ENHANCED VIABILITY OF FREEZE-DRIED *LACTOBACILLUS ACIDOPHILUS* USING SUPERCOOLING PRETREATMENT

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI ‘I AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

FOOD SCIENCE

DECEMBER 2021

By

Yu Wang

THESIS Committee:

Soojin Jun, Chairperson

Yong Li

Kacie Ho

ACKNOWLEDGMENTS

I would like to express my deepest appreciation to my advisor, Dr. Soojin Jun, for all his support and guidance throughout my education. He gave me the opportunity to be a part of the Food Processing lab when I started my food science master's program. I had countless valuable experiences and with his help, I have grown both academically and as a person. As I start a new chapter in my life, I will engrain the lessons I have learned and remember this life-changing adventure.

I would like to thank Dr. Yong Li, for the constructive comments, and kindly allowing me to work in the Food Microbiology Lab. The knowledge about food microbiology from his teachings have had substantial influence on my research and have engaged me into the food microbiology world. I would like to thank Dr. Kacie Ho, for her insightful teachings and kindly always offering her help. I would also express my appreciation to Dr. Yongsoo Kim and Dr. Jinzeng Yang, for their warm encouragement, heartfelt words of suggestions, and for kindly offering me to work in their labs.

I would like to convey my sincere gratitude to my previous and current lab mates. Especially Grace, who trained me lab techniques when I was just starting my experiments, and Dongyoung, Taiyoung and Samudra, they have always been supportive and offered advice in difficult moments, I could not have completed this dissertation without them, and my lab members Yijing and Jinwen for their kindness and help. I will always be grateful and remember our fun time spent together in the Food Processing Lab.

In addition, I would like to thank my best friend, I could not have completed this dissertation without the support of him, Shijie Huang, who provided stimulating discussions as

well as happy distractions to rest my mind outside of my research. I will definitely get a massive fish I promise.

Finally, I will bring my biggest thanks to my family, for all the support they have shown me through three-year of distance learning. This completion would not have been possible without you, I love you forever!

ABSTRACT

Probiotics are living microorganisms that can act as intestinal microflora to offer health benefits to the host. In recent years, the global demand for probiotics has increased significantly, leading to the expansion of the probiotics market size. Most probiotic fortified foods in the market contain strains of *Lactobacilli* and/or *Bifidobacterium*. Among these strains, *Lactobacillus acidophilus* (*L. acidophilus*) stands out with its wide application range; regulates functions in the gastrointestinal tract (GI) system and is used in therapeutic applications.

Probiotics are usually made into dried or frozen culture concentrates for lower distribution costs and to extend storage life. However, probiotics are sensitive to the environment and can be easily killed during a non-optimized manufacturing process. Therefore, suitable drying technologies should be applied to maintain the viability and functionality of probiotics. Freeze-drying is often considered the most appropriate drying method for biological materials and has been used to manufacture probiotic powders for decades. However, during the freeze-drying process, the initial freezing step forms ice crystals, which damage cell structure and decrease cell viability. Therefore, additional protective agents are commonly used to stabilize the cell suspension. The concentrations and combinations of protective agents are different for each probiotic strain, and the optimum protective effects need to be determined on a case-by-case basis.

As an alternative to solely relying on cryoprotectant additives, we propose to protect probiotics by strengthening their natural defenses. When *Lactobacillus* culture shifts from its optimum growth temperature to a lower one, a cold-shock response is induced, triggering the production of surface layer protein (SLP). With the help of SLP, cells gradually acclimate to the lower temperature environment. According to previous research, within growth-limited stress, the

lower the temperature, the more protective SLP will be secreted by *L. acidophilus*. Supercooling is a food processing technique which uses a storage temperature lower than the initial freezing temperature, thus limiting the development of ice crystals, to maintain the qualities of fresh foods. An oscillating magnetic field (OMF) controls ice nucleation and maintains supercooling status. OMF directly reorients and vibrates water molecules and/or breaks hydrogen bonds between water molecules, which in turn prevents ice crystallization. Herein, we investigated the use of OMF-assisted (10 mT, 30 HZ) supercooling to treat *L. acidophilus* for 12 hours and aimed at achieving more SLP to improve the viability of *L. acidophilus*.

OMF-assisted supercooling pretreatment effectively increased the production of SLP. Supercooled samples exhibited thicker SLPs than those in control groups and had significantly improved cell viability (the highest viability of 78% was observed with the supercooling pretreatment). These results show the protective effect of the SLP during freeze-drying and indicate that OMF-assisted supercooling pretreatment is helpful for enhancing the viability of *L. acidophilus.*

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1

INTRODUCTION

Probiotics are living microorganisms that are intended to provide health benefits when consumed. These microorganisms can colonize as intestinal microflora and have well reported health benefits in the gastrointestinal tract (GI) system and have been effectively applied as therapeutics (Azad et al. 2018, Schwenger et al. 2015, Falagas et al. 2007). Health awareness of consumers is on the rise, resulting in the expansion of the global probiotics market size. From 2020 to 2027, the probiotics market size is projected to increase from 2.3 to 4.15 billion USD, and *Lactobacillus* counts more than half the volume in the market (Kunal Ahuja & Kritika Mamtani 2021). *Lactobacillus acidophilus* (*L. acidophilus*) is one of the typical species in the genus *Lactobacillus*, which is found in human and animal intestines. When present with a certain amount, the *L. acidophilus* can modulate the functional microflora in the GI and immune system (Foysal et al. 2020).

According to the requirement of the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), functional foods with probiotics should contain a minimum of 10^7 viable probiotics per gram of product at the time of consumption (Doleyres & Lacroix 2005; Adhikari et al. 2000; Ouwehand & Salminen 1998). However, many existing products are unable to meet this regulation when consumed (Kailasapathy & Rybka 1997). Therefore, the viability of probiotics should be maintained well during and after the manufacturing process.

Probiotic culture concentrates are usually in dried or frozen form to ensure a long-term shelf life and reduce distribution costs. Among various drying methods such as spray drying, freeze-drying, and fluidized bed drying, freeze-drying is often considered the most appropriate

drying method for biological materials. Despite this, freeze-drying has limitations as ice crystals formed during the first freezing step can damage the bacterial cells and lower cell viability (Morgan et al., 2006).

In order to protect probiotics during freezing, additional cryoprotective agents are mixed with probiotics before freeze-drying. Skim milk and sugar are two cryoprotectants that are normally used for freeze-drying most lactic acid bacteria (LAB) cultures. To date, some evidence has shown the protective effects of many cryoprotectants (Reddy et al. 2009; Chen et al. 2019; Wang et al. 2021; Shu et al. 2018); however, optimum protection depends on the concentrations and combinations of cryoprotectants, which vary across bacteria strains and should be determined individually. Instead of evaluating the protective effects of different cryoprotectants, we propose to strengthen the defense system of probiotics and let them protect themselves. In general, when a cell culture shifts from its optimum growth temperature to a lower one, cells are in a transient growth arrest and general protein synthesis is severely inhibited. However, surface layer protein (SLP) production is triggered during this process and cells gradually become acclimated to the lower temperature (Phadtare et al. 2004; Barria et al. 2013; Gualerzi et al. 2003; De Maayer et al. 2014). Supercooling is a food processing technique used to maintain fresh food qualities. During the supercooling process, food samples are preserved under their initial freezing temperatures without ice crystals (Stonehouse & Evans 2015). Oscillating magnetic field (OMF) controls ice nucleation by re-orienting and vibrating water molecules and/or breaking hydrogen bonds between water molecules. In this study, with the assistance of OMF, *L. acidophilus* can be maintained under supercooling status for a certain time. During this period, *L. acidophilus* responses to the cold stress by producing SLP, which protects the cells from the following freezing step.

This research aimed to investigate supercooling as a novel pretreatment in the viability enhancement of freeze-dried *L. acidophilus*. Specific objectives leading to these goals were:

Objective 1: Test and optimize the supercooling conditions for pretreatment of *L. acidophilus* prior to freeze-drying

Objective 2: Analyze the correlation between cell viability and SLP under development against cold stress

Objective 3: Explore the potential of the developed supercooling pretreatment for freeze-dried probiotics supplements in commercial production scales

REFERENCES

Adhikari, K., Mustapha, A., Grün, I., & Fernando, L. (2000). Viability of microencapsulated *bifidobacteria* in set yogurt during refrigerated storage. Journal of dairy science, 83(9), 1946-1951.

Åvall-Jääskeläinen, S. & Palva, A. (2005). *Lactobacillus* surface layers and their applications. FEMS Microbiology Reviews, 29(3), 511-529.

Barria, C., Malecki, M., & Arraiano, C. (2013). Bacterial adaptation to cold. Microbiology, 159(Pt 12), 2437-2443.

Beveridge, T. J. (1994). Bacterial s-layers. Current Opinion in Structural Biology, 4(2), 204-212.

Beveridge, T. J. & Graham, L. L. (1991). Surface layers of bacteria. Microbiological reviews, 55(4), 684-705.

Brasca, M., Morandi, S., Lodi, R., & Tamburini, A. (2007). Redox potential to discriminate among species of lactic acid bacteria. Journal of Applied Microbiology, 103(5), 1516-1524.

De Maayer, P., Anderson, D., Cary, C., & Cowan, D. A. (2014). Some like it cold: understanding the survival strategies of psychrophiles. EMBO reports, 15(5), 508-517.

Doleyres, Y. & Lacroix, C. (2005). Technologies with free and immobilised cells for probiotic bfidobacteria production and protection. International Dairy Journal, 15(10), 973-988.

Elser, W. J., Thomas, R. A., & Ste en, G. I. (1935). The desiccation of sera and other biological products (including microorganisms) in the frozen state with the preservation of the original qualities of products so treated. The Journal of Immunology, 28(6), 433-473.

Fonseca, F., B eal, C., & Corrieu, G. (2001). Operating conditions that a ect the resistance of

lactic acid bacteria to freezing and frozen storage. Cryobiology, 43(3), 189-198.

Gibson, G. R., Probert, H. M., Van Loo, J., Rastall, R. A., & Roberfroid, M. B. (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutrition research reviews, 17(2), 259-275.

Gibson, G. R. & Roberfroid, M. B. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. The Journal of nutrition, 125(6), 1401-1412.

Gualerzi, C. O., Giuliodori, A. M., & Pon, C. L. (2003). Transcriptional and posttranscriptional control of cold-shock genes. Journal of molecular biology, 331(3), 527-539.

Jones, P. G., VanBogelen, R. A., & Neidhardt, F. C. (1987). Induction of proteins in response to low temperature in escherichia coli. Journal of bacteriology, 169(5), 2092-2095.

Kailasapathy, K. & Rybka, S. (1997). *L. acidophilus* and *bifidobacterium* spp.-their therapeutic potential and survival in yogurt. Australian Journal of Dairy Technology, 52(1), 28.

Kunal Ahuja & Kritika Mamtani. (2021, May). *Probiotics Market Size By Ingredients (Lactobacilli, Bifidobacterium, Streptococcus, Bacillus), By End-Use (Human, Animal), By Application (Food and Beverages {Dairy Products, Non-Dairy Products, Cereals, Baked Food, Fermented Meat Products, Dry Food}, Dietary Supplements {Food, Nutritional, Specialty Nutrients, Infant Formula}, Animal Feed*) (Report ID: GMI418). https://www.gminsights.com/industry-analysis/probiotics-market

Morgan, C. A., Herman, N., White, P., & Vesey, G. (2006). Preservation of micro-organisms by drying; a review. Journal of microbiological methods, 66(2), 183-193.

Ouwehand, A. C. & Salminen, S. J. (1998). The health e ects of cultured milk products with viable

and non-viable bacteria. International Dairy Journal, 8(9), 749-758.

Pano , J.-M., Thammavongs, B., & Gu eguen, M. (2000). Cryoprotectants lead to phenotypic adaptation to freeze{thaw stress in *Lactobacillus delbrueckii* ssp. Bulgaricus cip 101027t. Cryobiology, 40(3), 264-269.

Phadtare, S. et al. (2004). Recent developments in bacterial cold-shock response. Current issues in molecular biology, 6(2), 125-136.

Rogers, L. (1914). The preparation of dried cultures. The Journal of Infectious Diseases, (pp. 100-123).

S ara, M. & Sleytr, U. B. (2000). S-layer proteins. Journal of bacteriology, 182(4), 859-868.

Segal, G. & Ron, E. Z. (1998). Regulation of heat-shock response in bacteria. Annals of the New York Academy of Sciences, 851(1), 147-151.

Stonehouse, G. & Evans, J. (2015). The use of supercooling for fresh foods: A review. Journal of Food Engineering, 148, 74-79.

Wickerham, L. & Flickinger, M. (1946). Viability of yeasts preserved two years by the lyophil process. Brewers Digest, 21, 48T-52T.

Wickerham, L. J. & Andreasen, A. (1942). The lyophil process: Its use in the preservation of yeasts. Wallerstein Labs. Communs, 5, 165-169.

Chapter 2

LITERATURE REVIEW

2.1. Introduction

This chapter includes the historical development of probiotics, probiotics survival during processing and storage, including the present challenges and solutions, as well as the impacts of cold stress on bacterial cells. In addition, fundamentals of the supercooling pretreatment for the enhanced cell viability, i.e. how supercooling strengthens bacterial self-defense and helps them survive against the freezing threat, will be discussed in detail.

2.1.1. Historical context and development of probiotics

The first cultured dairy products can trace back to the beginning of civilization as they are referenced in the Holy Bible and early sacred books of Hinduism. In the Holy Bible, Abraham offered the Lord with "veal, buns and sour milk". Some traditional soured milk and cultured dairy products such as Kefir, leben, dahi and koumiss, were consumed even before the bacteria was acknowledged and are still popular now. In 1906, Tissier (1906) evaluated the clinical benefits of *bifidobacteria* in breast-fed infants and found intestinal infections in infants could be regulated by modulating the flora. This discovery established the concept that bacteria can directly impact human health. In the early 1920s, Kopeloff & Kirby (1929) proposed an argument on the benefits of bacteria in yogurt. They found that the long life of Bulgarian peasants is associated with their habit of drinking yogurt, which contains *Lactobacillus* species. In the 1930s, Minoru Shirota found that LAB could survive in the digestive system with high viability; he stated that a prime gut microflora is the best therapy to prevent many diseases (Alvarez-Olmos & Oberhelman 2001). Thereafter probiotics have been promoted as promising protective factors for the digestive system as they can resist pathogens like *Escherichia coli* (Resta-Lenert & Barrett 2003), *Salmonella*

(Thirabunyanon & Thongwittaya 2012), *Listeria* species (De Waard et al. 2002), and *Helicobacter pylori* (V´ıtor & Vale 2011).

