. plantarum* WCFS1 using the MasterPure^{TM} DNA Purification Kit (MCD85201, Epicentre, Madison, Wisconsin) following the manufacturer's instruction and starting with an isolated colony on step 6A of the kit's protocol. The isolated colony was obtained from a frozen stock of *L. plantarum* WCFS1 after streaking onto MRS medium.

It was attempted to amplify the *arcA* gene coding for arginine deiminase using primers arcA forward and reverse (5'- AGR CTN CTN TTY GAY GAY AT -3' and 5'- RTT RCT NCC RTC RTT NGT YTC YTC -3', respectively) as designed by Spano and others (2004). This study attempted to identify the *arcA* gene in *L. plantarum* WCFS1 in order to have a positive control for further studies on the presence of arginine deiminase pathway enzymes in probiotic *L. plantarum* strains. A temperature gradient (50 to 60 ˚C) PCR was run to determine the ideal annealing temperature for the primers. The PCR reaction consisted of 94°C for 3 minutes followed by 30 cycles of 94°C for 1 min., 40 seconds of annealing, and 72°C for 40 s, followed by 72°C for 5 minutes. The 20 µl reaction mixture consisted of 10 µl Mastermix (Bio-rad, 166-5009), 2 µl of the 20 µM forward and reverse primers, 1 µl of genomic DNA at a concentration of 10 µM, and 0, 0.2, 0.4, 0.6, or 0.8 µl of 50 mM $MgCl_2$. The presence of amplicons in each sample was confirmed by agarose gel electrophoresis. Amplicon bands were extracted from 1 % agarose gels using the MinElute Gel Extraction Kit (28604, Qiagen, US). Gel extracted and amplified bands were purified using the QIAquick

PCR Purification Kit (Qiagen) prior to sequencing. Purified DNA samples were re-suspended in RNAse and DNAse free water. The purified products were sequenced by Eton Bioscience, Inc. (Research Triangle Park, NC) using Sanger Sequencing and both the forward and reverse primers. The sequences obtained were aligned using EMBOSS Needle Pairwise Sequence Alignment (EMBL-EBI, Hinxton, South Cambridgeshire, England). A blastx (NIH, NCBI) search of the overlapping portion of each sequence was performed to determine the protein that the sequence likely encoded.

3.4 Results

Overall, the treatments that appeared to improve LP-A survival in the model system were supplemented with arginine, mixture, mixture plus, and casamino acids. Survival of LP-A was not improved by the addition of glutamine and only slightly by the addition of glutamate and citrulline at 4 ˚C after 32 days of incubation (Figure 3.1). The mixture, mixture plus, casamino acids, tryptone, and arginine all appear to improve survival compared to the control by day 32. However, after 64 days of incubation at 4 ˚C all cell counts fell to within the range of 2 and 3 $\log_{10}$ CFU g^{-1} (Figure 3.1). The effect of amino acid supplementation on survival was consistent on day 64 compared to day 32, except arginine fell off to a survival similar to the control.

When incubated in the CJ model system at 21°C for 24 h prior to 4°C incubation (Figure 3.1B), cell counts were higher than those held at 4 °C (Figure 3.1A) for every time point analyzed (Figure 3.1). The effect of the amino acid treatments, relative to each other, on survival of LP-A were similar to the samples incubated at 4°C. The difference in the

magnitude of the effect of the amino acids on cell count compared to the control was less in the samples incubated at 21-4°C than the samples incubated at 4°C on day 16 and 32. The treatments best supporting survival of LP-A were arginine, mixture, mixture plus, and casamino acids with the others grouping closer to the control. This was consistent on day 32 and 64. By day 64, citrulline and tryptone supplementation did appear to be improving survival compared to the control, glutamine and glutamate, but not as well as supplementation with the mixture, mixture plus, casamino acids, and arginine.

Within the 4ºC temperature profile, the difference between survival in the best performing treatment on day 32 (arginine), with a cell count of 4.6 $\log_{10}$ CFU mL^{-1}, and the control and day 64 (casamino acids), with a cell count of 2.3 $\log_{10}$ CFU mL^{-1}, and the control was just under 3 $\log_{10}$ CFU mL^{-1} and about 1 $\log_{10}$ CFU mL^{-1}, respectively. Within the 21°C-4°C temperature profile, the difference between survival in the best performing treatment on day 32 (arginine), with a cell count of 6.9 $\log_{10}$ CFU mL^{-1}, and the control and day 64 (arginine), with a cell count of 6.1 $\log_{10}$ CFU mL^{-1}, and the control was about 1 $\log_{10}$ CFU mL^{-1} and just over 1 $\log_{10}$ CFU mL^{-1}, respectively.

When tested in the acidified cucumber pickles, as opposed to the CJ model system, arginine supplementation and temperature profile (of the CJ rehydration media and acidified cucumber pickles) significantly affected the long term survival of probiotic LP-A. Survival was modeled in JMP using ANOVA (α=0.05) with cell counts in $\log_{10}$ CFU g^{-1} on days 0, 32, 64, and 128 as the response variable and the full factorial of Component (brine or cucumber), Temperature, Arginine, and Day/storage time (days 0, 32, 64, or 128) as effects (Figure

A.9A). In this model, Day ($P<0.0001$), Arginine ($p<0.0001$), Temperature ($p<0.0001$), Component ($p=0.0183$), Day*Arginine ($p<0.0001$), Day*Temperature ($p<0.0001$), and Day*Arginine*Temperature ($p=0.0033$) are significant. A LSMeans differences Student's t-test of Component shows that the cell count in the cucumber is significantly higher than in the brine with averages of 6.27 and 5.98 $\log_{10}$ CFU mL^{-1}, respectively. The ANOVA was rerun in JMP with component as a by variable and including the brine day 16 data. The effect of Arginine, Temperature, and Day were now analyzed separately for the cucumber and brine components (Figure A.9D and E). For both the brine and cucumber component, the 3-way interaction is insignificant with p-values of 0.1231 and 0.0977, respectively. Day, Day*Arginine, and Day*Temperature were significant for both cucumber and brine datasets with all p-values being < 0.0005. The LSMeans Differences Tukey HSD test was performed on the three-way interaction term (Day*Arginine*Temperature) for the cucumber and brine data separately. Significant differences between treatments are documented in Table 3.3. Within the cucumber dataset, this analysis showed that arginine significantly improves survival of LP-A in the samples that were only at 4°C during rehydration in CJ and in the acidified cucumbers after storage for 32 and 64 days. However, there is no significant effect of arginine on day 128. Within the brine dataset, the Tukey HSD test indicates that arginine significantly improved LP-A survival in the samples when the acidified cucumbers were incubated at 4°C on days 64 and 128 regardless of the incubation temperature of the CJ pre-adaptation and on day 32 when the CJ rehydration is performed at 4°C.

It was attempted to utilize primers published for amplification of the arcA gene in *L. plantarum* (Spano and others, 2004) in order to find this gene in *L. plantarum* WCFS1. Although this is not a commercially available probiotic strain, it was decided to perform the preliminary studies on this culture. In a study by Spano and others (2004), the *L. planatum* strain that they found could perform the ADI pathway was identified as *L. plantarum* WCFS1 using 16s rRNA sequencing. The amplicon obtained from L. plantarum WCSF1 for arcA presented an estimated molecular weight of 450 bp (Figure B.2), smaller than that reported by Spano and others (2004). The PCR product was extracted, re-amplified with a Tm of 60 ˚C and analyzed by gel electrophoresis on a 1.7 % agarose gel. There were three bands of 675, ~600 and ~500 bp on the second gel (Figure B.3) and they were extracted, purified and sequenced. Alignment of the sequence obtained with the higher molecular weight (Table A.1E) suggested such gene codes for a *divIVA* of *L. plantarum*, which encodes for a septum site-determining protein, with a 100% identity score using Blastx from the Blast Network Service (NCBI). A Needleman-Wunsch alignment of this amplified sequence and the *arcA* sequence (GenBank accession number AY379068) determined in the study by Spano and others (2004) (Table A.1A) gives a 43% identity score. The sequence (Table A.1) from the band with the lowest molecular weight was identified (99%) using Blastx as the gene *secA* of *L. plantarum*, which encodes for a protein translocase subunit. This sequence showed 32% similarity to the *arcA* sequenced in the study by Spano and others (2004). The identity of the sequence (Table A.1B and A.1C) from the band with an intermediate molecular weight of 600 bp was inconclusive. The sequences from the medium band from

the forward and reverse primer showed very low similarity when aligned using EMBOSS Needle. Blastx identified (51%) the sequence from the reverse primer as a hypothetical protein of *L. plantarum* and the forward primer as *recR* which encodes for a recombination protein. The chromatogram from the reverse primer of the medium size band appeared to have more noise than all other samples sequenced.

Analysis of the bacteria on the surface of the probiotic cucumber slices' skin and mesocarp using SEM suggested that arginine may affect the ability to produce biofilms (Figure 3.3). Within both treatments, there were portions of the slices that showed no bacteria, where the bacteria appeared evenly distributed throughout the surface that was being inspected, or where the cells seemed to be in a biofilm or at least aggregating among themselves or with other materials. However, the occurrence of cells that appeared to be in a biofilm or aggregating together seemed higher in treatment 21, 4, 20, the samples supplemented with arginine.

3.5 Discussion

The effect of amino acid supplementation on the survival of LP-A was investigated because LAB are known to be fastidious and the probiotic products they are usually associated with, dairy, have significantly higher levels of protein. Additionally, amino acids can be used by LAB to control intracellular pH, produce ATP or redox power, and to deal with stress (Fernandez and others, 2006). For example, Vrancken and others (2011) observed that *L. plantarum* IMDO 130201 increased expression of genes related to peptide and amino acid metabolism under low pH conditions.

The goal of this study was to determine the effect of amino acid supplementation on survival in acidified cucumber to contribute to the development of a process that could be implemented to produce a probiotic pickle product. Studies on the effect of nutrients in cucumber fermentation have previously been done. Tripuraneni (2011) found that supplementation of brine with biotin or valine or a combination significantly increased LAB growth and lactic acid production. Costilow and others (1953) determined that all of the vitamins and amino acids required for *Lactobacillus plantarum* growth are available in sufficient quantities in cucumber fermentations. However, the influence of the nutritional content of pickles on the survival of probiotic lactic acid bacteria has not been investigated.