Probiotics benefit human health by providing enough intestinal microflora, protecting the gastrointestinal tract (GI) system, helping metabolism, improving nutrient absorption, nutritional values of food, and enhancing the host immune system (Azad et al. 2018; Cani & Van Hul 2015; Lourens-Hattingh & Viljoen 2001; Teitelbaum & Walker 2002). Other therapeutic applications have also been reported, including treatments for urinary tract (Schwenger et al. 2015) and vaginitis infection (Falagas et al. 2007) and antitumor activity of probiotics in the gut and cervix (So et al. 2017). With the continuously evolving and commercialization of new probiotic strains, we must comprehensively assess new probiotic strains' safety. With appropriate models (*in vitro* and *in vivo* animal models) and tests, new probiotics should be thoroughly evaluated before application (Hag & Poondla 2021; Pradhan et al. 2020).

Consumer's health awareness is contributing to the rising demand for probiotics. The global probiotics market size was estimated to be USD 2.3 billion in 2020, and is projected to reach USD 4.15 billion by 2027 (Kunal Ahuja & Kritika Mamtani 2021)). There are various probiotic supplements in the market, which contain functional microorganisms either in fermented products or lyophilized form. According to Shah (2007) and Chow (2002), the most common commercial strains are of the following genera: *Lactobacillus*, *Streptococcus*, and *Bifidobacterium*. Clinical effects of *Lactobacillus* strains have been observed with *L. rhamnosus* and *L. acidophilus* (NCFB1478) (Song et al. 2015). Food applications like Cheddar (Gardiner et al. 1998), gouda (Gomes et al. 1995), and Argentinean Fresco cheese (Vinderola et al. 2000) also contains probiotic LAB, which can offer certain advantages as an effective vehicle of some probiotic organisms to the consumer. *Lactobacillus* has been used to prevent diarrheal disease associated with using

antibiotics. Recently, *Lactobacillus casei* strain Shirota has been considered as an effective treatment or the prevention of antibiotic-associated diarrhea in patients (Wong et al. 2014).

2.1.2. Probiotic survival during processing and storage

According to the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), probiotics are a group of bacteria defined as living microorganisms, which when consumed in adequate amounts (10^7 CFU g^{-1}), provide health benefits to the host (Hill et al. 2014; AFRC 1989). Therefore, it is important to achieve high viability of probiotics during processing and shelf life (Da Cruz et al. 2010). The viability of probiotics is limited by production protocols, microbiological and regulatory barriers. Factors associated with production processes are mainly processing temperature, packaging material, and storage conditions.

2.1.3. Probiotics survival during processing

Freeze-drying

Commercially available probiotics are usually in dried or frozen culture concentrate form to obtain long-term shelf life and for industrial or home to use into food. These culture concentrates can be consumed as food products (fermented or non-fermented) or dietary supplements (powder, capsule, tablet). However, viability loss during the drying process remains a big challenge (Santivarangkna et al. 2006). Therefore, drying technologies should be appropriately applied, to maintain the viability and functionality of probiotics. Among various drying methods such as spray drying, freeze-drying, and fluidized bed drying, freeze-drying is often considered the most appropriate drying method for biological materials and has been used to manufacture probiotic powders for decades (Chen & Mustapha 2012; Elser et al. 1935; Rogers 1914; Wickerham & Andreasen 1942; Wickerham & Flickinger 1946). During the freeze-drying process, the cell suspension is frozen in the initial freezing step, then the ice is removed by sublimation in the

primary drying step. Next, secondary drying removes the unfrozen water by desorption. Ice crystallization from the initial freezing step damages cell structures and decreases cell viability (Nireesha et al. 2013; Cook et al. 2012; Morgan et al. 2006; Wang et al. 2004). Besides cold stress, osmolar stress is also a factor that impacts cell viability (Burgain et al. 2011). Moreover, undesired store conditions such as high humidity, inappropriate temperature, and oxygen also decrease the cell viability (Zayed & Roos 2004; Gbassi et al. 2009; Yao et al. 2017).

Therefore, to protect probiotics during freeze-drying and storage, additional cryoprotective agents are added before freeze-drying (Haffner et al. 2016; Khem et al. 2016; Hubalek 2003; Selmer-Olsen et al. 1999). Cryoprotective agents are numerous and can be classified depend on the molecular weight (Meryman, 1971). Sugars like glucose, trehalose, and sorbitol are commonly used as low-molecular-weight cryoprotectants. During the dehydration process, they contribute to decomplexing hydrogen bonds with proteins and polar groups of the cellular membrane, thus, to restrain structural denaturation and other damages. (Meng et al. 2008; Crowe et al. 1996). Skim milk, soybean powder, and maltodextrin are considered as high- molecular-weight cryoprotective agents. Skim milk and soybean powder preserve the cellular membrane by forming a coating layer outside the cell surface throughout hydrophobic interactions (Choi et al. 2018; Khem et al. 2016; Abadias et al. 2001). Polysaccharide maltodextrin contributes to reducing the mobility of the cells in the glassy state (Semyonov et al. 2010).

Drying medium and protective agents

Skim milk is a commonly used freeze-drying medium for most LAB cultures; it provides cells a protective coat, stabilizes the cell membrane and prevents cellular injury, and porous structure was created in the freeze-dried products, making rehydration easier (Zayed & Roos 2004, Teixeira et al. 1996, Zayed & Roos 2004). Skim milk also provides a strengthened intrinsic

protective effect by synergistically acting with other protective agents that contain an amino group, a secondary group or both (de Valdez et al. 1985; Tripathi & Giri 2014).

Sugars and sugar alcohols are also favored protective agents and have been used in many freeze-dried LAB manufactured products. These primarily include sugar alcohols (sorbitol, inositol) and non-reducing sugars (trehalose).

Sorbitol has antioxidation properties, which protects cell membrane from interaction damage; it also preserves cell functionality by forming a sorbitol-protein structure (Linders et al. 1997b a, Wisselink et al. 2002). Carvalho et al. (2003) evaluated the protective effects of sorbitol and found no significant viability differences during freeze-drying but observed a protective effect of sorbitol on the survival of *Lb. bulgaricus*, *Lb. Plantarum*, *Lactobacillus rhamnosus*, Ent. faecalis, and *Enterococcus durans* during storage. Trehalose is also considered an effective protective sugar for freeze-dried probiotic bacteria (Crowe et al. 1996). It helps to maintain the protein functionality by forming a glassy matrix, and it can bind dried protein and acts as a water substitute, to prevent the disruption of protein (Bell & Hageman 1996, Arakawa et al. 1991). Leslie et al. (1995), Castro et al. (1997) and Teitelbaum & Walker (2002) found that trehalose and other carbohydrates are also able to lower the transition temperature of the dry membrane by replacing the water between the lipid head groups. This function helps to prevent phase transition and leakage upon rehydration. Leslie et al. (1995) stated trehalose is helpful to protect *Escherichia coli* and *Bacillus thuringiensis* during freeze-drying and storage. However, Linders et al. (1997a) claimed no positive effect when using trehalose as the protective agent in fluidized bed drying. Carvalho et al. (2002) also evaluated the protective effect of trehalose during freeze-drying and storage. They claimed there is no remarkable effect of trehalose than other carbohydrates (myo-inositol, sorbitol, and fructose) in the freeze-dried *Lb. Plantarum*, and trehalose shows low

viability by the end of storage period in *L. rhamnosus* case. Crystallization is the major the limitation of trehalose. Once trehalose is crystallized, the formation of hydrogen bonds between protein and sugar decreased, thus causing a poor protective effect of sugars during drying and subsequent storage (Carpenter et al. 1992; Arakawa et al. 1991).

It is hard to evaluate the experimental data since there is more than one factor influences the final results, (i) some investigations were centered on survival during the drying process instead of storage afterward. (ii) activity of probiotic bacteria was more concerned than that of viability. (iii) drying medium varies, and water was more used than complex medium. (iv) probiotic strains, the concentration of protective agents, and drying methods were employed differently. Up to date, evidence has shown the suitable selection of protective agents (Leslie et al. 1995; Conrad et al. 2000; Zayed and Roos 2004; Meng et al. 2008). It is possible to maintain the viability of probiotics during the storage of the freeze-dried process. However, to have optimum protection, the protective agents, including the concentration and species for each probiotic bacteria strain, should be determined for the specific product.

2.1.4. Cold stress

As mentioned above, selected probiotic strains are supposed to go through adverse conditions encountered in industrial processes such as starter handling (freeze-drying, spray drying, etc.) and subsequence storage. During the industrial production of probiotic starter cultures, bacteria strains are subjected to various stress from either growth environment like temperature, PH, nutrition, osmotic pressures, or manufacturing processes such as mechanical or hypothermic osmotic, oxidative stresses. These stresses bring severe damages to the bacteria strains, such as viability loss, membrane damage, and cell morphological changes (Panoff et al. 2000; Brasca et al. 2007; Fonseca et al. 2001). However, stress is one of the most important driving forces for

bacterial evolution and acclimation to new varieties. This phenomenon is called “stress acclimation”, stress acclimation time varies from strains and stress type; for osmotic shock, it usually takes several minutes, while it takes several hours for cold shock (Segal & Ron 1998; Rosen & Ron 2002). Among various stresses, cold stress is unique since it exists in all kinds of manufacturing processes; cold conditions are either required in probiotic strains handling and production or their cultures collection.

In most LAB, when the cell culture rapidly shifts from its optimum growth temperature to a lower one, a specific set of genes is induced, general protein synthesis is severely inhibited. During the initial acclimation phase of cold shock, surface layer protein (SLP) is triggered. Cells become acclimate to the lower temperature (Phadtare et al. 2004; Barria et al. 2013; Gualerzi et al. 2003; De Maayer et al. 2014). Some detailed cold shock responses were observed: (i) membrane fluidity decreased, influencing membrane-assisted functions like transportation and protein secretion. (ii) RNA and DNA secondary structures were stabilized, reducing mRNA translation efficiency and transcription efficiency. (iii) some proteins may show slow or inefficient folding. (vi) ribosome prior acclimation helps cells get used to low temperature (Phadtare et al. 2004). Moreover, cold stress influences on bacteria metabolisms, Monedero et al. (2007) found in *Lactobacillus* casei; there is a relationship between cold stress and metabolic regulation. The adaptive responses allowed bacteria to gradually acclimate the stress conditions by generating transient physiological modifications of the membrane’s properties and cell proteome pattern. Frece et al. (2005) demonstrated that SLP has a protective function which is important in *L. acidophilus* M92 strain establishment in the GI system. Song et al. (2014) stated that *L. plantarum* adapts to low temperature by activating potentially cryoprotective cspP gene and inducing protein; they suggested that low temperature pretreatment aids the ability to survive under subsequent

freeze-thaw processes and freeze-drying. In general, exposure to cold conditions before freezing helps LAB develop freezing tolerance.

Surface layer protein (SLP)

Structures outside the bacteria membrane are usually found in archaebacteria (Sleytr et al. 2001; K¨onig 1988). Regardless of the variations in the structure and chemistry of prokaryotic envelopes, surface layers are coevolved with these diverse structures. For example, in certain Archaea, surface layers relate to the plasma membrane and considered as bilayers. In Gram-negative bacteria, the surface layer is connected to the lipopolysaccharide and regarded as a part of the outer membrane. In Gram-positive bacteria and Archaea, surface layers are located on the cell surface; they were assembled with a wall matrix composed of peptidoglycan or pseudomurein.

Surface layers are usually checked with an electron microscope such as transmission electron microscope (TEM) and scanning electron microscope (SEM). A common feature of the surface layer is its corrugate inner surface and smooth outer surface. Fig.2.1 shows the cell wall structure of *Lactobacillus* strains interpreted based on experiments (Sch¨arZammaretti & Ubbink 2003; Delcour et al. 1999). The cell membrane outer layer has various polymers such as polysaccharides and lipoteichoic acids, SLP bounds to the rigid peptidoglycan containing layer via secondary cell wall polymers; even the surface proteins form a close packing, a significant fraction of the surface is open to the outside. The molecular weight of SLP is between 40 to 200 kDa (Vidgr´en et al. 1992; Sleytr et al. 2012); in *Lactobacillus* strains, the molecular mass of SLP is between 40 and 55 kDa and nonglycosylated (Goh et al. 2009).

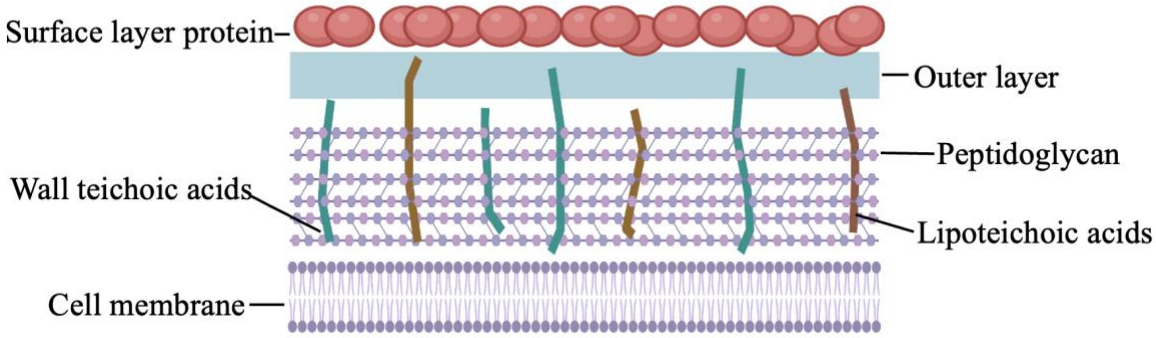


Figure 2.1 A structural model of the cell wall of *Lactobacillus* strains.

Pum & Sleytr (2009) and Egelseer et al. (2008) suggested that SLP has a uniform structure and physicochemical properties, which can spontaneously couple with constituent subunits. There is considerable potential in using SLP covered *Lactobacilli* in health-related probiotics applications. Also, the application of SLP as a carrier has been approved by Hyn¨onen & Palva (2013); in the article, *Lactobacilli,* which is covered with SLP, is an ideal carrier of medical molecules, such as antigens, adhesion molecules, and immunomodulation agents. Choi et al. (2018) investigated cold adaptation on the viability enhancement of freeze-dried *Lactobacillus*; they found 10 °C pretreatment has a positive effect in maintaining the high viability of *Lactobacillus*. Choi et al. (2019) decreased cold adaptation temperature to -5 °C; they found *Lactobacillus* produce more SLP and show higher viability. Thus, they suggest that SLP maintains the high viability of *Lactobacillus* during freeze-drying and storage; lower pretreatment temperature was the key to introduce more SLP.

2.2. Supercooling pretreatment in enhancing cell viability

As mentioned above, SLP has protective effects on *L. acidophilus* during freeze-drying, within growth-limited stress, the lower the temperature, the more SLP will be introduced. Herein, we pretreated *L. acidophilus* under supercooling states for 12 hours, aiming to get more SLP. Figure 2.2 shows the freezing curve of water; it is well-known that freezing including three steps; (1): precooling, (2): phase transition, and (3) tempering to storage temperature. In step (1), the temperature decreased by removing sensible heat; before reaching the freezing point, the products remain in the unfrozen phase with a certain degree of supercooling (ΔT). Ice nucleation occurs at a certain point of supercooling, ice nucleation occurs, ice crystals begin to form, resulting in a rapidly increasing temperature. The temperature increased and remained at freezing temperature; ice crystals grow till the end of step (2). When most of the water is converted into solid ice, the temperature of the product is gradually decreased, most of the water is converted into solid ice. The temperature of the product gradually decreases to the storage temperature (3).

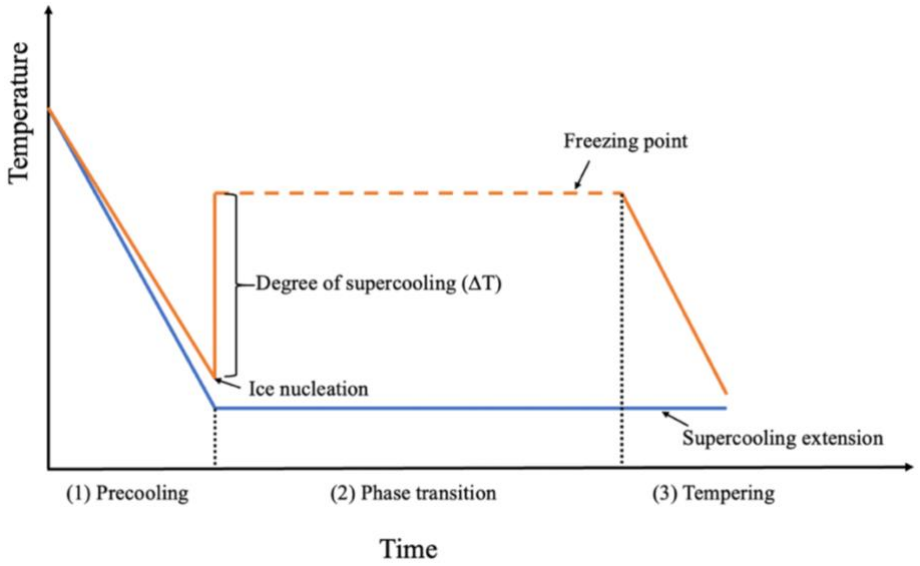


Figure 2.2 Freezing curve of water during freezing and supercooling processes.

The degree of supercooling is defined as the difference between the freezing point and nucleation temperatures; the larger the degree of supercooling, the smaller ice crystals are produced during freezing (Zhu et al. 2005). With the increasing emphasis on ice nucleation, either by inhibiting or promoting supercooling degree, some technologies such as high-pressure, electric and magnetic fields (MFs), and ultrasound, have been investigated to control ice nucleation (James et al. 2015; Cheng et al. 2017; Zheng & Sun 2006; Zhan et al. 2018). Among these technologies, MFs have been applied in industrial freezing. A Japanese company ABI (ABI Co., Ltd., Chiba, Japan), commercialized a novel patented freezing system named "CAS (Cells Alive System) freezing" CAS uses static and oscillating MFs to maintain water molecules in a supercooled state. Ryoho Freeze Systems Co., Ltd. (Nara, Japan) commercializes "Proton freezers," which uses static MFs and electromagnetic waves. In summary, MFs have an impact on ice nucleation and supercooling by working on the water, such as orientating, vibrating, and spinning water molecules. However, scientific mechanisms behind MFs have not been completely elucidated and still require further research (Zhao et al. 2015; Zhan et al. 2018).

2.2.1. Oscillating magnetic fields (OMF) on the control of ice nucleation

Thus, in order to explore the use of MFs, it is crucial to know the magnetic properties. Magnetic properties in the substances are achieved by quantum electrodynamics, which are result from the orbital motion and electrons spinning in the atoms. Even though nuclei regulate spin, it is thousands of times lower than in nuclear. Therefore, electrons are the critical factor that identifies the magnetic properties of the substance. Specifically, the orbital motion of electrons produces tiny atomic current loops around the nucleus; then, a magnetic moment is induced. Similarly, the electrons spinning produces a magnetic moment. Water is a typical demagnetized substance. In water, paired electrons filled the atomic orbitals in contrary directions, indicating the

orbital moments canceled each other; in the same way, electron spin moments canceled each other as well. Thus, water is only considered to have a magnetic moment with an external magnetic field. With external MF, the orbital motion of electrons is changed, inducing magnetic moments in the opposite way of the applied MFs. According to Lenz's law, this is a typical energy conservation law (Otero et al., 2016). The magnetic force put on a diamagnetic substance is proportional to the external MF and the magnetic susceptibility. Water has relatively low magnetic susceptibility ($X_{\text{mass}} = -9.051\times10^{-9}\text{m}^3\text{kg}^{-1}$ at 20 °C) (Jha et al. 2017b), thus, water will become weakly magnetized under weak MF, and strong MF (≥ 10 T) are able to make visible changes on water (Chen & Dahlberg 2011). Weak MFs did not make visible changes on water, while still change water properties. Some water properties, such as the viscosity, the electric conductivity, the refractive index, and the surface-tension force, and so on, are changed with exposure to MFs as low as 1 mT (Semikhina & Kiselev 1988; Pang & Deng 2008; Pang et al. 2012). Strong MFs (≥ 10T) make visible changes on water, such as water droplets against the gravity and levitation in the air (Beaugnon & Tournier 1991; Ikezoe et al. 1998) and some deformations on the water surface (Chen & Dahlberg 2011; Pang & Deng 2008).

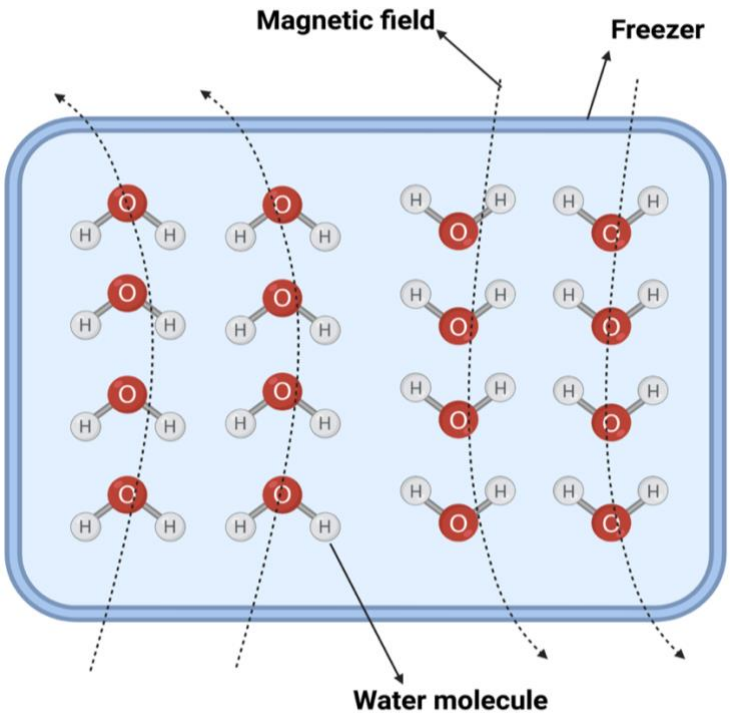


Figure 2.3 A schematic of OMF working on water molecules.

In order to investigate water activities under external MFs, computational simulation techniques have been used. Recent studies are more using Monte Carlo and molecular dynamics simulations in the water system. The predominant mechanism can be summarized as MFs affect the hydrogen bond network, then influence the interaction between water molecules, finally, new water cluster are formed, and water properties are changed (Zhou et al. 2000; Chang & Weng 2006; Toledo et al. 2008; Szcze´s et al. 2011). However, the conclusion varies from simulations. Specifically, Zhou et al. (2000) carried out a Monte Carlo simulation; they found the effects of MFs are dependent on intensities, MFs range from 100 to 200 mT affect water properties. However, there is no effects when the intensity of MFs is lower than 50 mT. They stated that external MFs weakened the hydrogen bonds and increased the number of water molecules. Chang & Weng (2006) used molecular dynamics simulation, in which they observed increasing hydrogen bonds number

(approximately 0.34%) with MF strength increased from 1 to 10 T; in other words, MFs help strengthen hydrogen bonds between water molecules, thus, constrain the water in liquid state. Toledo et al. (2008) tested MFs range from 45-65 mT, they suggested that the hydrogen bonds can be inter-cluster and intra-cluster, and MFs broke large water clusters into small water clusters; thus, intra-cluster hydrogen bonds are weakened, and stronger inter-cluster hydrogen bonds are formed. Szcze´s et al. (2011) reported that water properties are changed with exposing to weak MFs (15 mT), and a strengthened hydrogen bond network causes the changes. In summary, external MFs influence water properties, and the predominant mechanism is related to hydrogen bonds. In most cases, MFs can be static magnetic field (SMF) or oscillating magnetic field (OMF). SMF means the magnets are achieved with different configurations and sizes. OMF refers to magnets that are generated with Helmholtz coils or ferric core-based electromagnets. As mentioned above, OMF directly work on water by re-orientating and vibrating the water molecules and/or break hydrogen bonds between water molecules, thus, to maintain supercooling state and control ice crystallization (Jha et al. 2017a; Otero et al. 2017) (Fig.2.3).

Some interesting research showed the effect of OMF in maintaining supercooling state and controlling ice nucleation. Semikhina & Kiselev (1988) evaluated the impact of OMF (0.88 mT up to 200 Hz) in bi-distilled water; they found a sizeable supercooling degree with the presence of OMF. Zhan et al. (2019) used sodium chloride and poly dimethyl ammonium chloride as samples. They found that the supercooling degree was increased with the increasing OMF frequency (10.0 mT at 100–250 Hz). Liu et al. (2017) evaluated the effects of OMF (0–7.2 mT at 50 Hz) on carrot strips. They found that OMF is efficient in decreasing the cells's lowest noncrystallization temperature, and OMF also help to maintain the physics properties of carrot strips. Her et al. (2019) found honeydew cubes can be maintained under supercooling state (-5.5 °C) with OMF (8.0 mT

at 1 Hz). However, with a limited database, the mechanisms of OMF are still not precise, and controversy exists at the same time. Some researchers claimed that OMF did not influence freezing processes. Purnell et al. (2017) stated the effects of OMF-assisted supercooling various food species, MF intensity, cooling rate, and storage conditions. There is no overall pattern for OMF-assisted supercooling. Optimum OMF parameters have to be determined individually. Naito et al. (2012) investigated the effect of weak OMF (30 Hz, 0.5 mT) in distilled water and saline solution. However, no significant difference was observed whether there is MF or not.

In conclusion, MF-assisted supercooling shows some promising results in controlling ice nucleation and maintain supercooling state; there is considerable potential of OMF on supercooling. However, the mechanisms behind it are still controversial. In order to apply MFs into large-scale applications, the mechanisms behind MFs on supercooling should be clarified, in which a more comprehensive range of MFs should be investigated in the future.

2.3. Conclusion and thesis overview

Ice crystallization during the freezing process poses a severe threat to the viability of probiotics. Cryoprotective agents, such as sugars, skim milk, and soybean powder, were commonly used as a mixture of chemicals. However, to have the optimum protection, the protective agents, including the concentration and species for each probiotic bacteria strain, should be determined case-by-case. In order to overcome the complex combination problems from the chemical mixture, surface layer protein (SLP), is introduced. SLP production is one of the cold-shock responses. When the cell culture shifts from its optimum growth temperature to a lower one, SLP improves cells cold tolerance and helps cells become acclimated to low temperature. In general, cell freezing tolerance can be enhanced by exposure to cold conditions before freezing. Supercooling pretreatment maintains cells in low temperature without ice nucleation, thus, inducing SLP to help

L. acidophilus acclimate to the low temperature and survive in the following freezing step. This thesis provides a novel solution to the conventional cryoprotectants problems from an engineering perspective that; *L. acidophilus* was pretreated with OMF-assisted supercooling. Furthermore, the viability and cell surface changes are discussed. Results showed that supercooling pretreatment is efficient in the viability enhancement of freeze-dried *L. acidophilus*.

REFERENCES

Abadias, M., Benabarre, A., Teixid o, N., Usall, J., & Vinas, I. (2001). Effect of freeze drying and protectants on viability of the biocontrol yeast candida sake. International journal of food microbiology, 65(3), 173-182.

AFRC, R. F. (1989). Probiotics in man and animals. Journal of applied bacteriology, 66(5), 365-378.

Alvarez-Olmos, M. I. & Oberhelman, R. A. (2001). Probiotic agents and infectious diseases: a modern perspective on a traditional therapy. Clinical infectious diseases, 32(11), 1567-1576.

Azad, M., Kalam, A., Sarker, M., & Wan, D. (2018). Immunomodulatory e ects of probiotics on cytokine profiles. BioMed research international, 2018.

Barria, C., Malecki, M., & Arraiano, C. (2013). Bacterial adaptation to cold. Microbiology, 159(Pt 12), 2437-2443.

Beaugnon, E. & Tournier, R. (1991). Levitation of water and organic substances in high static magnetic fields. Journal de Physique III, 1(8), 1423-1428.

Bell, L. N. & Hageman, M. J. (1996). Glass transition explanation for the effect of polyhydroxy compounds on protein denaturation in dehydrated solids. Journal of food science, 61(2), 372-375.

Brasca, M., Morandi, S., Lodi, R., & Tamburini, A. (2007). Redox potential to discriminate among species of lactic acid bacteria. Journal of Applied Microbiology, 103(5), 1516-1524.

Burgain, J., Gaiani, C., Linder, M., & Scher, J. (2011). Encapsulation of probiotic living cells: From laboratory scale to industrial applications. Journal of food engineering, 104(4), 467-

483.

Carpenter, J., Arakawa, T., & Crowe, J. (1992). Interactions of stabilizing additives with proteins during freeze-thawing and freeze-drying. Developments in biological standardization, 74, 225-38.

Carvalho, A. S., Silva, J., Ho, P., Teixeira, P., Malcata, F. X., & Gibbs, P. (2002). Survival of freeze-dried *Lactobacillus plantarum* and *Lactobacillus rhamnosus* during storage in the presence of protectants. Biotechnology letters, 24(19), 1587-1591.

Castro, H., Teixeira, P., & Kirby, R. (1997). Evidence of membrane damage in *Lactobacillus bulgaricus* following freeze drying. Journal of Applied Microbiology, 82(1), 87-94.

Chang, K.-T. & Weng, C.-I. (2006). The effect of an external magnetic eld on the structure of liquid water using molecular dynamics simulation. Journal of Applied physics, 100(4), 043917.

Chen, H., Tian, M., Chen, L., Cui, X., Meng, J., & Shu, G. (2019). Optimization of composite cryoprotectant for freeze-drying *bifidobacterium bifidum* bb01 by response surface methodology. Artificial cells, nanomedicine, and biotechnology, 47(1), 1559-1569.

Chen, M. & Mustapha, A. (2012). Survival of freeze-dried microcapsules of galactosidase producing probiotics in a soy bar matrix. Food microbiology, 30(1), 68-73.

Chen, Z. & Dahlberg, E. D. (2011). Deformation of water by a magnetic field. The Physics Teacher, 49(3), 144-146.