The study of the effect of amino acid supplementation on survival of LP-A in an acidified CJ model system incubated at 4 °C or 21 °C for 24 h followed by 4 °C suggested that the effect of temperature is more relevant than amino acid supplementation. However, there seems to be a positive effect from the supplementation with arginine, an amino acid mixture, an amino acid mixture plus, and casamino acids as compared to the control. The effect of arginine supplementation was also investigated in acidified cucumber chips with positive results. Glutamine and glutamate did not improve the survival of LP-A in this system. These results are consistent with the study by Teixeira and others (2014) because arginine improved the survival of the probiotic cultures at pH 3.5, a pH similar to that in the acidified cucumber systems, while glutamine and glutamate had mostly no effect at this particular pH.

Tryptone was less effective than the casamino acids under both temperature profiles. This may indicate that LP-A in this system prefers free amino acids to peptides. The reasoning that arginine improves survival similarly to the free amino acid mixture and the free amino acid mixture that contains an equivalent concentration of arginine as the arginine treatment cannot be determined from this study. If arginine is the only amino acid that is being used to improve survival, then the survival in the mixture suggests that the concentration required is much lower than what is supplemented in the arginine treatment. The mixture treatment only contains 0.2 g/L as compared to 3.5 g/L in the arginine treatment. The fact that the mixtures result in higher cell counts on day 64 in the 4°C treatment may indicate otherwise, but without replication, the reality of this difference can't be determined. The probiotic culture could be using amino acids other than arginine from the mixtures to improve survival. To determine if this is the case, a treatment with all the free amino acids except arginine should be tested.

The ANOVA of survival as a result of the full factorial of Storage time, Arginine, and Temperature profile with component as a by variable was performed (Figure A.7D and E) with significant differences between treatments documented in table 3.3. Supplementation with 25 mM arginine in the acidified cucumbers showed a trend similar to that observed in the model system with arginine being more effective in the treatments incubated at 4°C than those incubated at 21°C prior to 4°C. Without increasing the temperature of incubation at any point, arginine supplementation in the acidified cucumbers resulted in cell counts of 7.2 and 6.4 $\log_{10}$ CFU mL^{-1} after 32 and 64 days of storage, respectively (Figure 3.2). These

levels are promising in that increasing the initial cell count could result in a probiotic pickle product with a 2-month shelf-life. These results were comparable to the survival in the samples that were incubated at an elevated temperature without arginine supplementation on days 32 and 64. The ability to maintain the product at 4ºC for the whole process may be beneficial to a processor working within a refrigerated facility.

If a four-month shelf life is desired, elevating the storage temperature of the probiotic pickles before refrigeration is required. After this length of storage, cell counts fall to about 2.5 $\log_{10}$ CFU mL^{-1} (0 mM arginine) and 4 $\log_{10}$ CFU mL^{-1} (25 mM arginine) in the samples where the acidified cucumbers are stored at 4°C only, regardless of CJ adaptation temperature (table 3.3). On the other hand, if the acidified cucumbers are incubated at 21ºC for 24 h prior to refrigeration, cell counts on day 128 are approximately 6 $\log_{10}$ CFU mL^{-1} (0 mM arginine) and 7 $\log_{10}$ CFU mL^{-1} (25 mM arginine) (table 3.3).

In the ANOVA of survival as effected by the full factorial of Temperature profile, Arginine, Storage Time, and Component (Figure A.7A), survival of LP-A in the cucumbers was significantly higher than survival in the brine (Figure A.7C). Although, when the pickles are inoculated, the culture is transferred into the product by pipetting an aliquot of the CJ inocula into the brine that surrounds the cucumbers, survival of LP-A within the cucumbers was significantly higher. This could indicate that the cells are somehow attaching to the cucumber and prefer this to being suspended in the brine or that the cells that happen to adhere to or adsorb to or penetrate the cucumber by random chance are surviving better or a combination of the two theories. All main effects and two-way interactions other than those

containing Component are significant. Therefore, the survival of LP-A is significantly higher in the cucumber portion of the product regardless of the treatment and there is no treatment that increases this difference between the counts in the brine and the cucumber.

Arginine was chosen to be investigated in the acidified cucumber chip system because this amino acid resulted in the highest survival at 4 °C on day 32 and at 21→4 °C on days 32 and 64 along with the casamino acids, the amino acids mixture, and the amino acids mixture plus. Arginine was chosen as opposed to the amino acid mixtures, given that supplementation with one amino acid is simpler relative to regulatory concerns and more cost effective. It must be noted that arginine supplementation to a pickled cucumber product would require GRAS approval by the FDA, and comes with health concerns associated with the formation of biogenic amines in the presence their precursors and LAB, including *L. plantarum* (Liu and others, 1998; Arena and others, 1999; Arena and others, 2001; De Angelis et el., 2002; Spano and others, 2004) and production of citrulline which is an ethyl carbamate precursor, a known animal carcinogen (Spano and others, 2004). Ethyl carbamate is a concern in fermented foods (Weber and others, 2008) but its rate of formation is reduced as the temperature is decreased (Stevens and others, 1993). The effect of suboptimal growth temperature on arginine catabolism in LABs has not been studied. It is commonly studied at growth-promoting temperatures (Arena and others, 1999; Vrancken and others, 2011; Liu and others, 1995; Vrancken and others, 2009). However, the activity of arginine deiminase from *Enterococcus faecium* peaked at around 48°C and decreased as temperature increased and decreased from this point with about one third of the activity seem at 48°C when

incubation temperature was 20°C and 70°C. In this study, the probiotic cultures are only exposed to growth-promoting temperatures for 24 h. However, the fact that improved survival of LP-A is observed, even in the refrigerated samples that are supplemented with arginine warrants investigation of its metabolism.

Arginine has been approved as GRAS for use in cover brines that is injected into beef subprimals or mixed into beef patties. In the letter for approval, a risk assessment of human arginine consumption by Shao and others (2008) is cited. In this study, researchers found no evidence of adverse effects of arginine consumption and so the researchers conservatively set the observed safe level of supplemental arginine at 115 mM (20 g/L) for normal healthy adults. They also included that arginine is naturally present in meat products at a level of around 1.5% (1.7 g arginine per 4 oz. meat, 86.1 mM). At their usage rate, 0.068 g arginine would be consumed per 4 oz. of meat. The amount that was added to the probiotic pickles in this study would result in consumption of 0.12 g arginine per 28 g serving of pickles, which is over 15 times less than the amount of arginine, added and already present, that is allowed in a food product according to 21CFR172.320. However, within 21CFR172.320, there are specific guidelines to the use and supplementation of amino acids and addition to a pickled cucumber does not meet the qualifications. Since arginine would be a new additive for a pickled cucumber product, supplementation would require FDA approval.

Although literature supports the fact that consumption of arginine will not cause any adverse health effects, there is still the concern that *Lactobacillus plantarum* has been observed to produce the biogenic amines, putrescine and agmatine, from arginine (Spano and

others, 2004). The first metabolite in the ADI pathway is arginine and this pathway can be performed by LABs to resist acid stress (De Angelis and others, 2002). In this study, it was observed that arginine significantly improves survival of LP-A in the refrigerated samples. Therefore, one hypothesis is that the probiotic culture is improving its acid tolerance by catabolizing arginine through the ADI pathway. The ADI pathway also results in production of a biogenic amine precursor, ornithine. Lactic acid bacteria, including certain strains of *L. plantarum*, can produce putrescine from ornithine (Arena and others, 2001). Agmatine, another biogenic amine, can be synthesized from arginine via arginine decarboxylase in other *Lactobacillus* spp. (Romano and others, 2012; Arena and others, 2001; Wunderlichova and others, 2014). The ability to perform the ADI pathway and to produce biogenic amines varies between strains and is correlated with the environment in which the strain is found (Romano and others, 2012; Spano and others, 2004). The environmental dependence is likely a result of horizontal gene transfer, which lactic acid bacteria are known to perform in order to adapt and evolve (Makarova and others, 2006; Kleerebezem and others, 2003). It has also been observed that the ability to perform the ADI pathway correlates with being a hetero- as opposed to a homofermenter (Liu and others, 1998). Therefore, a pickled cucumber product that is supplemented with arginine and *L. plantarum* cultures should be tested for the presence of relevant metabolites. Even if the culture is shown to lack the relevant genes, the microorganisms that are native to the cucumber may also pose a concern. Therefore, both genetic studies of the probiotic and chemical analyses of the pickled cucumbers should be performed. Due to the fact that the metabolism of arginine via the ADI

pathway is strain specific, it was attempted to determine if the genes encoding for the ADI pathway were present in *L. plantarum* WCSF1, a strain that has been investigated for probiotic qualities in multiple human studies (van den Nieuwboer and others, 2016). A gene search on JGI IMG/M (Walnut Creek, CA, USA) of arginine deiminase (EC 3.5.3.6) within the *Lactobacillus* spp. does not return any *L. plantarum* strains as containing this gene sequence. Attempts to amplify *arcA*, *arcB* and *arcC* from *L. plantarum* WCSF1 were also unsuccessful. Rather than using the degenerative primers that were used to amplify these genes in *L. plantarum* in the study by Spano and others (2004), primers could have been designed based on the sequences these authors published.

Visualizing the samples using the SEM was done in order to determine if there was an effect of arginine on the adsorption of probiotic cultures to the cucumber chips. Based on the slices observed, it appeared that probiotic cultures in the samples supplemented with arginine more often appear aggregated or in biofilms. Therefore, further investigation of arginine involvement in aggregation or biofilm formation should be performed. The catabolism of arginine by certain *L. plantarum* strain can occur via the ADI pathway, which results in the production of ATP and a consumption of intracellular protons, deacidifying the cytoplasm. This could indirectly improve the cell's ability to produce extracellular polymeric substances and biofilms because of the availability of energy for biosynthesis and increase in pH to one more accommodating to enzyme activity. The ideal pH for exopolysaccharide production by *L. delbrueckii* subsp. *Bulgaricus* was 6.2 (Aslim and others, 2005), by *L. pentosus* LPS26 was 6.0 (Sanchez and others, 2006).