Cheng, L., Sun, D.-W., Zhu, Z., & Zhang, Z. (2017). Emerging techniques for assisting and accelerating food freezing processes: A review of recent research progresses. Critical reviews

in food science and nutrition, 57(4), 769-781.

Choi, I. S., Kim, H. M., Wi, S. G., Chun, H. H., Hwang, I. M., & Park, H. W. (2018). Effect of cation influx on the viability of freeze-dried *Lactobacillus brevis* wikim0069. Applied Sciences, 8(11), 2189.

Choi, I. S., Ko, S. H., Kim, H. M., Chun, H. H., Lee, K. H., Yang, J. E., Jeong, S., & Park, H. W. (2019). Shelf-life extension of freeze-dried *Lactobacillus brevis* wikim0069 using supercooling pretreatment. LWT, 112, 108230.

Chow, J. (2002). Probiotics and prebiotics: a brief overview. Journal of renal nutrition, 12(2), 76-86.

Cook, M. T., Tzortzis, G., Charalampopoulos, D., & Khutoryanskiy, V. V. (2012). Microencapsulation of probiotics for gastrointestinal delivery. Journal of controlled release, 162(1), 56-67.

Crowe, L. M., Reid, D. S., & Crowe, J. H. (1996). Is trehalose special for preserving dry biomaterials Biophysical journal, 71(4), 2087-2093.

Da Cruz, A. G., Faria, J. d. A. F., Saad, S. M. I., Bolini, H. M. A., Sant, A. S., Cristianini, M., et al. (2010). High pressure processing and pulsed electric fields: potential use in probiotic dairy foods processing. Trends in food science & technology, 21(10), 483-493.

De Maayer, P., Anderson, D., Cary, C., & Cowan, D. A. (2014). Some like it cold: understanding the survival strategies of psychrophiles. EMBO reports, 15(5), 508-517.

de Valdez, G. F., De Giori, G. S., de Ruiz Holgado, A. P., & Oliver, G. (1985). Effect of drying medium on residual moisture content and viability of freeze-dried lactic acid bacteria.

Applied and Environmental Microbiology, 49(2), 413-415.

De Waard, R., Garssen, J., Bokken, G., & Vos, J. (2002). Antagonistic activity of *Lactobacillus casei* strain shirota against gastrointestinal *listeria monocytogenes* infection in rats. International journal of food microbiology, 73(1), 93-100.

Delcour, J., Ferain, T., Deghorain, M., Palumbo, E., & Hols, P. (1999). The biosynthesis and functionality of the cell-wall of lactic acid bacteria. Lactic acid bacteria: genetics, metabolism and applications, (pp. 159-184).

Egelseer, E.-M., S ara, M., Pum, D., Schuster, B., & Sleytr, U. B. (2008). Genetically engineered s-layer proteins and s-layer-specific heteropolysaccharides as components of a versatile molecular construction kit for applications in nanobiotechnology. In NanoBioTechnology (pp. 55-86). Springer.

Falagas, M., Betsi, G., & Athanasiou, S. (2007). Probiotics for the treatment of women with bacterial vaginosis. Clinical microbiology and infection, 13(7), 657-664.

Fonseca, F., B eal, C., & Corrieu, G. (2001). Operating conditions that affect the resistance of lactic acid bacteria to freezing and frozen storage. Cryobiology, 43(3), 189-198.

Frece, J., Kos, B., Svetec, I.-K., Zgaga, Z., Mr sa, V., & Su skovi c, J. (2005). Importance of s-layer proteins in probiotic activity of *Lactobacillus acidophilus* m92. Journal of Applied Microbiology, 98(2), 285-292.

Fujimura, K. E., Slusher, N. A., Cabana, M. D., & Lynch, S. V. (2010). Role of the gut microbiota in defining human health. Expert review of anti-infective therapy, 8(4), 435-454.

Gardiner, G., Ross, R., Collins, J., Fitzgerald, G., & Stanton, C. (1998). Development of a probiotic

cheddar cheese containing human-derived *Lactobacillus* paracaseistrains. Applied and environmental microbiology, 64(6), 2192-2199.

Gbassi, G. K., Vandamme, T., Ennahar, S., & Marchioni, E. (2009). Microencapsulation of *Lactobacillus plantarum* spp in an alginate matrix coated with whey proteins. International journal of food microbiology, 129(1), 103-105.

Goh, Y. J., Azc arate-Peril, M. A., O'Flaherty, S., Durmaz, E., Valence, F., Jardin, J., Lortal, S., & Klaenhammer, T. R. (2009). Development and application of a uppbased counterselective gene replacement system for the study of the s-layer protein slpx of *Lactobacillus acidophilus* ncfm. Applied and environmental microbiology, 75(10), 3093-3105.

Gomes, A. M., Malcata, F., Klaver, F., & Grande, H. (1995). Incorporation and survival of bifidobacterium sp. strain bo and *Lactobacillus acidophilus* strain ki in a cheese product.

Gualerzi, C. O., Giuliodori, A. M., & Pon, C. L. (2003). Transcriptional and posttranscriptional control of cold-shock genes. Journal of molecular biology, 331(3), 527-539.

Haffner, F. B., Diab, R., & Pasc, A. (2016). Encapsulation of probiotics: insights into academic and industrial approaches. AIMS Materials Science, 3(1), 114-136.

Hag, S. & Poondla, N. (2021). Genetically engineered probiotics. In Probiotic Research in Therapeutics (pp. 295{328). Springer.

Her, J.-Y., Kang, T., Hoptowit, R., & Jun, S. (2019). Oscillating magnetic field (omf) based supercooling preservation of fresh-cut honeydew melon. Transactions of the ASABE, 62(3), 779-785.

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R.

B., Flint, H. J., Salminen, S., et al. (2014). Expert consensus document: The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature reviews Gastroenterology & hepatology, 11(8), 506.

Hubalek, Z. (2003). Protectants used in the cryopreservation of microorganisms. Cryobiology, 46(3), 205-229.

Hyn¨onen, U. & Palva, A. (2013). *Lactobacillus* surface layer proteins: structure, function and applications. Applied microbiology and biotechnology, 97(12), 5225-5243.

Ikezoe, Y., Hirota, N., Nakagawa, J., & Kitazawa, K. (1998). Making water levitate. Nature, 393(6687), 749-750.

Jha, P. K., Sadot, M., Vino, S. A., Jury, V., Curet-Ploquin, S., Rouaud, O., Havet, M., & Le-Bail, A. (2017a). A review on effect of dc voltage on crystallization process in food systems. Innovative Food Science & Emerging Technologies, 42, 204-219.

Jha, P. K., Xanthakis, E., Jury, V., & Le-Bail, A. (2017b). An overview on magnetic field and electric field interactions with ice crystallisation; application in the case of frozen food. Crystals, 7(10), 299.

Kang, T., Hoptowit, R., & Jun, S. (2020). Effects of an oscillating magnetic field on ice nucleation in aqueous iron-oxide nanoparticle dispersions during supercooling and preservation of beef as a food application. Journal of Food Process Engineering, 43(11), e13525.

Khem, S., Small, D. M., & May, B. K. (2016). The behaviour of whey protein isolate in protecting *Lactobacillus* plantarum. Food chemistry, 190, 717-723.

K¨onig, H. (1988). Archaeobacterial cell envelopes. Canadian Journal of Microbiology, 34(4), 395-406.

Kopelo , N. & Kirby, G. H. (1929). The relation of focal infection to mental disease. Journal of Mental Science, 75(309), 267-270.

Kunal Ahuja & Kritika Mamtani. (2021, May). *Probiotics Market Size By Ingredients (Lactobacilli, Bifidobacterium, Streptococcus, Bacillus), By End-Use (Human, Animal), By Application (Food and Beverages {Dairy Products, Non-Dairy Products, Cereals, Baked Food, Fermented Meat Products, Dry Food}, Dietary Supplements {Food, Nutritional, Specialty Nutrients, Infant Formula}, Animal Feed*) (Report ID: GMI418). https://www.gminsights.com/industry-analysis/probiotics-market

Leslie, S. B., Israeli, E., Lighthart, B., Crowe, J. H., & Crowe, L. M. (1995). Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. Applied and environmental microbiology, 61(10), 3592-3597.

Linders, L. J., de Jong, G. I., Meerdink, G., & van't Riet, K. (1997a). Carbohydrates and the dehydration inactivation of *Lactobacillus* plantarum: the role of moisture distribution and water activity. Journal of Food Engineering, 31(2), 237-250.

Linders, L. J., Wolkers, W. F., Hoekstra, F. A., & van't Riet, K. (1997b). Effect of added carbohydrates on membrane phase behavior and survival of dried*Lactobacillus* plantarum. Cryobiology, 35(1), 31-40.

Liu, B., Song, J., Yao, Z., & Bennacer, R. (2017). Effects of magnetic field on the phase change cells and the formation of ice crystals in biomaterials: Carrot case. Journal of Thermal Science and Engineering Applications, 9(3).

Lourens-Hattingh, A. & Viljoen, B. C. (2001). Yogurt as probiotic carrier food. International dairy journal, 11(1-2), 1-17.

Meng, X., Stanton, C., Fitzgerald, G., Daly, C., & Ross, R. (2008). Anhydrobiotics: The challenges of drying probiotic cultures. Food Chemistry, 106(4), 1406-1416.

Meryman, H. (1971). Cryoprotective agents. Cryobiology, 8(2), 173-183.

Monedero, V., Maz e, A., Bo¨el, G., Zu´n˜iga, M., Beaufils, S., Hartke, A., & Deutscher, J. (2007). The phosphotransferase system of *Lactobacillus* casei: regulation of carbon metabolism and connection to cold shock response. Journal of molecular microbiology and biotechnology, 12(1-2), 20-32.

Morgan, C. A., Herman, N., White, P., & Vesey, G. (2006). Preservation of microorganisms by drying; a review. Journal of microbiological methods, 66(2), 183-193.

Naito, M., Hirai, S., Mihara, M., Terayama, H., Hatayama, N., Hayashi, S., Matsushita, M., & Itoh, M. (2012). Effect of a magnetic eld on drosophila under supercooled conditions. PloS one, 7(12), e51902.

Nireesha, G., Divya, L., Sowmya, C., Venkateshan, N., Lavakumar, V., et al. (2013). Lyophilization/freeze drying-an review. International journal of novel trends in pharmaceutical sciences, 3(4), 87-98.

O¨ nneby, K., Pizzul, L., Bjerketorp, J., Mahlin, D., H akansson, S., & Wessman, P. (2013). Effects of di-and polysaccharide formulations and storage conditions on survival of freeze-dried sphingobium sp. World Journal of Microbiology and Biotechnology, 29(8), 1399-1408.

Otero, L., Rodr guez, A. C., P erez-Mateos, M., & Sanz, P. D. (2016). Effects of magnetic fields

on freezing: Application to biological products. Comprehensive Reviews in Food Science and Food Safety, 15(3), 646-667.

Pang, X.-F. & Deng, B. (2008). The changes of macroscopic features and microscopic structures of water under influence of magnetic eld. Physica B: Condensed Matter, 403(19-20), 3571-3577.

Pang, X.-F., Deng, B., & Tang, B. (2012). Influences of magnetic field on macroscopic properties of water. Modern Physics Letters B, 26(11), 1250069.

Pano , J.-M., Thammavongs, B., & Gu eguen, M. (2000). Cryoprotectants lead to phenotypic adaptation to freeze{thaw stress in *Lactobacillus* delbrueckii ssp. Bulgaricus cip 101027t. Cryobiology, 40(3), 264-269.

Phadtare, S. et al. (2004). Recent developments in bacterial cold-shock response. Current issues in molecular biology, 6(2), 125-136.

Pradhan, D., Mallappa, R. H., & Grover, S. (2020). Comprehensive approaches for assessing the safety of probiotic bacteria. Food Control, 108, 106872.

Pum, D. & Sleytr, U. B. (2009). S-layer proteins for assembling ordered nanoparticle arrays. In Nanobioelectronics-for Electronics, Biology, and Medicine (pp. 167-180). Springer.

Purnell, G., James, C., & James, S. J. (2017). The effects of applying oscillating magnetic fields during the freezing of apple and potato. Food and Bioprocess Technology, 10(12), 2113-2122.

Reddy, K. B. P. K., Awasthi, S. P., Madhu, A. N., & Prapulla, S. G. (2009). Role of cryoprotectants on the viability and functional properties of probiotic lactic acid bacteria during freeze drying. Food Biotechnology, 23(3), 243-265.

Resta-Lenert, S. & Barrett, K. (2003). Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive escherichia coli (eiec). Gut, 52(7), 988-997.

Rosen, R. & Ron, E. Z. (2002). Proteome analysis in the study of the bacterial heatshock response. Mass Spectrometry Reviews, 21(4), 244-265.

Santivarangkna, C., Kulozik, U., & Foerst, P. (2006). Effect of carbohydrates on the survival of *Lactobacillus* helveticus during vacuum drying. Letters in applied microbiology, 42(3), 271-276.

Sch¨ar-Zammaretti, P. & Ubbink, J. (2003). The cell wall of lactic acid bacteria: surface constituents and macromolecular conformations. Biophysical Journal, 85(6), 4076-4092.

Schwenger, E. M., Tejani, A. M., & Loewen, P. S. (2015). Probiotics for preventing urinary tract infections in adults and children. Cochrane Database of Systematic Reviews, (12).

Segal, G. & Ron, E. Z. (1998). Regulation of heat-shock response in bacteria. Annals of the New York Academy of Sciences, 851(1), 147-151.

Selmer-Olsen, E., Birkeland, S.-E., & S rhaug, T. (1999). Effect of protective solutes on leakage from and survival of immobilized *Lactobacillus* subjected to drying, storage and rehydration. Journal of Applied Microbiology, 87(3), 429-437.

Semikhina, L. & Kiselev, V. (1988). Effect of weak magnetic elds on the properties of water and ice. Soviet Physics Journal, 31(5), 351-354.

Semyonov, D., Ramon, O., Kaplun, Z., Levin-Brener, L., Gurevich, N., & Shimoni, E. (2010). Microencapsulation of *Lactobacillus* paracasei by spray freeze drying. Food Research International, 43(1), 193-202.

Shah, N. P. (2007). Functional cultures and health bene ts. International dairy journal, 17(11), 1262-1277.

Shu, G., Wang, Z., Chen, L., Wan, H., & Chen, H. (2018). Characterization of freezedried *Lactobacillus acidophilus* in goat milk powder and tablet: Optimization of the composite cryoprotectants and evaluation of storage stability at di erent temperature. LWT, 90, 70-76.

Sleytr, U. B., S ara, M., Pum, D., & Schuster, B. (2001). Characterization and use of crystalline bacterial cell surface layers. Progress in Surface Science, 68(7-8), 231-278.

So, S. S., Wan, M. L., & El-Nezami, H. (2017). Probiotics-mediated suppression of cancer. Current opinion in oncology, 29(1), 62-72.