Goh and others (2013) observed biofilm formation by diluting an overnight *E. coli* into M63 minimal salt medium supplemented with arginine at 0.05% (2.9 mM), 0.1% (5.7 mM), 0.2% (11.5 mM), and 0.4% (23.0 mM) and incubated at 37 °C for 24 h. Biofilm formation was significantly higher than the control with 11.5 mM arginine, but not at any other concentration tested. Valine, leucine, lysine, phenylalanine, proline, and threonine also increased biofilm formation at 0.2% (17.1 mM) and 0.4% (34.1 mM), 0.05% (3.8 mM) and higher, 0.05% (3.4 mM), 0.1 (6.1 mM), 0.1 (8.7 mM) and higher, and 0.1% (8.4 mM) and higher, respectively. In a study of biofilm formation by *Staphylococcus aureus,* it was observed that deletion of the arginine transporter gene inhibited arginine transport and reduced polysaccharide intercellular adhesion, but did not affect biofilm formation (Zhu and others, 2007). In this study, supplementation with the mixture of free amino acids in the model system resulted in a similar level of survival at all time points as supplementation with arginine. The concentration of arginine in the sample supplemented with the mixture of free amino acids was 0.2 g/L as opposed to ~3.6 g/L in the sample supplemented with only arginine. It could be that arginine is not needed in such large concentrations to support survival. It could also be that other amino acids or a combination of other amino acids were benefitting the probiotics in some way.

It cannot be known as to the origin of some of the substances that are seen on the cucumber. For example, the substance that is present in many of the images, but very concentrated in figure 3.3G and 3.3H is not seen on the cucumber fruit in papers by Smith and others (1979), Samuels and others (1993), or Reina and others (2002). Especially when

looking at figure 3D, it appears that the cells are preferentially binding to the wax. It does seem like the cells are attaching to this substance, especially in figure 3.3G, H, and possibly K where there are high densities of cells. Also, there are no cells seen attached to the raw surface of the cucumber where there are tears in the wax, indicating that the cells preferentially bind to the wax surrounding the fruit. In the studies by Smith and others (1979) and Samuels and others (1993), the surfaces of fresh cucumbers were observed using SEM. In the study by Reina and others (2002), the cucumbers were submerged in water with $1x10^8$ CFU mL^{-1} *S. aureus* and *L. monocytogenes* for 30 or 60 minutes and then washed with distilled water on a platform shaker before taking samples for SEM pictures. In this study, uninoculated acidified cucumbers were not examined and so it cannot be concluded from where this substance is originating. Based on comparison to SEM pictures of cucumbers in inoculated water (Reina and others, 2002), it could be from any ingredient in the brine, some part of the drying medium of the lyophilized cultures, or a product of the probiotic cultures metabolism. It is unlikely that it is solely an artifact of the arginine since it is seen in samples without arginine as well. The long filamentous substance in figure 3.3D is similar to an image in a paper by Chung and Toh (2014) who labeled it as a biofilm of *Staphylococcus aureus* on the surface of a catheter. This substance was only seen on the cucumber chips from the samples supplemented with arginine. The round objects that are lighter in color and appear to be protruding from the surface in picture F are likely trichomes, based on figure 1B published by Smith and others (1979). Smith and others (1979) published SEM

pictures of the surface of pickling cucumber fruit, observing stomata, trichomes, scars from trichomes that are no longer attached, and epidermal cells.

3.6 Conclusion

The effect of amino acid supplementation and temperature profile on survival of the *L. plantarum* probiotic culture in the CJ model system clearly shows that altering the temperature profile is more effective that amino acid supplementation. The general trend observed is that arginine, casamino acids, and the free amino acid mixtures improve survival compared to the non-supplemented control. Investigation of arginine in the acidified cucumber chips shows that arginine significantly improves survival under certain temperature. Future work should include quantification of the metabolites in the arginine deiminase pathway and the biogenic amines that could potentially be synthesized from arginine in probiotic acidified cucumbers. This would show if the culture is using the ADI pathway to survive in this system and if arginine supplementation poses a health concern. Additionally, the biofilm formation rate of the probiotic culture in an acidified cucumber system supplemented with arginine should be investigated further to determine if the trends observed using SEM are real.

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Table 3.1. Amino acid concentration and sources used in acidified cucumber juice for each treatment.

Amino Acid Treatment Number	Amino Acid Supplement	Concentration in the equilibrated acidified CJ	Source, catalog number
1	L-Citrulline	20.4 mM	Sigma, C7629
2	L-Arginine·HCl	20.2 mM	MP Biomedicals, 02100743
3	L-Glutamine	20.1 mM	Sigma, G3202
4	L-Glutamic acid	20.1 mM	Sigma, G8415
5	Tryptone	1%	Bacto, 211699
6	Casamino acids	1%	Bacto, 223050
7	Free amino acid mixture	2%	Sigma, R7131
8	Mixture, arginine, glutamate, citrulline, glutamine	2%, 23.6 mM, 19.6 mM, 20.4 mM, 20.1 mM	See above
9	Tryptophan[a]	0.24 mM	NA
	Threonine[a]	1.6 mM	NA
	Isoleucine[a]	1.6 mM	NA
	Leucine[a]	2.2 mM	NA
	Lysine[a]	2.0 mM	NA
	Methionine[a]	0.40 mM	NA
	Cysteine[a]	0.33 mM	NA
	Phenylalanine[a]	1.2 mM	NA
	Tyrosine[a]	0.61 mM	NA
	Valine[a]	1.9 mM	NA
	Arginine[a]	2.5 mM	NA
	Histidine[a]	0.64 mM	NA
	Alanine[a]	2.7 mM	NA
	Aspartic acid[a]	3.1 mM	NA
	Glutamic acid[a]	13.0 mM	NA
	Glycine[a]	3.2 mM	NA
	Proline[a]	1.3 mM	NA
	Serine[a]	1.9 mM	NA

[a] Estimated concentration in the cucumber according to the USDA ARS National Nutrient Database for Standard Reference Release 28 (Beltsville, MD, USA) for 11205, Cucumber, with peel, raw. Treatment 9 is the control where no amino acids were added.

Table 3.2. Concentration of amino acids in the free amino acid solution in treatments 7 and 8.

Individual Amino Acid	Concentration in R7131[a] (mM)	Concentration in Treatment 7 (mM)	Concentration in Treatment 8 (mM)
L-Arginine	55	1.1	21
L-Asparagine·H2O	21.5	0.43	0.43
L-Aspartic acid	7.5	0.15	0.15
L-Citrulline	0	0	20
L-Cysteine	20.5	0.41	0.41
L-Glutamic acid	7	0.14	20
L-Glutamine	0	0	20
L-Glycine	6.5	0.13	0.13
L-Histidine	4.85	0.097	0.097
Hydroxy-L-proline	7.5	0.15	0.15
L-Isoleucine	19	0.38	0.38
L-Leucine	19	0.38	0.38
L-Lycine·HCl	11	0.22	0.22
L-methionine	5	0.10	0.10
L-Phenylalanine	0.955	0.0191	0.0191
L-Proline	8.5	0.17	0.17
L-serine	14.5	0.29	0.29
L-Threonine	8.5	0.17	0.17
L-Tryptophan	1.25	0.025	0.025
L-Tyrosine	6.5	0.13	0.13
L-Valine	8.5	0.17	0.17

[a]RPMO 1640 Amino Acids Solution (50x) (Sigma-Aldrich), which was supplemented in the acidified CJ of treatment 7 and 8 at 2%.

Table 3.3. Cell count in the acidified cucumbers as a result of arginine supplementation and temperature profile.

					Date of Analysis				
					0	16	32	64	128
Treatment ID	**CJ Rehydration**	**Jar Temperature Profile**	**Arginine (mM)**	**Component**	**Cell Count (log_{10} CFU mL^{-1})**				
21,4,0	21°C (24h)	4°C	0	C	8.20 ± 0.07^{A}	NA	6.34 ± 0.43^{CDE}	4.99 ± 0.45^{EFG}	2.71 ± 0.63^{H}
				B	8.20 ± 0.07^{A}	7.18 ± 0.13^{ABCD}	5.92 ± 0.48^{DE}	4.53 ± 0.47^{EF}	2.00 ± 0.00^{G}
21,4,20	21°C (24h)	4°C	20	C	8.20 ± 0.07^{A}	NA	7.06 ± 0.31^{ABCD}	6.51 ± 0.65^{BCDE}	4.09 ± 0.91^{GH}
				B	8.20 ± 0.07^{A}	7.44 ± 0.26^{ABCD}	6.71 ± 0.36^{ABCD}	6.11 ± 0.66^{BCD}	3.99 ± 0.97^{F}
4,21,0	4°C (18h)	21°C (24h)_4°C	0	C	8.20 ± 0.12^{AB}	NA	7.66 ± 0.24^{ABCD}	6.90 ± 0.45^{ABCD}	5.96 ± 0.56^{DEF}
				B	8.20 ± 0.12^{AB}	7.7 ± 0^{ABC}	7.2 ± 0.14^{ABCD}	6.75 ± 0.28^{ABCD}	6.11 ± 0.26^{CD}
4,21,20	4°C (18h)	21°C (24h)_4°C	20	C	8.20 ± 0.12^{AB}	NA	7.98 ± 0.05^{ABC}	7.53 ± 0.027^{ABCD}	6.91 ± 0.14^{ABCD}
				B	8.20 ± 0.12^{AB}	7.92 ± 0.03^{AB}	7.69 ± 0.18^{ABC}	7.11 ± 0.027^{ABCD}	6.91 ± 0.38^{ABCD}
4,4,0	4°C (18h)	4°C	0	C	8.20 ± 0.12^{AB}	NA	4.36 ± 0.15^{FGH}	3.11 ± 1.57^{H}	2.39 ± 0.54^{H}
				B	8.20 ± 0.12^{AB}	6.82 ± 0.42^{ABCD}	4.19 ± 0.30^{EF}	3.10 ± 1.56^{FG}	2.00 ± 0.00^{G}

Table 3.3. Continued

4,4,20	4°C (18h)	4°C	20	C	8.20 ± 0.12^{AB}	NA	7.18 ± 0.52^{ABCD}	6.42 ± 0.47^{ABCDE}	4.10 ± 0.19^{FGH}
				B	8.20 ± 0.12^{AB}	7.55 ± 0.37^{ABCD}	6.78 ± 0.41^{ABCD}	6.29 ± 0.07^{BCDE}	4.16 ± 1.03^{F}

Temperature of incubation is listed for the CJ rehydration and the acidified cucumbers with the time of incubation in parentheses. Underscore indicates a change of incubation temperature to the temperature following the underscore. Data with different superscripted letters represent cell counts that are significantly different from one another. Cucumber (C) and brine (B) data were analyzed separately.

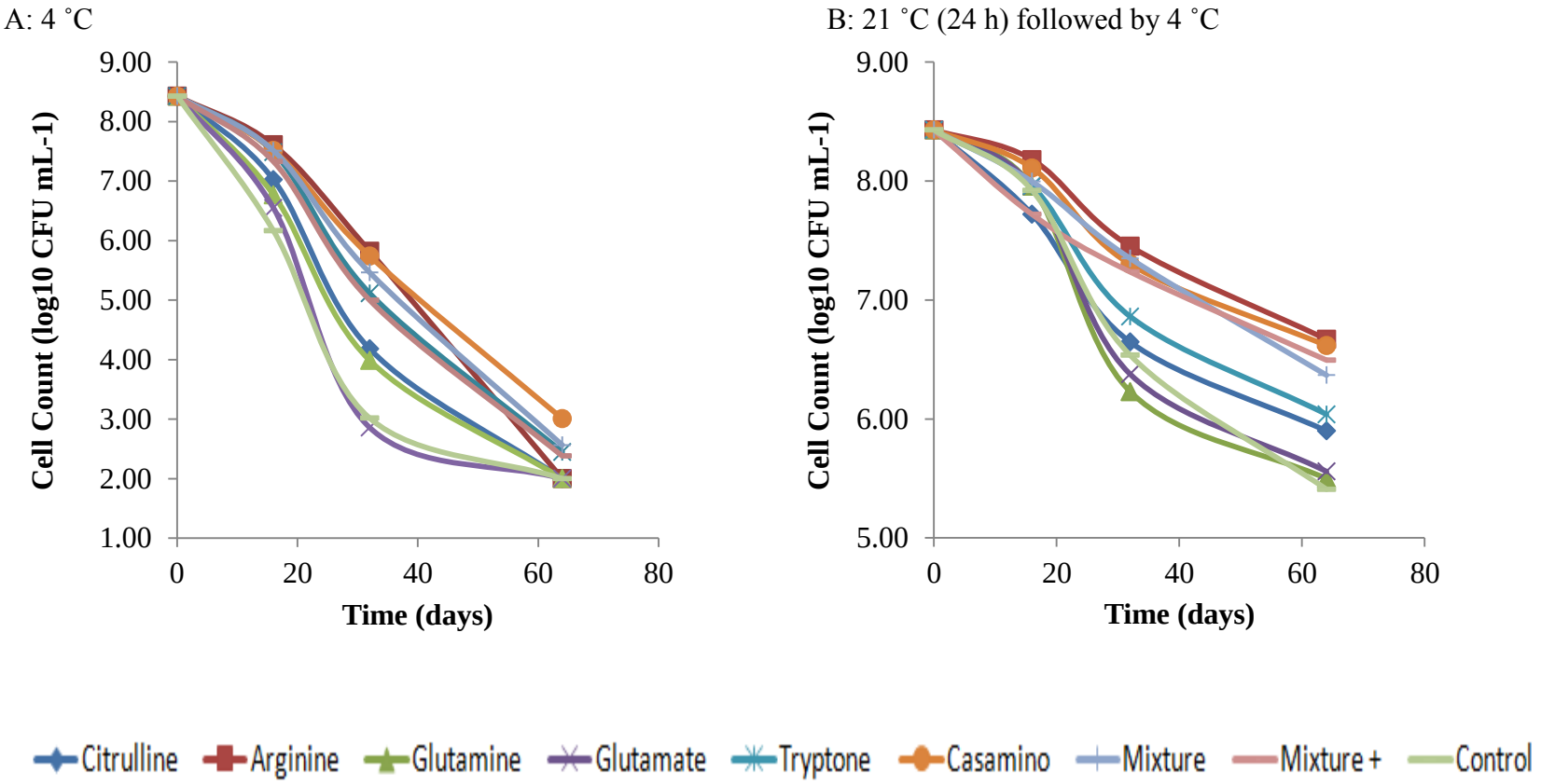


Figure 3.1. Effect of temperature profile and supplementation of amino acids on survival of LP-A in the model system (acidified CJ).

A) 4°C; B) 21°C for 24 h followed by 4°C.

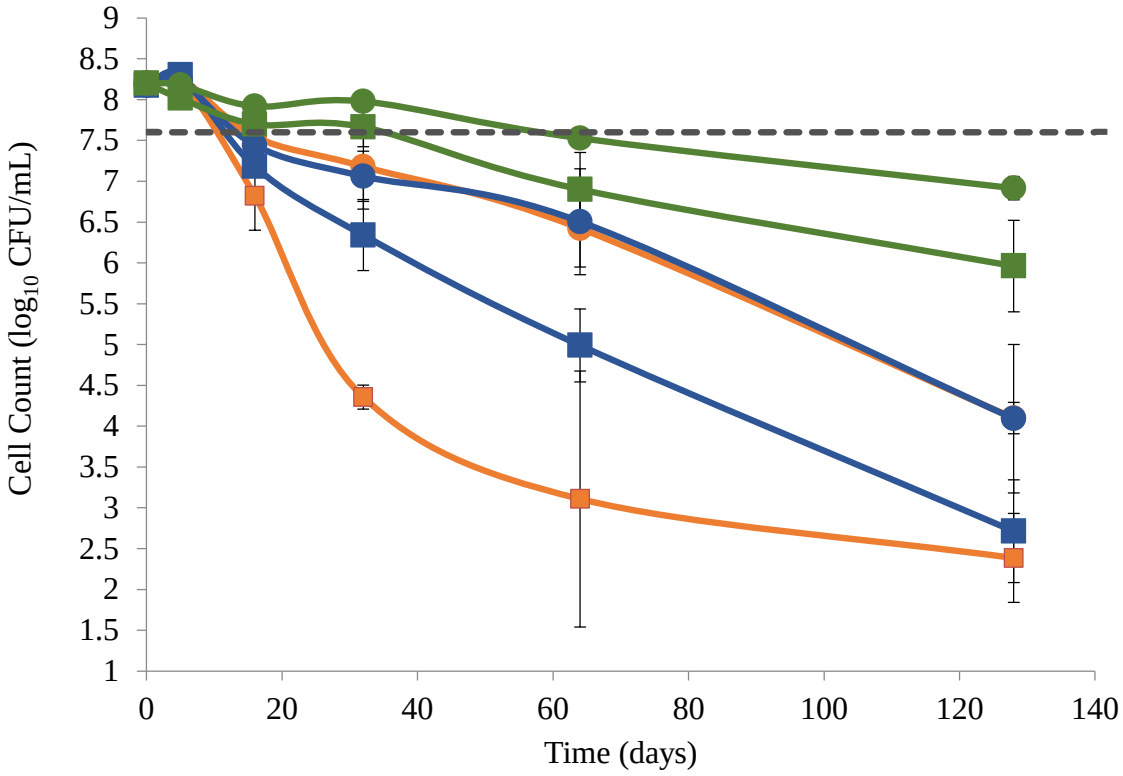
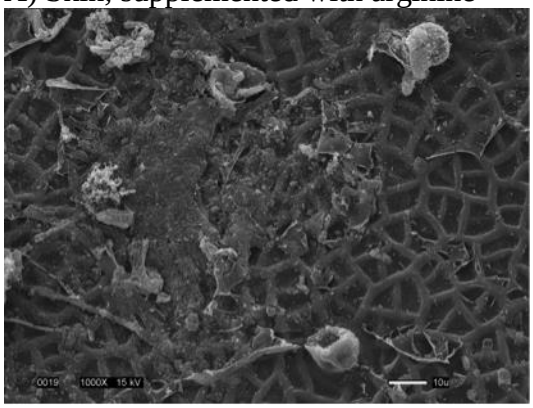


Figure 3.2. Effect of arginine supplementation and temperature profile on survival of LP-A in acidified cucumbers as a function of time.

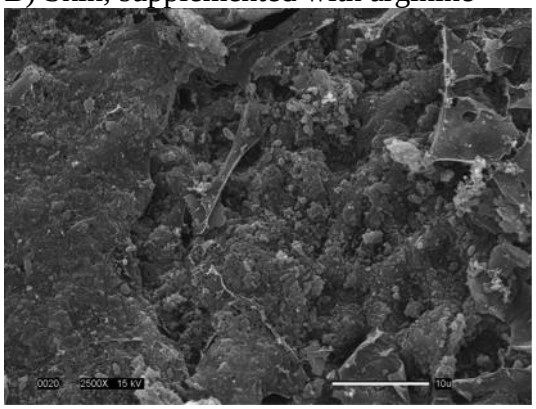
Orange: 4°C CJ inoculum and 4°C jar; Green: 4°C CJ pre-adaptation and 21°C (24 h) → 4°C product storage; Blue: 21°C CJ pre-adaptation and 4°C product storage. Squares: 0 mM arginine; Circles: 25 mM arginine. The green dotted line represents the cell count required for the product to be considered probiotic.

Figure 3.3. SEM images of probiotic pickled cucumber chip skin (A, B, C, D, E, F) and mesocarp (G, H, I, J, K, L) with (A, B, C, D, G, H) and without arginine (E, F, I, J, K, L).