Song, M., Yun, B., Moon, J.-H., Park, D.-J., Lim, K., & Oh, S. (2015). Characterization of selected *Lactobacillus* strains for use as probiotics. Korean journal for food science of animal resources, 35(4), 551.

Song, S., Bae, D.-W., Lim, K., Gri ths, M. W., & Oh, S. (2014). Cold stress improves the ability of *Lactobacillus* plantarum l67 to survive freezing. International journal of food microbiology, 191, 135-143.

Szcze s, A., Chibowski, E., Ho lysz, L., & Rafalski, P. (2011). Effects of static magnetic field on water at kinetic condition. Chemical Engineering and Processing: Process Intensi cation, 50(1), 124-127.

Teitelbaum, J. E. & Walker, W. A. (2002). Nutritional impact of pre-and probiotics as protective gastrointestinal organisms. Annual review of nutrition, 22(1), 107-138.

Teixeira, P., Castro, H., & Kirby, R. (1996). Evidence of membrane lipid oxidation of spray-dried

Lactobacillus bulgaricus during storage. Letters in Applied Microbiology, 22(1), 34-38.

Thirabunyanon, M. & Thongwittaya, N. (2012). Protection activity of a novel probiotic strain of bacillus subtilis against salmonella enteritidis infection. Research in veterinary science, 93(1), 74-81.

Tissier, H. (1906). The treatment of intestinal infections by the method of transformation of bacterial intestinal flora. CR Soc. Biol, 60, 359-361.

Toledo, E. J., Ramalho, T. C., & Magriotis, Z. M. (2008). Influence of magnetic field on physical-chemical properties of the liquid water: Insights from experimental and theoretical models. Journal of Molecular Structure, 888(1-3), 409-415.

Tripathi, M. K. & Giri, S. K. (2014). Probiotic functional foods: Survival of probiotics during processing and storage. Journal of functional foods, 9, 225-241.

Vinderola, C. G., Prosello, W., Ghiberto, D., & Reinheimer, J. A. (2000). Viability of probiotic (bifidobacterium, *Lactobacillus acidophilus* and *Lactobacillus* casei) and nonprobiotic microflora in argentinian fresco cheese. Journal of Dairy Science, 83(9), 1905-1911.

V´ıtor, J. M. & Vale, F. F. (2011). Alternative therapies for helicobacter pylori: probiotics and phytomedicine. FEMS Immunology & Medical Microbiology, 63(2), 153-164.

Wang, G., Luo, L., Dong, C., Zheng, X., Guo, B., Xia, Y., Tao, L., & Ai, L. (2021). Polysaccharides can improve the survival of lactiplantibacillus plantarum subjected to freeze-drying. Journal of Dairy Science, 104(3), 2606-2614.

Wang, Y.-C., Yu, R.-C., & Chou, C.-C. (2004). Viability of lactic acid bacteria and *bifidobacteria* in fermented soymilk after drying, subsequent rehydration and storage. International journal

of food microbiology, 93(2), 209-217.

Wisselink, H., Weusthuis, R., Eggink, G., Hugenholtz, J., & Grobben, G. (2002). Mannitol production by lactic acid bacteria: a review. International Dairy Journal, 12(2-3), 151-161.

Wong, S., Jamous, A., O'Driscoll, J., Sekhar, R., Weldon, M., Yau, C. Y., Hirani, S. P., Grimble, G., & Forbes, A. (2014). A *Lactobacillus* casei shirota probiotic drink reduces antibiotic-associated diarrhoea in patients with spinal cord injuries: a randomised controlled trial. British Journal of Nutrition, 111(4), 672-678.

Yao, M., Wu, J., Li, B., Xiao, H., McClements, D. J., & Li, L. (2017). Microencapsulation of *Lactobacillus* salivarious li01 for enhanced storage viability and targeted delivery to gut microbiota. Food hydrocolloids, 72, 228-236.

Zayed, G. & Roos, Y. H. (2004). Influence of trehalose and moisture content on survival of *Lactobacillus* salivarius subjected to freeze-drying and storage. Process Biochemistry, 39(9), 1081-1086.

Zhan, X., Sun, D.-W., Zhu, Z., &Wang, Q.-J. (2018). Improving the quality and safety of frozen muscle foods by emerging freezing technologies: A review. Critical reviews in food science and nutrition, 58(17), 2925-2938.

Zhan, X., Zhu, Z., & Sun, D.-W. (2019). Effects of extremely low frequency electromagnetic field on the freezing processes of two liquid systems. Lwt, 103, 212-221.

Zhao, L., Ma, K., & Yang, Z. (2015). Changes of water hydrogen bond network with different externalities. International journal of molecular sciences, 16(4), 8454-8489.

Zheng, L. & Sun, D.-W. (2006). Innovative applications of power ultrasound during food freezing

processes a review. Trends in Food Science & Technology, 17(1), 16-23.

Zhou, K., Lu, G., Zhou, Q., Song, J., Jiang, S., & Xia, H. (2000). Monte carlo simulation of liquid water in a magnetic eld. Journal of Applied Physics, 88(4), 1802-1805.

Zhu, S., Ramaswamy, H. S., & Le Bail, A. (2005). Ice-crystal formation in gelatin gelduring pressure shift versus conventional freezing. Journal of Food Engineering, 66(1), 69-76.

Chapter 3

Enhanced viability of freeze-dried *Lactobacillus acidophilus* using supercooling pretreatment

ABSTRACT

Viability loss during the freezing process remains a big challenge for freeze-dried lactic acid bacteria. Oscillating magnetic field (OMF)-assisted supercooling pretreatment was studied and validated for the improving the viability of freeze-dried *L. acidophilus*. OMF-assisted supercooling decreased supercooling temperature to -7 °C without ice crystallization, while one of the reference groups without OMF assistance was frozen at -7 °C. Supercooling pretreatment increased surface layer protein (SLP) expression and improved cell viability. The SLP on supercooling pretreated cells was thicker than those without supercooling pretreated cells. Supercooling pretreatment significantly increased the viability of *L. acidophilus* after freeze-drying compared to those that did not undergo a pretreatment. The highest viability of 78% was observed after freeze-drying with supercooling pretreatment. SLP expression during pretreatment protected cells in the freezing process and helped maintain cell viability. These results suggested that OMF-assisted supercooling pretreatment enhanced the viability of freeze-dried *L. acidophilus*.

3.1. Introduction

Probiotics are living microorganisms which confer health benefits to the host by providing enough intestinal microflora. In the gastrointestinal tract (GI) system, probiotics regulate various functions, such as metabolism, digestion, immune system enhancement, and antitumor activities in gut and cervix (Azad et al. 2018; Cani & Van Hul 2015; Lourens- Hattingh & Viljoen 2001; Teitelbaum & Walker 2002; So et al. 2017). In therapeutic applications, probiotics effectively treat infection such as urinary tract infection (Schwenger et al. 2015) and vaginitis infection (Falagas et al. 2007). Increasing health awareness of consumers increased the demand for probiotics, resulting in the expansion of probiotics market size, according to Kunal Ahuja & Kritika Mamtani (2021), the probiotics market size is projected to increase from 2.3 billion USD in 2020 and approach 4.15 billion USD by 2027. Most probiotic fortified foods in the market contain strains of *Lactobacilli* and/or *Bifidobacterium*. In particular, the global *Lactobacillus* probiotics market was valued at 1.6 billion USD in 2020 and is poised to witness a significant growth rate through 2027. The clinical effects of *Lactobacillus* strains have been reported and focus on digestive and immune health. Recently, *Lactobacillus casei* Shirota has been considered an effective measurement for preventing diarrhea in patients with antibiotic-associated diarrhea (Wong et al. 2014). Mazloom et al. (2013) suggested that *Lactobacillus* strains effectively decrease the blood glucose level and are expected to have therapeutic benefits for type 2 diabetes. Also, *Lactobacillus* maintained the physiological homeostasis and GI health by modulating functional microflora (Huang et al. 2020; Foysal et al. 2020).

However, probiotics are sensitive to the environment; their viability is limited by manufacturing technology, which is mainly associated with processing temperature, the oxygen level in packaging, humidity in storage conditions, and so on. Moreover, according to the

requirement of the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), functional foods with probiotic bacteria should contain a minimum of 10^7 viable probiotic bacteria per gram of product at the time of consumption (Doleyres & Lacroix 2005; Adhikari et al. 2000; Ouwehand & Salminen 1998). Therefore, it is essential to maintain the viability of probiotics during the manufacture process.

To obtain long shelf-life, probiotics are usually in dried or frozen culture concentrates. These culture concentrates can be easily used as food products that can be fermented or non-fermented and dietary supplements, like powder, capsule, and tablet. However, like mentioned above, viability loss during the drying process leaves a big challenge (Santivarangkna et al. 2006). Therefore, drying technology should be appropriately chosen to maintain the viability and functionality of probiotics. Among a variety of drying methods (spray drying, freeze-drying, fluidized bed drying, etc.), freeze-drying is the most competent drying method for biological materials, which has been manufactured on probiotic powders for decades (Chen & Mustapha 2012; Elser et al. 1935; Rogers 1914; Wickerham & Andreasen 1942; Wickerham &Flickinger 1946). Freeze-drying consists of three main steps, (i) initial freezing of the cell suspension, (ii) primary drying to remove the ice by sublimation, (iii) secondary drying to remove unfrozen water by desorption. However, ice crystals formed in the initial freezing step, damaging cell structure, decreasing cell viability. (Nireesha et al. 2013; Cook et al. 2012; Wang et al. 2004). Therefore, protective agents stabilize the cell suspension during freeze-drying (Hubalek 2003; Haffner et al. 2016). Sugars (glucose, trehalose, and sorbitol) contribute to decomplex water-forming hydrogen bonds with biomolecules, thus restraining structural denaturation and other damages (Meng et al. 2008; Crowe et al. 1996; Santivarangkna et al. 2006). Skim milk and soybean powder formed a coating layer outside the cell surface throughout hydrophobic interactions (Choi et al. 2018; Khem

et al. 2016; Abadias et al. 2001). Polysaccharide maltodextrin reduced the mobility of the cells in the glassy state (Semyonov et al., 2010). Different cryoprotectants are usually used as a mixture, which protects LAB in many ways. However, the optimum combination for each probiotic strain should be determined for the specific product.

LAB develops their freezing tolerance by exposure to cold conditions before freezing. In other words, when LAB culture shifts from its optimum growth temperature to a lower temperature, they are under cold stress, and the cold-shock response is induced. The cold-induced protein synthesis is produced, and cells are gradually acclimated to the low temperature (Phadtare et al. 2004; Barria et al. 2013; Gualerzi et al. 2003; De Maayer et al. 2014). Surface layer protein (SLP), which induced by cold stress, covers the cell surface, and protect LAB during freeze-drying (Frece et al. 2005), Choi et al. (2018) investigated cold adaptation at 10 °C to strengthen *L. acidophilus*, they found that cold-adapted cells was found to show positive effects on the storage stability. Later, Choi et al. (2019) used lower temperature (-5 °C) to treat *L. acidophilus*, compared with 10 °C; they found that *L. acidophilus* demonstrates a higher viability, and more SLP was observed. Thus, they state that more SLP maintains the higher viability of LAB during the freeze-drying process. That is, lower temperature pretreatment effectively improved the freezing tolerance of *L. acidophilus*.

Supercooling is a status during the freezing process, in which the temperature of the liquid or gas is below its freezing point without becoming a solid. External OMF has been investigated to control ice nucleation. It can directly re-orientating and vibrating water molecules, and/or break hydrogen bonds between water molecules, to improve supercooling degree and form small ice crystallization (Jha et al., 2017; Otero et al. 2017). Therefore, in this study, we used OMF-assisted supercooling technology, to decrease the pretreatment temperature to -7 °C without ice nucleation.

Cells without supercooling pretreatment were used as control (freezing at -18 °C, F (-18 °C), refrigerator at 7 °C, R (7 °C) and freezing at -7 °C, F (-7 °C). The effects of OMF-assisted supercooling pretreatment were evaluated on SLP production, cell wall thickness, and the viability of *L. acidophilus.*

3.2. Materials and Methods

3.2.1. Bacterial strain and growth conditions

Frozen *L. acidophilus* cultures were obtained from the Food Microbiology Lab, (University of Hawai'i at Manoa, Honolulu, HI). All experiments were conducted in a certified biosafety level II laboratory. *L. acidophilus* was cultured in De Man, Rogosa, and Sharpe (MRS) medium (Difco Laboratories, Sparks, MD, USA) at 37°C for 24h. The culture was then stored in 1.5 mL cryovial tubes (microcentrifuge tubes, sigma, USA) at -80 °C (VIP ECO, Phcbi, USA) as concentrated cellular biomass in 20% glycerol.

3.2.2. Pretreatment procedure

L. acidophilus was cultured in 50 ml of MRS broth (Difco™ Lactobacilli MRS Agar, VWR, USA) at 37°C for 24 h, then harvested by centrifugation (6000×g for 15 min at room temperature (25°C)). Then, the precipitated cells were washed with 0.9% sterile saline three times. Next, 50 ml of 10% sterile skim milk solution (skimmed milk, VWR, USA) was added, and the initial count number was plated in MRS agar (Difco™ Lactobacilli MRS Agar, VWR, USA). The cells were exposed to -7 °C (SC (-7 °C)) for 12 hours with OMF-assisted supercooling, then freeze-drying (XL50, VirTis, USA) for 48 h. Cells without supercooling pretreatment were used as control (freezing at -18 °C, F (-18 °C), refrigerator at 7 °C, R (7 °C) and freezing at -7 °C, F (-7 °C). The freeze-dried cells were transferred to sealed polyethylene tubes and stored at two temperature conditions (-18 °C and 25 °C), which analog storage conditions at freezing and room temperature

(25 °C), the viability of *L. acidophilus* was determined after 0, 7, and 14 days of storage by standard count method based on MRS agar medium.

OMF-assisted supercooling

The supercooling process was performed in a 7.1 ft^3 (0.2 m^3) commercial chest freezer (Haier, Qingdao, China) with a PID controller (D1S-2R-220, SESTOS Electronics H·K., Hong Kong). The freezer was stabilized overnight before the experiments. Table 3.1 shows the supercooling behaviors of samples stored at different storage temperature with/without OMF (10 mT, 30 HZ) for 12 hours. Some of OMF-assisted samples were supercooled and all the controls were nucleated, 80% samples can be supercooled at -7 °C when stored under OMF-assisted -10 °C storage temperature. Thus, the freezer temperature was set at -10 °C to obtain supercooling environment. Figure 3.1 shows a schematic of the fabricated supercooling chamber. Helmholtz coils were placed upon the Styrofoam-like box to generate OMF. The magnet was operated using a power supply based on an integrated gate bipolar transistor (IGBT) (SKYPER™, SEMIKRON Inc., Hudson, NH). A function generator (33220A, Agilent Technologies, Santa Clara, Cal.) was used to control the frequency and waveform of the magnetic field output (30 Hz at 50% duty cycle and pulse wave). The field strength of the applied OMF was measured using a Teslameter (F71, Lake Shore Cryotronics Inc., Westerville, OH). However, the current passes against the resistance of the winding coils, and the heat was produced by the electric current flow (Otero et al., 2017). A fan with an airflow rate of 1 $ft^3\ min^{-1}$ (0.03 $m^3\ min^{-1}$) was set on the bottom of the Styrofoam-like box to circulate the cold air into it.