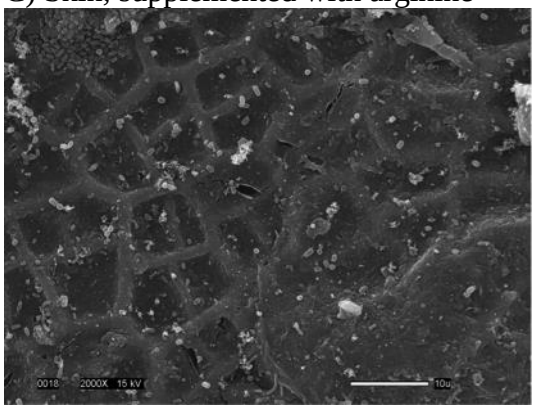
A) Skin, supplemented with arginine



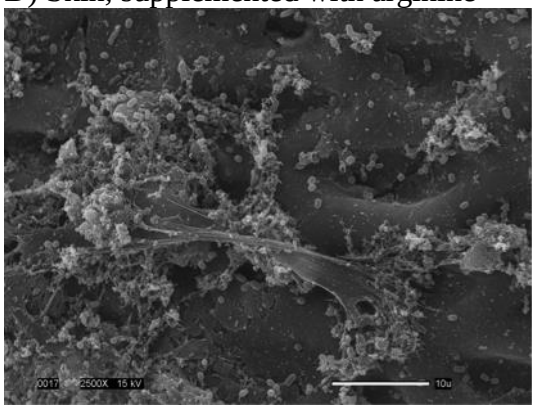
B) Skin, supplemented with arginine



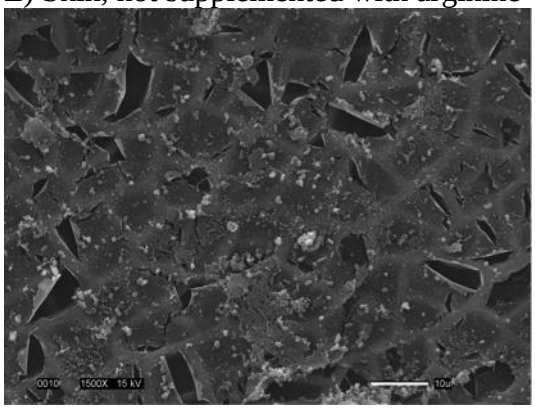
C) Skin, supplemented with arginine



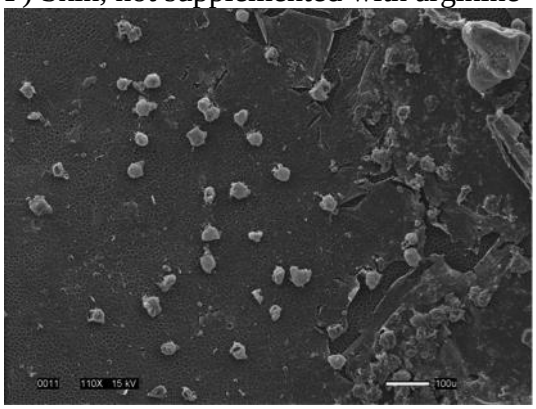
D) Skin, supplemented with arginine



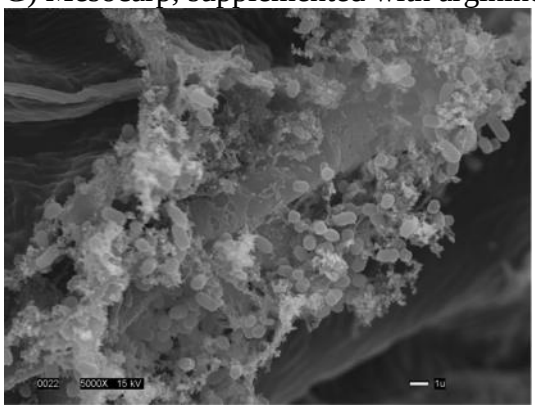
E) Skin, not supplemented with arginine



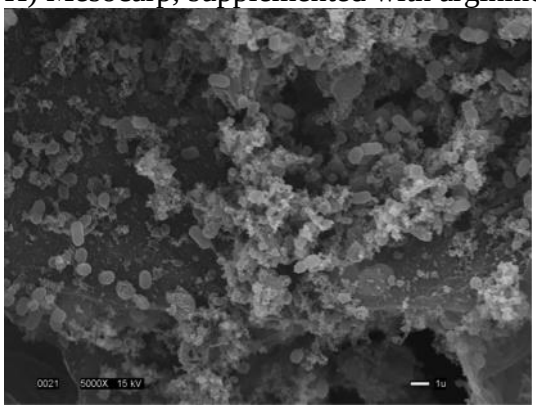
F) Skin, not supplemented with arginine



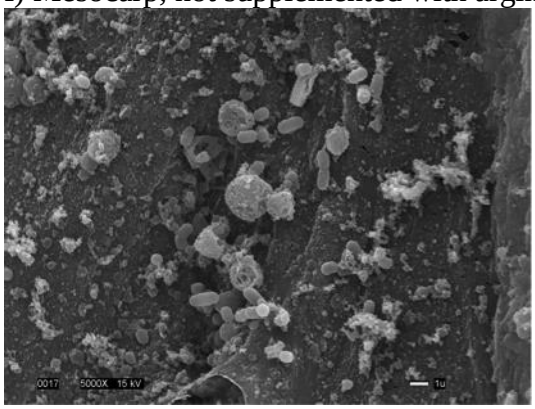
G) Mesocarp, supplemented with arginine



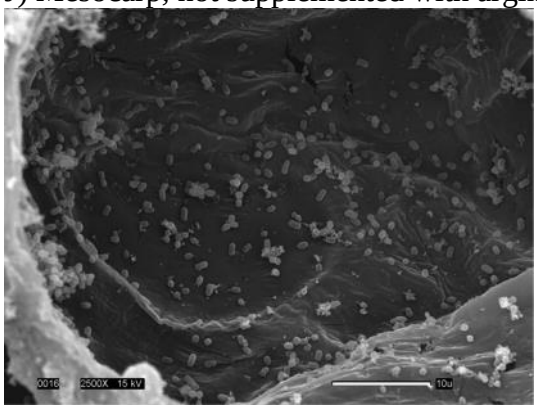
H) Mesocarp, supplemented with arginine



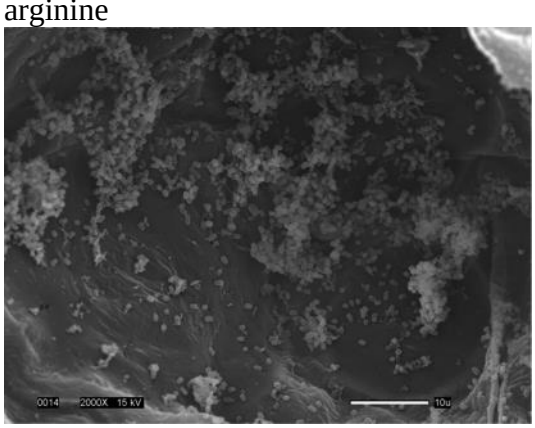
I) Mesocarp, not supplemented with arginine



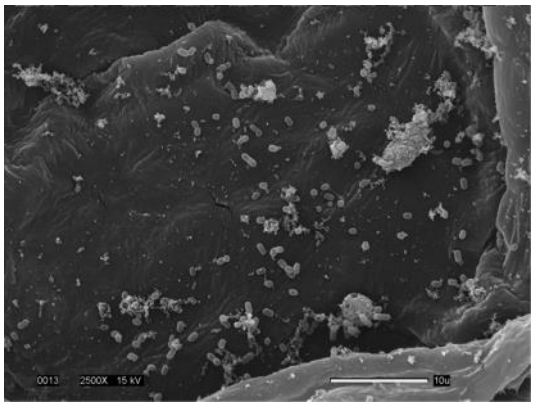
J) Mesocarp, not supplemented with arginine



K) Mesocarp, not supplemented with arginine



L) Mesocarp, not supplemented with arginine



APPENDICES

Appendix A: Supplemental Statistical Information

Supplemental Statistical Information for Chapter 2

Modeling of the effect of sodium benzoate concentration in the cover brine on survival of LP-A, LP-B, and LP-C

The survival of LP-A, PA-B, and LP-C in un-equilibrated cover brine supplemented with 0, 3, 6, or 9 mM or in a saline control was modeled in JMP using ANOVA. However, when survival was modeled by the effects of Sodium Benzoate, Strain, and Sodium benzoate*Strain, the Residual by Predicted plot indicated that the assumption of homogeneity of variance was violated (Figure A.1A). This is supported by the fact that the variability associated with the replicates of LP-C survival is much greater than for the replicates of LP-A and LP-B. In the ANOVA that models the survival of all strains together, the error associated with the cover brine treatments containing 0 and 3 mM sodium benzoate inoculated with LP-C is likely decreasing the power of the model to differentiate treatments. In this model, there is no significant difference in survival between cover brine treatments within strain, other than LP-C. The survival of LP-C in the 4 cover brines is significantly lower than that in the saline control (Figure A.1D). This is the opposite of what was seen in the ANOVA modeling the survival of each probiotic culture individually. These models show significant differences in cell counts between sodium benzoate concentrations for all three strains (Figure A.2). This is likely because the survival data of LP-A and PA-B have less error between replicates, artificially increasing the power to differentiate treatment

effects when it comes to probiotic strain LP-C, which has more error between replicates. The lettering in Figure 2.3 is representative of the analysis of the strains individually.

A)

Effect Tests

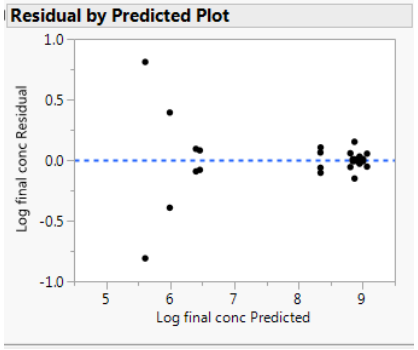
Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Sodium benzoate (mM)	4	4	4.726942	10.1717	0.0003*
Strain	2	2	35.447213	152.5546	<.0001*
Sodium benzoate (mM)*Strain	8	8	4.816870	5.1826	0.0031*

B)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	14	44.991025	3.21364	27.6612
Error	15	1.742682	0.11618	Prob > F
C. Total	29	46.733707		<.0001*

C)



D)

Level		Least Sq Mean
Saline,B	A	9.0671373
Saline,A	A	9.0065826
9,B	A	8.9503924
3,A	A	8.9488725
6,A	A	8.9440558
3,B	A	8.8725304
6,B	A	8.8717145
9,A	A	8.8442190
0,A	A	8.8074207
0,B	A	8.3429121
Saline,C	A	8.3414514
9,C	B	6.4611665
6,C	B	6.4000933
3,C	B	5.9926106
0,C	B	5.6096826

Figure A.1. JMP output from the ANOVA modeling the survival of LP-A, LP-B, and LP-C as a result of brine treatment (Sodium benzoate), strain (LP-A, LP-B, or LP-C), and brine treatment*strain.