Samples were placed in a holder, which was created using a 3D printer (Form2, Formlabs, MA). Thermocouple wires were taped on the bottom of the tubes to collect sample temperatures and temperature data was collected using T-type thermocouples (TT-T-40-SLE, Omega

Engineering, Inc., Stamford, Conn.) and recorded every 10s using a data acquisition unit (39704A, Agilent Technologies, Inc., Santa Clara, CA). In the process of time-temperature monitoring, the time at which the sample temperature was instantaneously increased to the freezing point was determined as the time of ice nucleation.

Table 3.1 Supercooling behavior of *L. acidophilus* with 50 ml 10% skim milk stored at different storage temperature with/without OMF intensities of 10 mT for 12 hours.

Storage temperature (°C)	OMF (10 mT)	Trail #	Supercool? (°C)	Initial temperature (°C)	Nucleation temperature (°C)	Freezing temperature (°C)	Time at nucleation (h)
-9	On	1	Yes, -6.01	19.94	-	-	-
		2	Yes, -5.74	20.10	-	-	-
		3	Yes, -6.56	21.12	-	-	-
		4	Yes, -5.74	20.98	-	-	-
		5	Yes, -6.56	19.61	-	-	-
	Off	1	No	22.08	-5.63	-1.00	2.14
		2	No	20.00	-6.11	-0.94	1.65
		3	No	22.93	-5.97	-1.21	2.11
		4	No	19.99	-4.32	-1.62	1.98
		5	No	21.98	-6.26	-0.99	1.72
-10	On	1	Yes, -8.11	21.15	-	-	-
		2	Yes, -7.00	20.15	-	-	-
		3	Yes, -6.82	19.62	-	-	-
		4	Yes, -7.39	21.23	-	-	-
		5	No	20.98	-7.10	-1.32	5.32
	Off	1	No	18.04	-7.00	-0.80	1.82
		2	No	19.95	-6.91	-1.22	1.93
		3	No	22.78	-7.12	-0.94	2.12
		4	No	19.82	-5.91	-1.82	1.98
		5	No	21.35	-6.23	-1.02	2.00
-11	On	1	No	21.03	-6.43	-1.11	4.14
		2	No	19.17	-8.21	-1.07	3.52
		3	Yes, -6.71	20.98	-	-	-
		4	No	19.87	-8.10	-1.52	8.31
		5	No	18.92	-6.91	-0.92	6.15
	Off	1	No	20.92	-5.93	-1.31	1.71
		2	No	19.90	-6.74	-0.98	1.19
		3	No	18.94	-4.37	-1.14	1.58
		4	No	18.78	-7.12	-1.24	1.32
		5	No	17.99	-5.17	-1.82	1.69

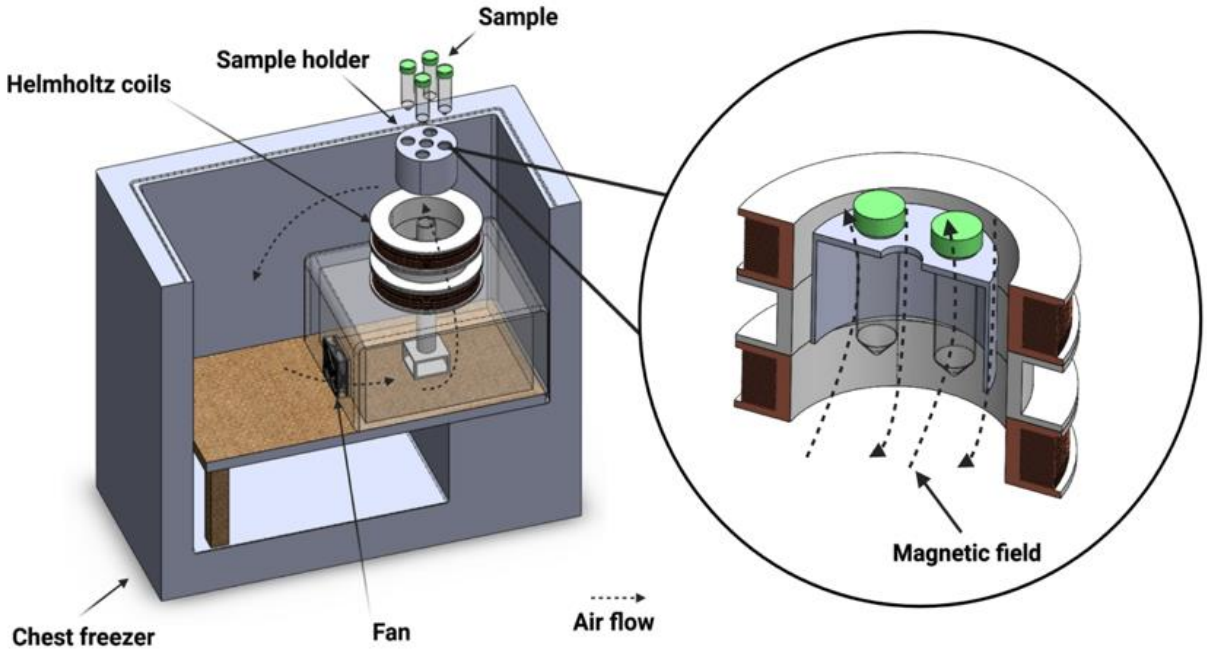


Figure 3.1 Schematic diagrams of the experimental apparatus.

Determination of L. acidophilus viability

The viable count of bacteria was conducted before and after freeze-drying separately. The collected cells or dried bacterial powder were randomly selected and added into the sterile saline solution for a series of dilutions. After that, bacterial suspension was injected into the MRS agar plate, and the plates were held in the incubator at 37 °C incubator for 48 h. The viability of *L. acidophilus* was determined by the standard count method based on MRS agar medium. A flow chart of the experiment process by different pretreatments is shown in Figure 3.2.

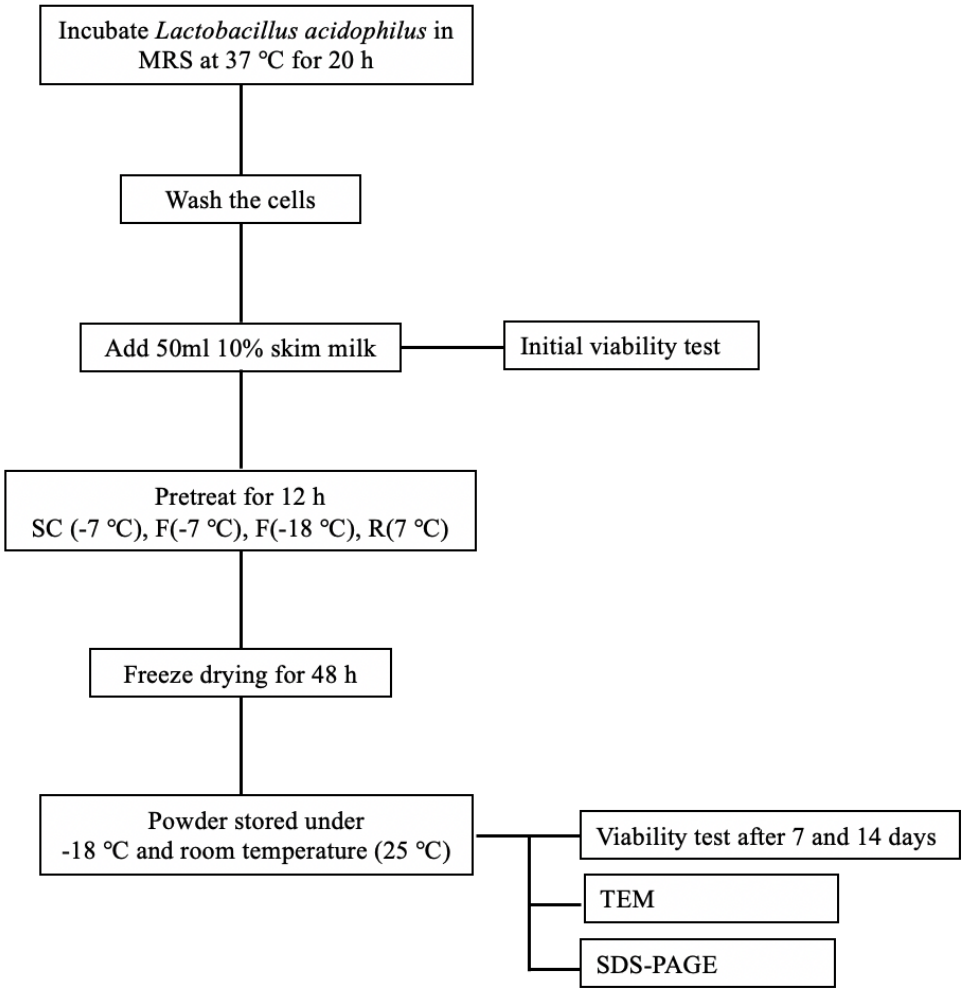


Figure 3.2 Flow chart of experiment process by different pretreatments.

3.2.3. Proteomic analysis

Electrophoresis

After incubation and wash, *Lactobacillus acidophilus* cells were mixed with 5 ml PBS, then samples were pretreated under -7 °C supercooling and -80 °C for 12 hours, respectively. Next, followed the procedures mentioned above. Different pretreated *Lactobacillus acidophilus* cells powder was dissolved in PBS (pH=7.2), 30 μl of DNAase (1mg ml^{-1}) and lysozyme (10mg ml^{-1}) were added to every 3 ml of *Lactobacillus acidophilus* suspension, respectively. Then, the suspensions were stored under room temperature (25 °C) for 30 min, then sonicated for 15 min in an ice-water bath. Next, the samples were centrifuged at 15 000 × *g* for 3 min, the supernatants containing SLP were left, subsequently, the SLP was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using 4–12 % NuPAGE Bis-Tris gels (Thermo Fisher, Carlsbad, CA, USA), followed by method of Laemmli (1970).

Transmission electron microscopy (TEM)

L. acidophilus cells were fixed with 2.5 % glutaraldehyde in 0.1M sodium cacodylate buffer with 2mM calcium chloride, pH 7.4 for 1-2 hours, washed in 0.1M cacodylate buffer, followed by post fixation with 1% OsO_4 in 0.1M cacodylate buffer for 1 hour. Tissue was dehydrated twice in an ethanol series of 30%, 50%, 70%, 85%, 95%, and three times in absolute ethanol; then the cells were substituted with propylene oxide and embedded in LX112 epoxy resin. Ultrathin (60-80 nm) sections were obtained on an RMC Powertome ultramicrotome, double-stained with uranyl acetate and lead citrate, viewed on a Hitachi HT7700 TEM at 100 kV, and photographed with an AMT XR-41B 2k x 2k CCD camera. The average thickness was determined from measurements for 5 bacteria at different regions of each sample, and on average 10 thickness points were took from each bacterium.

3.2.4. Statistical analysis

The experiment data is presented as the mean from five independent experiments and was analyzed using OriginPro 2021b (OriginLab Corporation, Northampton, MA, USA.). A one-way ANOVA was conducted on the variables along with Bonferroni Tests. The significant difference among the different treatments was determined at a confidence level of 95%.

3.3. Results and discussion

3.3.1. Effect of OMF-assisted supercooling

The cooling curves of the OMF-assisted supercooling and control samples are shown in Fig.3.3. *L. acidophilus* samples were cooled below their freezing point without ice nucleation, indicating that the samples were in supercooling status. OMF-assisted supercooling *L. acidophilus* samples were successfully preserved in supercooling status at -7 °C for 12 hours, whereas control samples were spontaneously nucleated within 2 hours. These results show that OMF effectively controlled ice nucleation and maintained sample under supercooling status. The intensity of OMF in this study was 10 mT at 30 Hz, which is relatively weak; however, it still impacted water properties. According to previous research, weak OMF influences water by re-orientating and vibrating water molecules, and/or break hydrogen bonds between water molecules (Otero et al., 2017). Similar research has been approved the effects of weak OMF on water (Semikhina & Kiselev 1988; Zhan et al. 2019; Liu et al. 2017; Her et al. 2019). Nevertheless, OMF is still a controversial topic, some researchers doubt its mechanisms (Purnell et al., 2017; Naito et al., 2012). However, in this study, OMF has been successfully maintained supercooling status up to 12 hours.

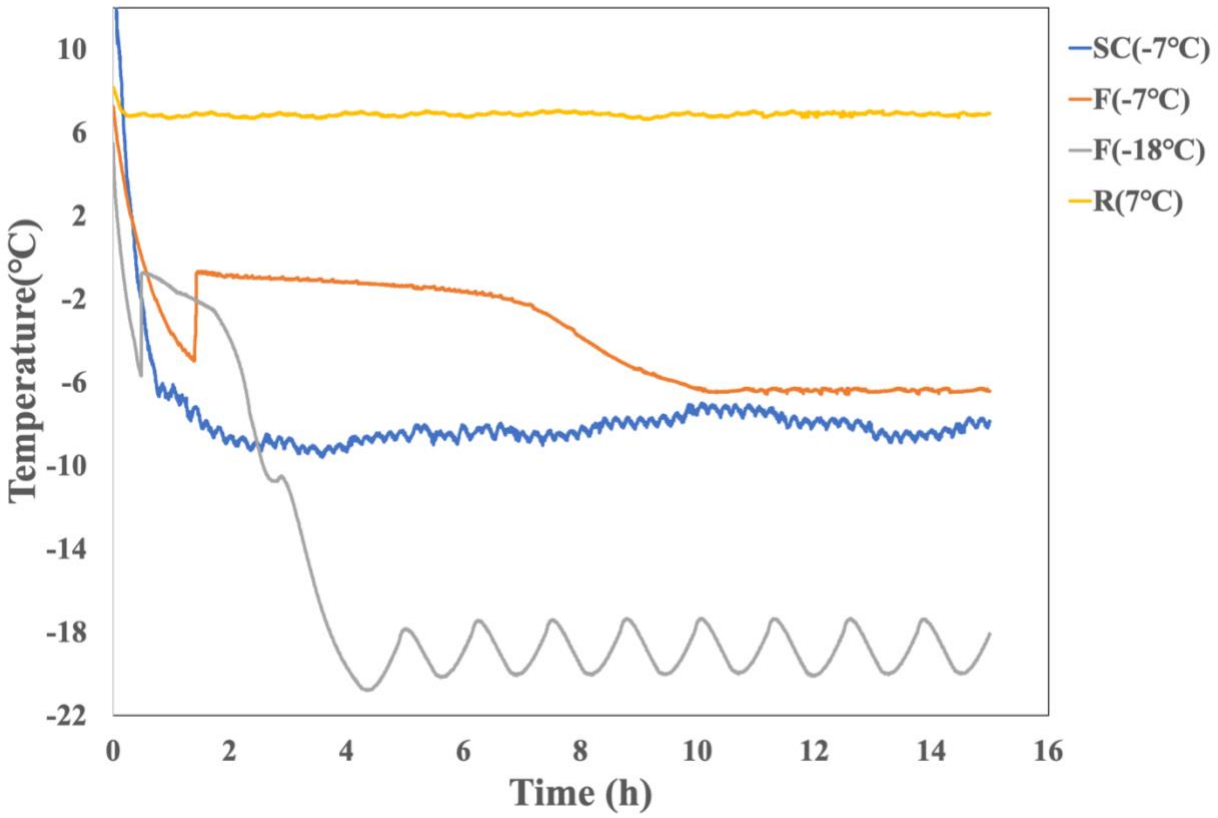


Figure 3.3 Temperature profiles of supercooling and control samples. The OMF-assisted supercooling samples were maintained in supercooling state for 12 h.