A) Residual by Predicted Plot showing a violation of the assumption of homogeneity of variance; B) ANOVA table; C) Effects Tests table; D) LSMeans Differences Tukey HSD test of brine treatment.

A)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	4	0.05369689	0.013424	8.7524
Error	5	0.00766892	0.001534	Prob > F
C. Total	9	0.06136581		0.0176*

Parameter Estimates

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Sodium benzoate (mM)	4	4	0.05369689	8.7524	0.0176*

B)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	4	0.62224275	0.155561	10.3000
Error	5	0.07551501	0.015103	Prob > F
C. Total	9	0.69775776		0.0124*

Parameter Estimates

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Sodium benzoate (mM)	4	4	0.62224275	10.3000	0.0124*

C)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	4	8.867873	2.21697	6.6796
Error	5	1.659498	0.33190	Prob > F
C. Total	9	10.527370		0.0306*

Parameter Estimates

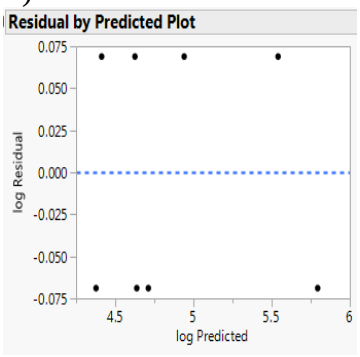
Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Sodium benzoate (mM)	4	4	8.8678725	6.6796	0.0306*

Figure A.2. JMP output from the ANOVA modeling the survival of A) LP-A, B) LP-B, C) LP-C as a result of brine treatment.

Modeling the effect of sorbic and acetic acid and incubation time of the CJ rehydration on long-term survival of LP-A in the acidified cucumbers

A)



B)

Summary of Fit	
RSquare	0.980404
RSquare Adj	0.862828
Root Mean Square Error	0.194792
Mean of Response	4.880183
Observations (or Sum Wgts)	8

C)

Analysis of Variance				
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	6	1.8983573	0.316393	8.3385
Error	1	0.0379438	0.037944	Prob > F
C. Total	7	1.9363011		0.2591

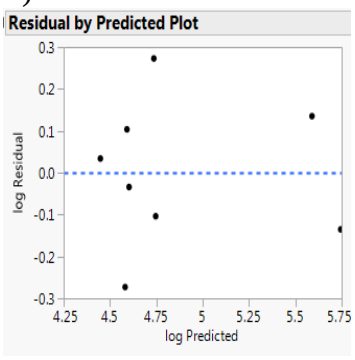
D)

Effect Tests					
Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
sorbic	1	1	0.66411703	17.5027	0.1494
time	1	1	0.04809216	1.2675	0.4624
sorbic*time	1	1	0.11413199	3.0079	0.3330
pH	1	1	0.36203010	9.5412	0.1993
sorbic*pH	1	1	0.64114562	16.8972	0.1519
time*pH	1	1	0.05727553	1.5095	0.4349

Figure A.3. JMP output from the ANOVA modeling the survival of LP-A in acidified cucumbers as a result of CJ adaptation modified by the addition of acetic acid (pH) and sorbic acid and by time of incubation (2 or 18 h).

A) Residual by Predicted Plot; B) Summary of Fit table; C) Analysis of Variance table; D) Effects Test table.

A)



B)

Summary of Fit

RSquare	0.891881
RSquare Adj	0.747722
Root Mean Square Error	0.264166
Mean of Response	4.880183
Observations (or Sum Wgts)	8

C)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	4	1.7269498	0.431737	6.1868
Error	3	0.2093513	0.069784	Prob > F
C. Total	7	1.9363011		0.0831

D)

Effect Tests

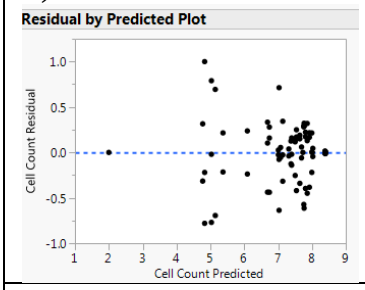
Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
sorbic	1	1	0.66366457	9.5103	0.0540
time	1	1	0.04809216	0.6892	0.4673
pH	1	1	0.37404748	5.3601	0.1035
sorbic*pH	1	1	0.64114562	9.1876	0.0563

Figure A.4. JMP output from the reduced ANOVA modeling the survival of LP-A in the acidified cucumbers as a result of CJ adaptation.

A) Residual by Predicted Plot; B) Summary of Fit table; C) Analysis of Variance table; D) Effects Test table.

Modeling the effect of temperature profile and trehalose on survival of LP-A in the model system

A)



B)

Summary of Fit

RSquare	0.963423
RSquare Adj	0.941809
Root Mean Square Error	0.360107
Mean of Response	7.234471
Observations (or Sum Wgts)	106

C)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	39	225.43207	5.78031	44.5746
Error	66	8.55869	0.12968	Prob > F
C. Total	105	233.99076		<.0001*

D)

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Storage Time	3	3	113.25976	291.1327	<.0001*
Temperature Profile	4	4	73.45844	141.6180	<.0001*
Storage Time*Temperature Profile	12	12	50.95431	32.7443	<.0001*
Trehalose	1	1	0.09044	0.6974	0.4067
Storage Time*Trehalose	3	3	0.20711	0.5324	0.6617
Temperature Profile*Trehalose	4	4	0.04877	0.0940	0.9840
Storage Time*Temperature Profile*Trehalose	12	12	0.54519	0.3504	0.9756

E)

Contrast

Test Detail

0,14	0
0,21-14	0
0,21-14-4	0
0,21-4	0
0,4	0
16,14	0.1667
16,21-14	-0.167
16,21-14-4	0
16,21-4	0.1667
16,4	-0.167
32,14	0.1667
32,21-14	-0.167
32,21-14-4	0
32,21-4	0.1667
32,4	-0.167
64,14	0.1667
64,21-14	-0.167
64,21-14-4	0
64,21-4	0.1667
64,4	-0.167
Estimate	1.046
Std Error	0.0949
t Ratio	11.023
Prob>|t|	1e-16
SS	15.757

SS	NumDF	DenDF	F Ratio	Prob > F
15.76	1	66	121.5059	<.0001*

Figure A.5. JMP output of the ANOVA modeling the full factorial of Incubation time, Temperature (profile), and Trehalose on survival of probiotic LP-A in the acidified CJ model system.

A) Residual by Predicted Plot; B) Summary of Fit table; C) Analysis of Variance table; D) Effects Test table; E) Contrast analysis of the difference between 21-14°C and 21-4°C with the difference between 14°C and 4°C.

Comparison of two different models in SAS

The relationship between cell count and storage time appeared to be linear in the experiment where the effect of temperature and trehalose were investigated in the model system (figure 2.5). Therefore, the temperature profile treatments were compared using the slopes of line fitted to the data. Statistical analysis of the data in this manner was comparable to analyzing the data as individual data points. Estimating the difference between the temperature profiles in both models gave the same results with very similar p-values.

Data analyzed as individual data points

```
proc glm data=data;
        class temp treh day;
        model count=temp|treh|day cj;
        lsmeans temp*treh*day;
        ods output lsmeans=lsm;
        estimate "14-4" temp 1 0 0 0 -1;
        estimate "21_4-4" temp 0 0 0 1 -1;
        estimate "21_14_4-4" temp 0 0 1 0 -1;
        estimate "21_14-4" temp 0 0 1 0 -1;
        estimate "14-21_4" temp 1 0 0 -1 0;
        estimate "14-21_14_4" temp 1 0 -1 0 0;
        estimate "14-21_14" temp 1 -1 0 0 0;
        estimate "21_4-21_14_4" temp 0 0 1 -1 0;
        estimate "21_4-21_14" temp 0 1 0 -1 0;
        estimate "21_14_4-21_14" temp 0 -1 1 0 0;
 run;
```

Dependent Variable: count

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	30	169.6246338	5.6541545	29.96	<.0001
Error	45	8.4935975	0.1887466		
Corrected Total	75	178.1182313			

R-Square	Coeff Var	Root MSE	count Mean
0.952315	6.409382	0.434450	6.778341

Source	DF	Type III SS	Mean Square	F Value	Pr > F
temp	4	93.22025423	23.30506356	123.47	<.0001
treh	1	0.11359711	0.11359711	0.60	0.4419
temp*treh	4	0.06257685	0.01564421	0.08	0.9873
day	2	56.92506324	28.46253162	150.80	<.0001
temp*day	8	27.71656582	3.46457073	18.36	<.0001
treh*day	2	0.18519546	0.09259773	0.49	0.6155
temp*treh*day	8	0.52950919	0.06618865	0.35	0.9405
cj	1	0.06242527	0.06242527	0.33	0.5681

Data analyzed as slopes of the lines as plotted in Figure 2.5

```
proc glm data=one;
        where day > 0;
        class temp treh cj;
        model count=temp*day/solution;
        estimate "slope14-slope4" temp*day -1 0 0 0 1;
        estimate "slope21-4-slope4" temp*day 0 0 0 -1 1;
        estimate "slope21-14-4-slope4" temp*day 0 0 -1 0 1;
        estimate "slope21-14-slope4" temp*day 0 -1 0 0 1;
        estimate "slope14-slope21-4" temp*day -1 0 0 1 0;
        estimate "slope14-slope21-14-4" temp*day -1 0 1 0 0;
        estimate "slope14-slope21-14" temp*day -1 1 0 0 0;
        estimate "slope21-4-slope21-14-4" temp*day 0 0 -1 1 0;
        estimate "slope21-4-slope21-14" temp*day 0 -1 0 1 0;
        estimate "slope21-14-4-slope21-14" temp*day 0 -1 1 0 0;
run;
```

Dependent Variable: count

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	165.4796489	33.0959298	183.30	<.0001
Error	70	12.6385824	0.1805512		
Corrected Total	75	178.1182313			

R-Square	Coeff Var	Root MSE	count Mean
0.929044	6.268689	0.424913	6.778341

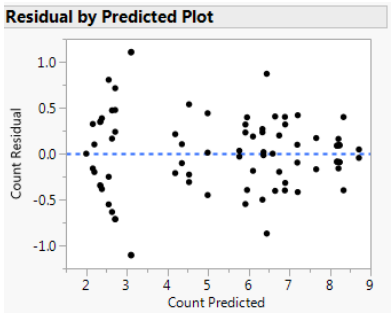
Source	DF	Type III SS	Mean Square	F Value	Pr > F
day*temp	5	165.4796489	33.0959298	183.30	<.0001