3.3.2. Effect of supercooling on the cell surface

SLP formation

Formation of SLPs in the cell surface was evaluated by SDS-PAGE after freeze-drying. As mentioned above, in the pretreat process, cells were mixed with PBS instead of skim milk. On the one hand, PBS avoided the background influences from skim milk, performed a clear protein profile; more importantly, cells were under a severer condition when mixed with PBS, which is a further proof of supercooling effects. Fig 3.4 shows that the whole protein patterns of *L. acidophilus* were different under different pretreatments. Even though SLP was detected on both control and supercooling cells, a more intense band of SLP was observed in supercooling

pretreated cells. Which is a clear sign that the effect of supercooling on the production of SLP. This confirmed that SLP is induced by freezing, but it is expressed stronger after exposure to a colder environment before freezing (Khaleghi et al., 2010; Khaleghi & Kermanshahi 2012).

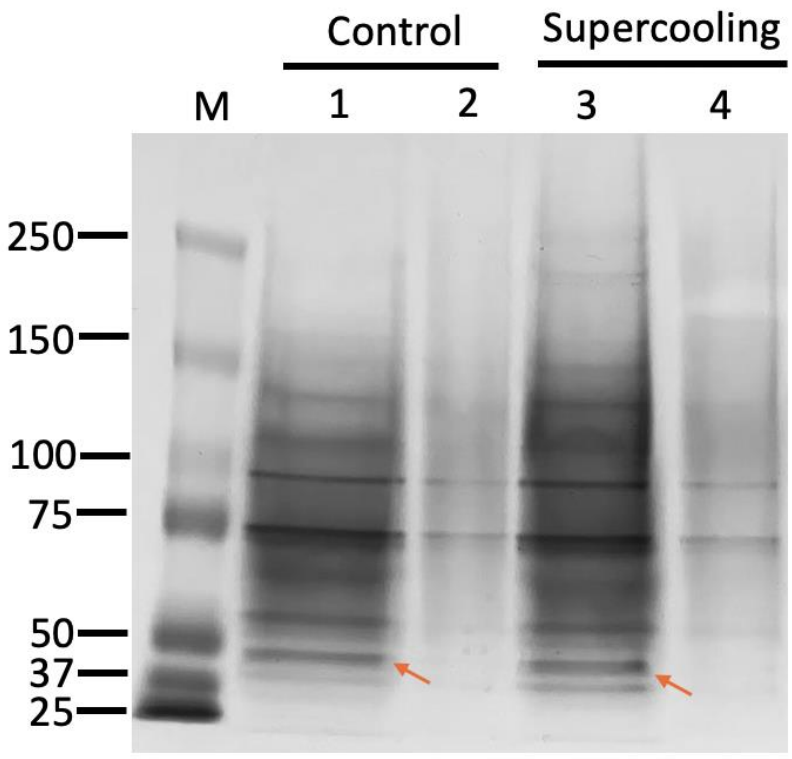


Figure 3.4 SDS-PAGE protein profiles of L. acidophilus. M: standard marker, Line 1: supernatant from the control group, line 2: pellets from the control group, line 3: supernatant from the supercooling group, line 4: pellets from the supercooling group.

Changes in cell wall thickness

Cell surface changes in *L. acidophilus* were evaluated by TEM (Table 3.2). SLP covered the cell surfaces of both supercooling and control cells. However, there was a significant difference ($F = 52.29$, $df = 234$, $P < 0.05$) in the thickness of surface layer between different groups, supercooling pretreatment increased the thickest layer to 10.53 ± 1.32 nm, followed by 8.04 ± 1.76

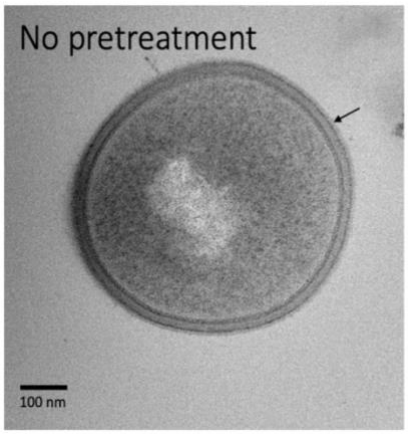
nm with frozen at -7 °C group; 7.12 ± 1.16 nm with frozen at -18 °C group, and 7.24 ± 1.77 nm with refrigerated at 7 °C group (Table. 3.1). Surface layer thickness increased in all groups; however, thickness increased the most in the supercooling group, as the thickness increase rate may indicate SLP production was influenced by temperature. For TEM and thickness change after pretreatment and during storage, please refer to Appendix A.

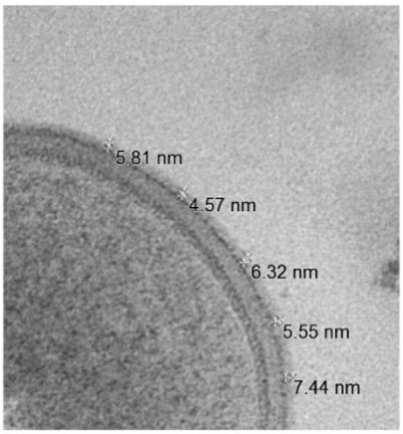
Table 3.2 Cell wall thickness analysis of different pretreatment L. acidophilus after freeze-drying. ($F = 52.29$, $df = 234$, $P < 0.05$). Different letters indicate that values are significantly different (Bonferroni Tests significant difference test). Cells without any treatment, No pretreatment, supercooling at -7 °C, SC (-7 °C), freezing at -18 °C, F (-18 °C), refrigerator at 7 °C, R (7 °C), freezing at -7 °C, F (-7 °C).

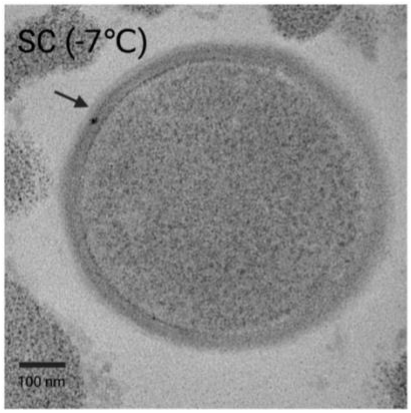
Treatment condition	Surface layer thickness (nm)	Increase rate (%)
No pretreatment	6.23 ± 1.24 d	-
SC (-7 ℃)	10.53 ± 1.32 a	69.02
F (-7 ℃)	8.04 ± 1.76 b	29.05
F (-18 ℃)	7.12 ± 1.16 cd	14.29
R (7 ℃)	7.24 ± 1.77 bc	16.21

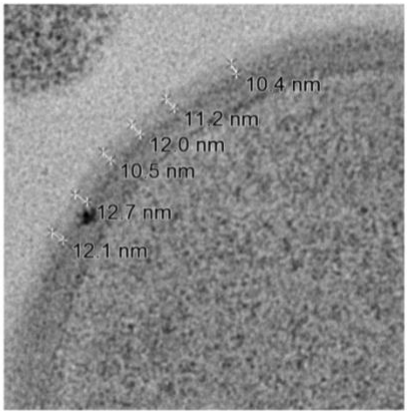
In industrial processes, probiotics are subjected to various adverse conditions (temperature, pH, nutrition, osmotic pressures, etc.), in which these stresses activate bacteria stress-sensing systems, bacteria respond by activating acclimation mechanisms, including protein induction and some physiological changes to acclimate to adverse environments (Grosu-Tudor et al. 2016). Among mentioned stresses, cold stress is special since it exists in all kinds of manufacturing processes. Palomino et al. (2016) and Khaleghi et al. (2010) found there is an increased release of

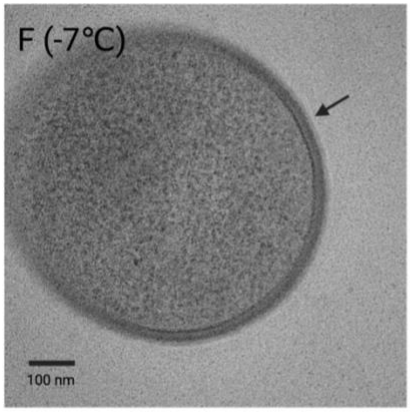
SLP when *L. acidophilus is* under stress conditions; they state that pre-stress process helps *L. acidophilus* produce more common and specific proteins. Thus, in this article, we believe that supercooling pretreatment helps *L. acidophilus* produce more SLP, and thicker surface layer protect *L. acidophilus* from the freezing threat.

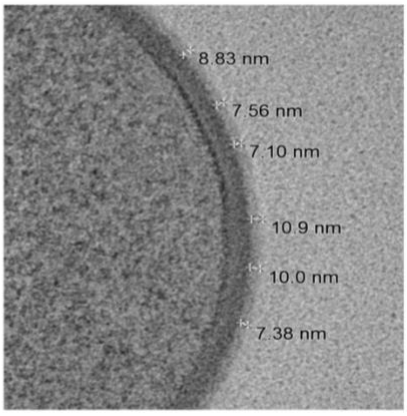

No pretreatment
100 nm


5.81 nm
4.57 nm
6.32 nm
5.55 nm
7.44 nm


SC (-7°C)
100 nm


10.4 nm
11.2 nm
12.0 nm
10.5 nm
12.7 nm
12.1 nm


F (-7°C)
100 nm


8.83 nm
7.56 nm
7.10 nm
10.9 nm
10.0 nm
7.38 nm

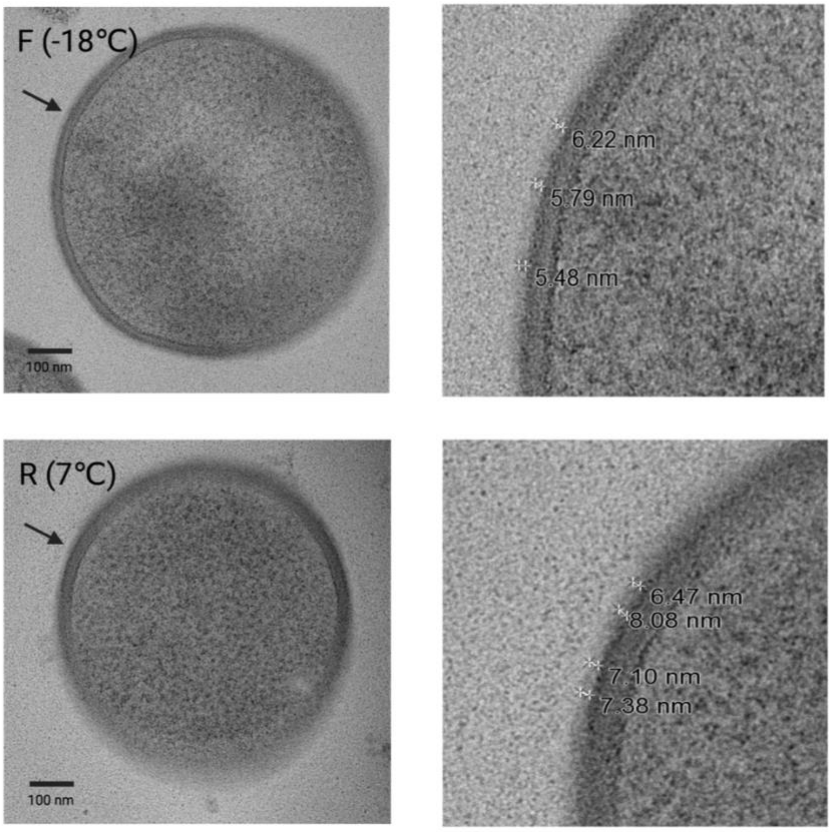


Figure 3.5 TEM images of L. acidophilus cells after freeze-drying. No pretreatment, cells without any treatment; cells with -7 °C supercooling pretreatment, SC (-7 °C); -7 °C frozen pretreatment, F (-7 °C); -18 °C frozen pretreatment, F (-18 °C); 7 °C refrigerated pretreatment, R (7 °C). The layer at the surface of the cell wall represents SLP (black arrow), scale bars indicate 100 nm, images on the right show the close view of the arrow.

3.3.3. Effect of supercooling pretreatment on cell viability enhancing

After freeze-drying, the viability of freeze-dried *L. acidophilus* cells differed under different pretreatment conditions (Fig.3.6). Supercooling pretreatment shows the highest viability of *L. acidophilus*, 78.83% of *L. acidophilus* cells survived with supercooling pretreatment, while the viability of control group cells was lower than 60%. Specifically, cells were frozen when stored at -7 °C without OMF assisting (F (-7 °C)), the viability was only 53.38%. Viability in refrigerated group (R (7 °C)) was 54.34%, and frozen at -18 °C group (F (-18 °C)) was 44.57%. High viability

in the supercooling group indicates that supercooling pretreatment improved the viability of *L. acidophilus*. Based on the SDS-PAGE and TEM results, supercooling pretreatment increased SLP production and SLP help to maintain the viability of *L. acidophilus* during freeze-drying.