Parameter	Estimate	Standard Error	t Value	Pr > |t|
slope14-slope4	-0.08236719	0.00387375	-21.26	<.0001
slope21-4-slope4	-0.05348230	0.00387375	-13.81	<.0001
slope21-14-4-slope4	-0.06432949	0.00387375	-16.61	<.0001
slope21-14-slope4	-0.08111105	0.00399144	-20.32	<.0001
slope14-slope21-4	-0.02888489	0.00387375	-7.46	<.0001
slope14-slope21-14-4	-0.01803770	0.00387375	-4.66	<.0001
slope14-slope21-14	-0.00125614	0.00399144	-0.31	0.7539
slope21-4-slope21-14-4	-0.01084719	0.00387375	-2.80	0.0066
slope21-4-slope21-14	-0.02762875	0.00399144	-6.92	<.0001
slope21-14-4-slope21-14	-0.01678156	0.00399144	-4.20	<.0001

Parameter	Estimate	Standard Error	t Value	Pr > |t|
Intercept	8.428526123	0.10164578	82.92	<.0001
day*temp 14	-0.020776539	0.00357534	-5.81	<.0001
day*temp 21-14	-0.022032681	0.00358894	-6.14	<.0001
day*temp 21-14-4	-0.038814242	0.00357534	-10.86	<.0001
day*temp 21-4	-0.049661430	0.00357534	-13.89	<.0001
day*temp 4	-0.103143731	0.00357534	-28.85	<.0001

Modeling the effect of temperature profile on the survival of LP-A in acidified cucumbers

A)



B)

Summary of Fit

RSquare	0.97368
RSquare Adj	0.952714
Root Mean Square Error	0.527579
Mean of Response	5.141542
Observations (or Sum Wgts)	107

C)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	47	607.52092	12.9260	46.4395
Error	59	16.42206	0.2783	Prob > F
C. Total	106	623.94299		<.0001*

D)

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Trt	5	5	201.54296	144.8178	<.0001*
Day	3	3	210.59483	252.2033	<.0001*
Trt*Day	15	15	147.66965	35.3691	<.0001*
Component	1	1	0.15973	0.5739	0.4517
Trt*Component	5	5	1.54540	1.1104	0.3647
Day*Component	3	3	0.57939	0.6939	0.5595
Trt*Day*Component	15	15	1.44340	0.3457	0.9870

Figure A.6. JMP output of the ANOVA of the survival of LP-A as a result of Temperature profile (Trt), Component (brine vs. cucumber), and Storage time (Day).

Temperature profile includes temperature of the CJ rehydration and the acidified cucumbers. A) Residual by Predicted plot; B) Summary of Fit table; C) Analysis of Variance table; D) Effects Test table.

Discussion of mathematically determining the required initial probiotic LP-A cell count in order to achieve a probiotic pickle product

Calculating the initial cell count required based on the standard deviation observed in this study is unwarranted because the confidence interval of this standard deviation, due to the low number of replicates, is (0.04, 202.5) (Equation A1C).

(0.04, 202.5) (Equation A.1C).

Equation A.1C. $\left(\frac{s^2(df)}{\chi^2_{df,\frac{\alpha}{2}}}, \frac{s^2(df)}{\chi^2_{df,1-\frac{\alpha}{2}}}\right)$

$\left(\frac{0.45^2(1)}{5.024}, \frac{0.45^2(1)}{0.001}\right)$ = (0.04, 202.5)

Other assumptions that could be made in calculating the probiotic cell count required at 2 months is that the standard deviation is within some percentage of the standard deviation observed or that the standard deviation is consistent regardless of the treatment and take the average of the standard deviations for all treatments and increase the N to the number of replicates total. If it was assumed that the standard deviation is within 20% of the standard deviation observed, this would mean the cell count required at 2 months would be 8.4 $\log_{10}$ CFU g^{-1} cucumber (Equation A.2A).

Equation A.2A. $\mu = 7.5 + 1.645(0.45 * 1.2)$

$\mu = 8.39$

If this method is used and it is assumed that the survival rate will be 5.3%, the initial cell count required would be 9.66 $\log_{10}$ CFU mL^{-1} cucumber (equation 2b).

Equation A.2B. $log_{10}\left(\frac{10^{8.39}}{0.053}\right) = 9.66$

The average standard deviation at 2 months is 0.607 and the number of replicates is 21. Based on this pooled standard deviation, at 2 months, there should be 7.72 $\log_{10}$ CFU g^{-1} cucumber (Equation A.3A).

Equation A.3A. $\mu = 7.5 + 1.645\left(\frac{0.607}{\sqrt[2]{21}}\right)$

$$\mu = 7.72$$

Based on this method, the initial cell count required would be 9.00 $\log_{10}$ CFU mL^{-1} cucumber (Equation A.3B).

Equation A.3B. $\log\left(\frac{10^{7.72}}{0.053}\right) = 9.00$

This method of calculating the required initial cell count is likely not appropriate because the standard deviation of the average of the standard deviations for the 9 treatments on day 64 is 0.41 which is 91% of the standard deviation for the treatment of interest. Also, it is not known if there is an effect of treatment on the variability observed between replicates. The confidence interval of the population standard deviation in this case would be (0.22, 0.77) (Equation A.3C).

Equation A.3C. $\left(\frac{0.607^2(21-1)}{34.170}, \frac{0.607^2(21-1)}{9.591}\right)$

$$= (0.22, 0.77)$$

If the upper limit for the confidence interval for the population is used to calculate the cell count required at 2 months, that number is 8.77 $\log_{10}$ CFU g^{-1} cucumber (Equation A.4D). This would mean an initial cell count of 10 $\log_{10}$ CFU mL^{-1} cucumber is required (Equation A.4E).

Equation A.3D. $\mu = 7.5 + 1.645(0.77)$

$$\mu = 8.77$$

Equation A.3E. $\log\left(\frac{10^{8.77}}{0.053}\right) = 10.0$

There are various methods that could be used to determine what initial cell count is required. However, they are not validated and the best option would be to repeat the study enough times so that the upper limit of the population standard deviation is more realistic. For example, if the sample standard deviation is accurate and the experiment was repeated a total of 9 times, the upper limit of the population standard deviation confidence interval would be brought down to 1.65 and the required initial cell count would be 9.7 $\log_{10}$ CFU mL.

Supplemental Statistical Information for Chapter 3

Statistical analysis of the effect of arginine supplementation and temperature profile on survival of LP-A in acidified cucumbers.

A) R^2=0.96

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	47	406.19445	8.64244	33.3427
Error	63	16.32962	0.25920	Prob > F
C. Total	110	422.52407		<.0001*

Parameter Estimates

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Day	3	3	215.20025	276.7490	<.0001*
Component	1	1	0.98412	3.7967	0.0558
Day*Component	3	3	0.46116	0.5931	0.6219
Arginine_mM	1	1	34.57180	133.3787	<.0001*
Day*Arginine_mM	3	3	12.25710	15.7627	<.0001*
Component*Arginine_mM	1	1	0.01360	0.0525	0.8196
Day*Component*Arginine_mM	3	3	0.16476	0.2119	0.8878
OverallTemperatureProfile	2	2	66.47548	128.2319	<.0001*
Day*OverallTemperatureProfile	6	6	34.17436	21.9742	<.0001*
Component*OverallTemperatureProfile	2	2	0.17837	0.3441	0.7102
Day*Component*OverallTemperatureProfile	6	6	0.26572	0.1709	0.9837
Arginine_mM*OverallTemperatureProfile	2	2	9.68987	18.6919	<.0001*
Day*Arginine_mM*OverallTemperatureProfile	6	6	5.75140	3.6982	0.0033*
Component*Arginine_mM*OverallTemperatureProfile	2	2	0.07007	0.1352	0.8738
Day*Component*Arginine_mM*OverallTemperatureProfile	6	6	0.18958	0.1219	0.9933

B) R^2=0.96

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	24	404.75250	16.8647	81.6114
Error	86	17.77157	0.2066	Prob > F
C. Total	110	422.52407		<.0001*

Lack Of Fit

Parameter Estimates

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Day	3	3	215.37284	347.4101	<.0001*
Component	1	1	1.21932	5.9005	0.0172*
Arginine_mM	1	1	34.75203	168.1717	<.0001*
Day*Arginine_mM	3	3	12.18006	19.6472	<.0001*
OverallTemperatureProfile	2	2	66.59452	161.1318	<.0001*
Day*OverallTemperatureProfile	6	6	34.11384	27.5139	<.0001*
Arginine_mM*OverallTemperatureProfile	2	2	9.67253	23.4036	<.0001*
Day*Arginine_mM*OverallTemperatureProfile	6	6	5.76936	4.6532	0.0004*

C)

Component

Least Squares Means Table

Level	Least Sq Mean	Std Error	Mean
B	6.1096833	0.06270386	5.98359
C	6.3198744	0.06168144	6.26818

LSMeans Differences Student's t

α= 0.050 t= 1.98827

Level		Least Sq Mean
C	A	6.3198744
B	B	6.1096833

D) ANOVA of Brine data, R^2=0.96

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	23	209.39639	9.10419	34.9693
Error	31	8.07080	0.26035	Prob > F
C. Total	54	217.46719		<.0001*

Parameter Estimates

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Day	3	3	110.22873	141.1299	<.0001*
Arginine_mM	1	1	17.83999	68.5235	<.0001*
Day*Arginine_mM	3	3	6.26240	8.0180	0.0004*
OverallTemperatureProfile	2	2	34.11623	65.5204	<.0001*
Day*OverallTemperatureProfile	6	6	18.73377	11.9928	<.0001*
Arginine_mM*OverallTemperatureProfile	2	2	4.99125	9.5857	0.0006*
Day*Arginine_mM*OverallTemperatureProfile	6	6	2.87843	1.8427	0.1231

E) ANOVA of Cucumber data, R^2=0.96

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	23	195.11440	8.48323	32.8695
Error	32	8.25882	0.25809	Prob > F
C. Total	55	203.37323		<.0001*

Parameter Estimates

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Day	3	3	105.18584	135.8525	<.0001*
Arginine_mM	1	1	16.73686	64.8494	<.0001*
Day*Arginine_mM	3	3	6.15175	7.9453	0.0004*
OverallTemperatureProfile	2	2	32.49877	62.9606	<.0001*
Day*OverallTemperatureProfile	6	6	15.67194	10.1205	<.0001*
Arginine_mM*OverallTemperatureProfile	2	2	4.77380	9.2484	0.0007*
Day*Arginine_mM*OverallTemperatureProfile	6	6	3.06799	1.9812	0.0977

Figure A.7. JMP output of the ANOVA modeling the survival of LP-A in acidified cucumber chips as a result of Temperature profile, Storage time (Day), and Arginine supplementation.

A) JMP output of the ANOVA model with the full factorial of the effects, component, temperature profile, arginine, and storage time (day). B) Insignificant effects are removed from the model. C) Student's t-test of the component effect shows count in cucumber is significantly higher. D) JMP output of the ANOVA modeling the survival of LP-A in the brine. E) JMP output of the ANOVA modeling the survival of LP-A in the cucumber.

Appendix B - Investigation of the presence of the gene encoding the arginine deiminase enzyme in *L. plantarum* WCFS1

Table B.1. DNA sequences referenced in Chapter 3

A)
1 ccatatctca aaattgctca aaaagagcat gatttctttg ctcaaacatt gcgtgacaat
61 ggtgctgaaa ctgtttatat cgaaaatctt gcaacagaag tttttgaaaa atcatctgaa
121 acaaaagaag agtttttaag ccatttgttg catgaagcag gttaccgtcc aggacgtact
181 tatgatggat tgactgaata tttaacttca atgccaacaa aagatatggt tgaaaaagtc
241 tatgccggtg ttcgtaaaaa tgaattggat atcaaacgca cagcacttag tgacatggca
301 ggttctgatg cagaaaatta tttctacctc aacccattac caaatgctta cttcacacgt
361 gacccacaag cttcaatggg tgtcggaatg actattaata aaatgacttt cccagcacgt
421 caacctgaaa gcttgattac cgaatatgtg atggctaacc atccacgttt caaagacact
481 ccaatctggc gtgatcgtaa tcatactact cgtattgaag gtggtgatga attaattctt
541 aataagacaa ctgtagcaat cggggtttca gaacgtactt catctaaaac aattcaaaat
601 cttgctaaag aattatttgc aaatccactt tctacatttg atacagtgct tgcggttgaa
661 atccctcata accatgcaat gatgcacttg gacacagtat ttacaatgat taaccat

B)
1 gtcttctcag caataggagc ctccaatatg atcatgataa tcgaatgggg acggttagag
61 atcatagtaa tcgtcccatg gccgaggatg tcgttgggac catgatataa cacttcatct
121 tcagtggcga atgttatgta gtaggtatgt gtcatgaaga aaattccgtg tcgtcgtaga
181 cgttacagca caatcccccа acctgctatc aatgaactat gtgtctgtgt gaaacgcttg
241 cacacggtcc catagtccgt cgatcgctgc atgtgggata agttcagtga gtcaacgata
301 caataagctt tttggtcaga caattcataa ttccggctct tttgtccgcg aaatgaaagg
361 cctaataggg aataccatct tcatcgggca atgtcagatc aag

C)
1 gatgaggtga atttgttaca ggacttagtc gtggtgtggc gaacgacgcg ttgactacgg
61 tcgatgaaca gacacaaagt acgatgggaa aatatgctac gggattacgg tagaaactag
121 gtagttacat gtgtatccag aaccaattgc taagttgatt gatagttgta tgaacttgcc
181 cggcattggt ggcgagactg ccagccggct ggcgttttat acaattgata tgaacgttga
241 tgacgtcacc gagttcgcta agtcgctgat tgcggccaat cgggacctgc attgttgtag
301 tatttgcggc aatatcaccg aagatgatcc gtgtgtgatt tgtaaggata agtcgcgtga
361 tcaaagcacc gttctggttg ttgaggaaac caacgacggc ag

Table B.1 Continued

D) 1 gttacaatat tggatgaggt aatgattttc tagaacaaat tatcgagatt atcaaattac 61 cttgaagcaa aataaagacc tccagaacgg ttggactcga gtgaaggtaa gttgaagtat 121 ttcaatgaat tgaaagattc cttgaatcaa tctatcttgg tggctcaaga agctgctgat 181 aaagtgaaga cgaactctaa gaaggaagcc gacattatca cccgcgaagc tcagaaacaa 241 gcgtctgaca ttgtgagtga agcaaccgac aagtcaaacc aaatgattga cg
E) 1 ttacgtgaga acatggtggt ctaccgggaa gactggtgca gcgaccactt aattttgcca 61 tcattgatga agttgactca atcttaattg atgaagcccg gacaccgttg atcatttctg 121 gacaatcaga aggtacaacg ggaatgtata agcgggctga ccgttttgct aagacgttga 181 cgaaggatga agattacaag gttgacttgg aatctaagac ggtcgcatta ttagacgaag 241 ggattcggaa ggctgaaaaa tactttggct tggaaaacct ttacgatact gataatacgg 301 cgttgaacca ttacttggat gaagctttgc gtgcgaacta catcatgttg aaagataaag 361 actacgtgat ttcagacggt caggcgttga tcgttgattc ctttacgg

A) Amino acid sequence of the arginine deiminase enzyme in *L. plantarum* LP90 published by Spano and others (2004). B, C, D, and E) Sequences of DNA extracted from the gel that separated DNA amplicons that were a result of PCR with the arcA primers published by Spano and others (2004). B and C) Reverse and forward primer, respectively; band size ~600 bp. D and E) Overlapping regions of sequences from forward and reverse primers for band size ~500 bp and ~675 bp, respectively.

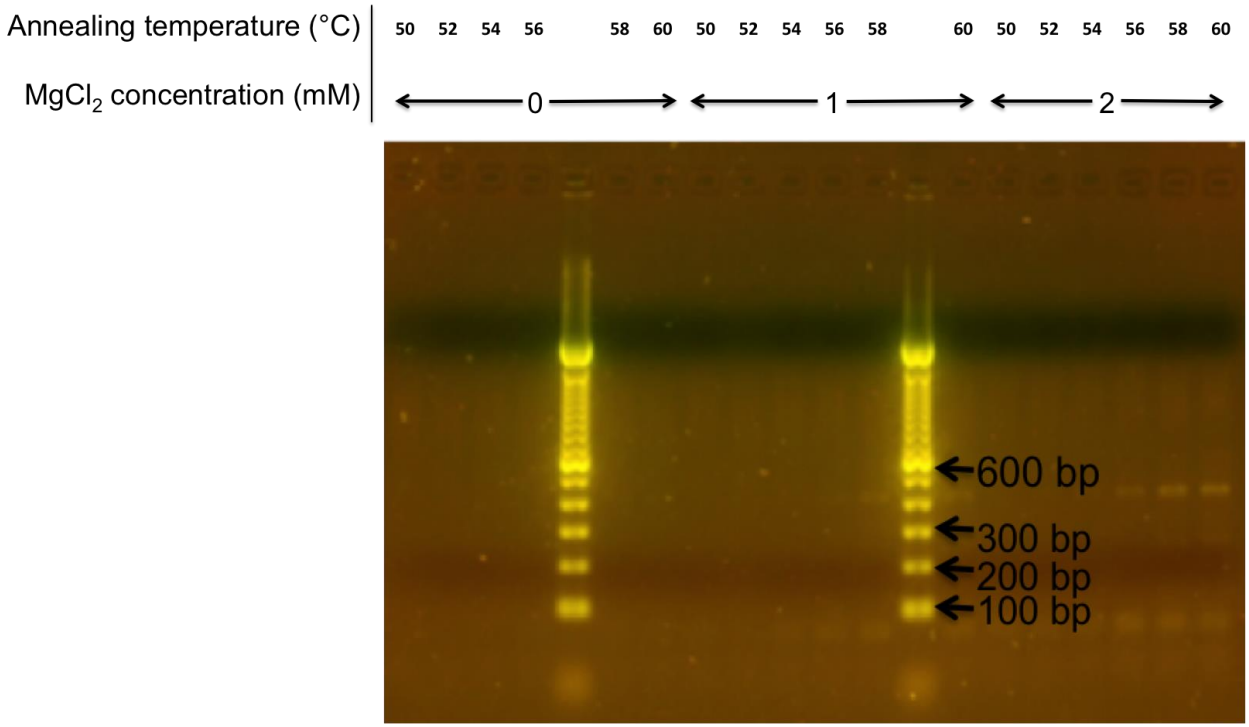


Figure B.1. PCR product of DNA extracted from *L. plantarum* WCFS1 cloned and amplified with the arcA degenerate primers using 3 different levels of $MgCl_2$ concentration (0, 1, 2 mM) and 6 different annealing temperatures (50, 52, 54, 56, 58, 60°C).

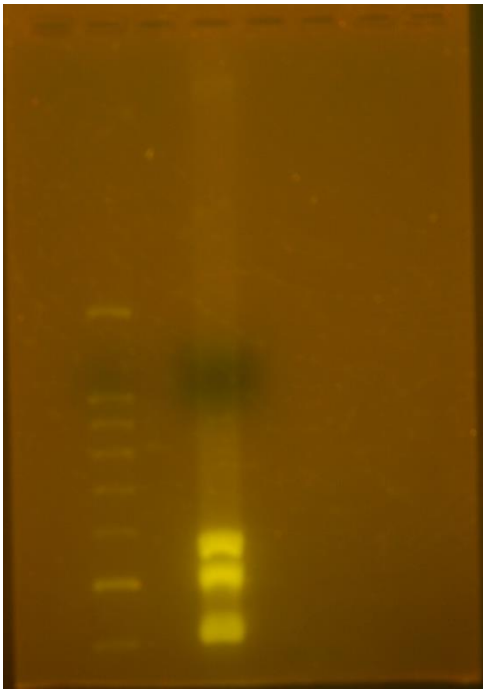


Figure B.2. Gel electrophoresis of the PCR product of *L. plantarum* WCFS1 using the arcA degenerate primers published by Spano and others (2004).

DNA from Figure A.8 was excised and purified from gel and re-run on this gel, resulting in 3 separate bands.