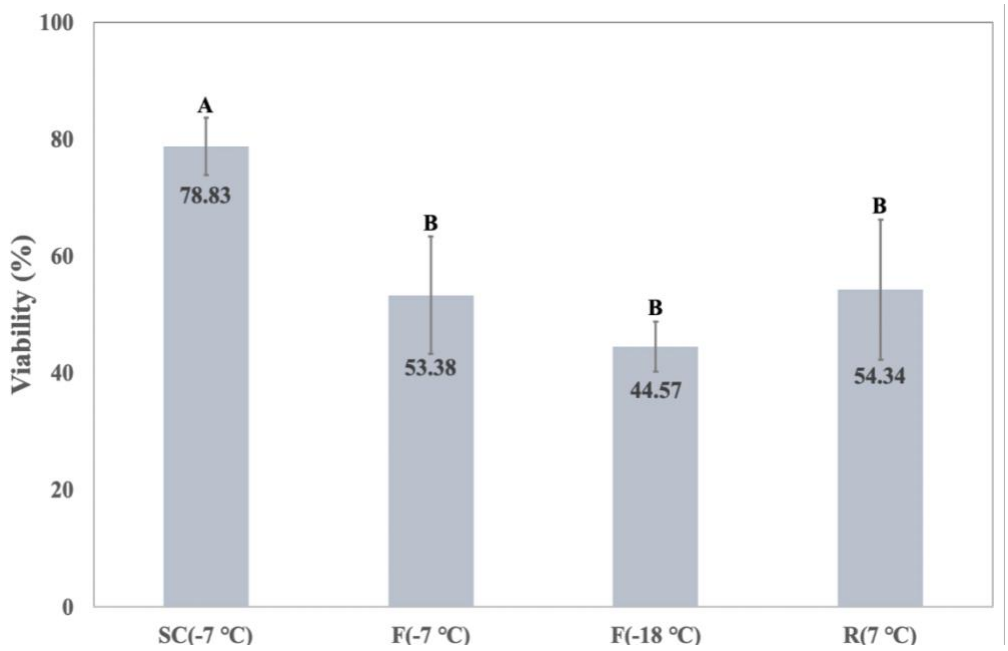


Figure 3.6 Viability of L. acidophilus after freeze-drying. SC (-7 °C): supercooling at -7 °C; F (-7 °C): freezing at -7 °C; F (-18 °C): freezing at -18 °C; R (7 °C): refrigerated at 7 °C.

However, the viability decreased dramatically within only 7 and 14 days (Fig. 3.7-3.10), which might be caused by the packaging. In this study, the freeze-dried powder was sealed in the centrifuge tubes, while during the storage period, many factors such as oxygen content, storage temperature, moisture content of powders, relative humidity, light, and so on, could pose threats to the viability (Lee & Salminen 2009; Mattila-Sandholm et al. 2002). Therefore, packaging in this project needs to be further improved.

The viability of *L. acidophilus* varied with storage temperature, in general, powder stored under -18 °C has higher viability than that stored under room temperature (25 °C). As shown in

Fig. 3.7 to 3.10, after 7 and 14-day preservation, the viability of *L. acidophilus* stored at -18 °C was slightly higher than those stored at room temperature (25 °C). This is because longevity of freeze-dried microbial cells depends on the storage temperature (Capela et al. 2006), and microorganisms survive better at a relatively low temperature (Amine et al. 2014). Under high temperatures, intracellular water tends to be more active and migrates more frequently, bacteria struggle with water transmission and intensive oxidation, which are detrimental to bacteria survival. Thus, high viability of freeze-dried *L. acidophilus* powder requires unnecessary contact with oxygen and moisture.

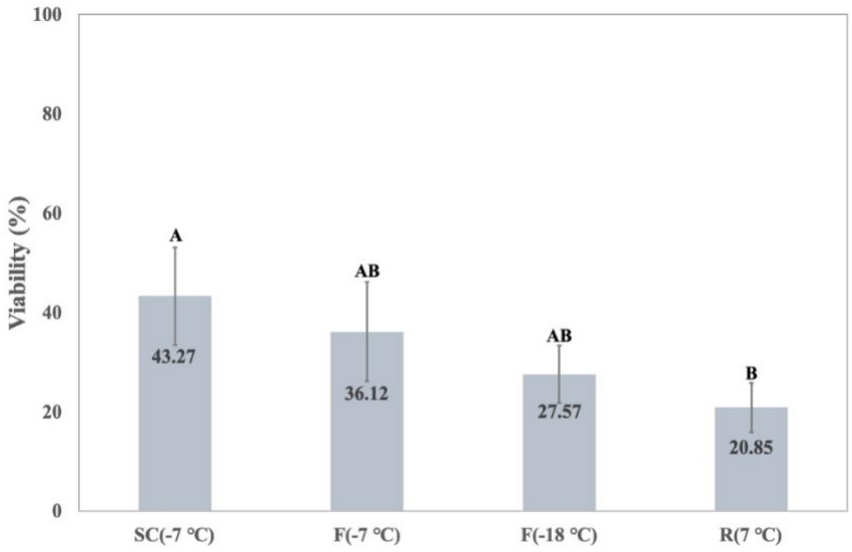


Figure 3.7 Viability of L. acidophilus after 7-day stored at -18 °C. SC (-7 °C): supercooling at -7 °C; F (-7 °C): freezing at -7 °C; F (-18 °C): freezing at -18 °C; R (7 °C): refrigerated at 7 °C.

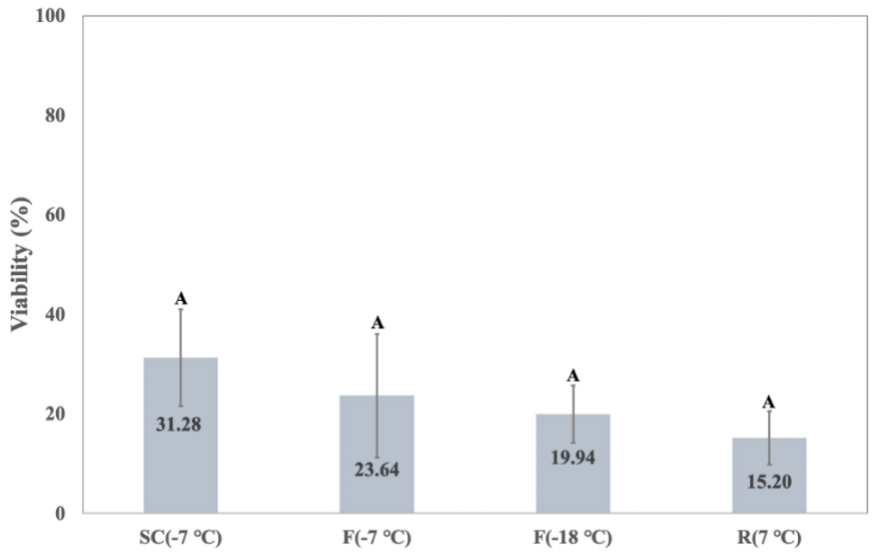


Figure 3.8 Viability of L. acidophilus after 14-day stored at -18 °C. SC (-7 °C): supercooling at -7 °C; F (-7 °C): freezing at -7 °C; F (-18 °C): freezing at -18 °C; R (7 °C): refrigerated at 7 °C.

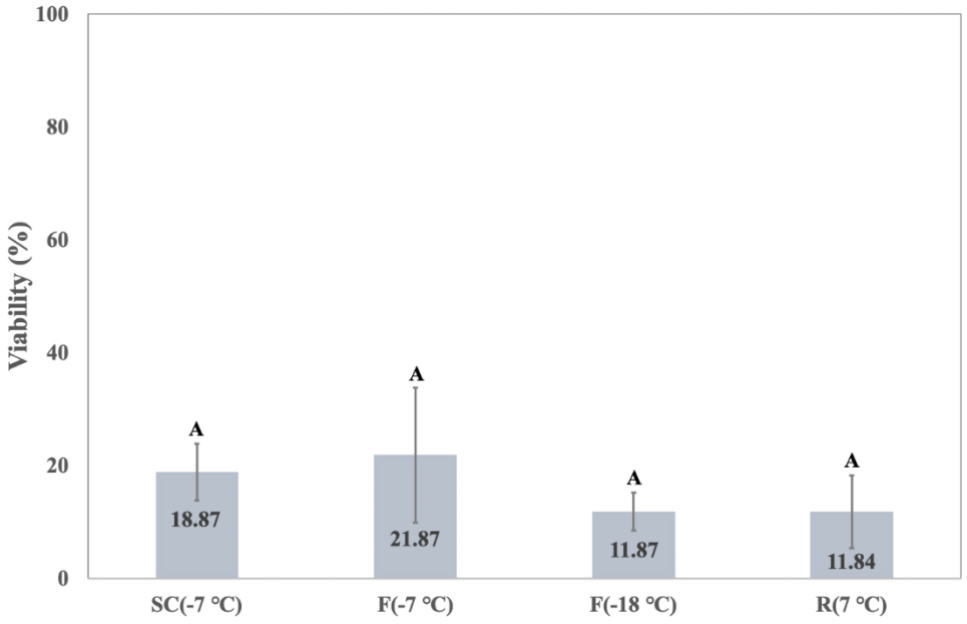


Figure 3.9 Viability of L. acidophilus after 7-day stored at room temperature (25 °C). SC (-7 °C): supercooling at -7 °C; F (-7 °C): freezing at -7 °C; F (-18 °C): freezing at -18 °C; R (7 °C): refrigerated at 7 °C.

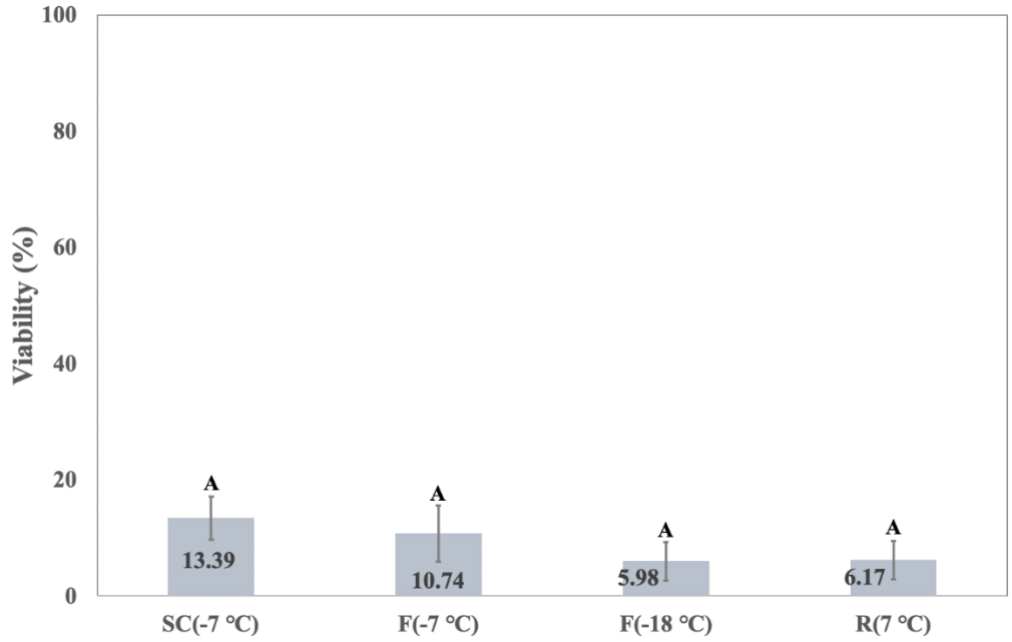


Figure 3.10 Viability of L. acidophilus after 14-day stored at room temperature (25 °C). SC (-7 °C): supercooling at -7 °C; F (-7 °C): freezing at -7 °C; F (-18 °C): freezing at -18 °C; R (7 °C): refrigerated at 7 °C.

3.4. Conclusion

This research successfully evaluated the application of OMF (10 mT at 30 Hz) in maintaining supercooling status for 12 hours. According to the results of SDS-PAGE and TEM, supercooling pretreated *L. acidophilus* has thicker SLP. In the next viability test, supercooling pretreated *L. acidophilus* has higher viability after freeze-drying, which further proved that SLP has positive impact in maintaining the viability of *L. acidophilus*. Thus, in this article, we believe that supercooling pretreatment helps *L. acidophilus* produces more SLP, and thicker SLP protect *L. acidophilus* from the freezing threat. This supercooling pretreatment has potential for viability enhancement on freeze-dried probiotics, which provides a novel solution of cryoprotectants chosen during the freezing process.

REFERENCES

Abadias, M., Benabarre, A., Teixid´o, N., Usall, J., & Vinas, I. (2001). Effect of freeze drying and protectants on viability of the biocontrol yeast candida sake. International journal of food microbiology, 65(3), 173-182.

Adhikari, K., Mustapha, A., Grün, I., & Fernando, L. (2000). Viability of microencapsulated *bifidobacteria* in set yogurt during refrigerated storage. Journal of dairy science, 83(9), 1946-1951.

Amine, K. M., Champagne, C. P., Salmieri, S., Britten, M., St-Gelais, D., Fustier, P., & Lacroix, M. (2014). Effect of palmitoylated alginate microencapsulation on viability of *bifidobacterium longum* during freeze-drying. LWT-food Science and Technology, 56(1), 111-117.

Azad, M., Kalam, A., Sarker, M., & Wan, D. (2018). Immunomodulatory effects of probiotics on cytokine profiles. BioMed research international, 2018.

Barria, C., Malecki, M., & Arraiano, C. (2013). Bacterial adaptation to cold. Microbiology, 159(Pt 12), 2437-2443.

Cani, P. D. & Van Hul, M. (2015). Novel opportunities for next-generation probiotics targeting metabolic syndrome. Current opinion in biotechnology, 32, 21-27.

Capela, P., Hay, T., & Shah, N. P. (2006). Effect of cryoprotectants, prebiotics and microencapsulation on survival of probiotic organisms in yoghurt and freeze-dried yoghurt. Food Research International, 39(2), 203-211.

Chen, M. & Mustapha, A. (2012). Survival of freeze-dried microcapsules of galactosidase

producing probiotics in a soy bar matrix. Food microbiology, 30(1), 68-73.

Choi, I. S., Kim, H. M., Wi, S. G., Chun, H. H., Hwang, I. M., & Park, H. W. (2018). Effect of cation influx on the viability of freeze-dried *Lactobacillus* brevis wikim0069. Applied Sciences, 8(11), 2189.

Choi, I. S., Ko, S. H., Kim, H. M., Chun, H. H., Lee, K. H., Yang, J. E., Jeong, S., & Park, H. W. (2019). Shelf-life extension of freeze-dried *Lactobacillus* brevis wikim0069 using supercooling pretreatment. LWT, 112, 108230.

Cook, M. T., Tzortzis, G., Charalampopoulos, D., & Khutoryanskiy, V. V. (2012). Microencapsulation of probiotics for gastrointestinal delivery. Journal of controlled release, 162(1), 56-67.

Crowe, L. M., Reid, D. S., & Crowe, J. H. (1996). Is trehalose special for preserving dry biomaterials Biophysical journal, 71(4), 2087-2093.

Dalvi-Isfahan, M., Hamdami, N., Xanthakis, E., & Le-Bail, A. (2017). Review on the control of ice nucleation by ultrasound waves, electric and magnetic fields. Journal of Food Engineering, 195, 222-234.

De Maayer, P., Anderson, D., Cary, C., & Cowan, D. A. (2014). Some like it cold: understanding the survival strategies of psychrophiles. EMBO reports, 15(5), 508-517.

Doleyres, Y. & Lacroix, C. (2005). Technologies with free and immobilised cells for probiotic bi dobacteria production and protection. International Dairy Journal, 15(10), 973-988.

Falagas, M., Betsi, G., & Athanasiou, S. (2007). Probiotics for the treatment of women with bacterial vaginosis. Clinical microbiology and infection, 13(7), 657-664.

Foysal, M. J., Fotedar, R., Siddik, M. A., & Tay, A. (2020). *Lactobacillus acidophilus* and l. plantarum improve health status, modulate gut microbiota and innate immune response of marron (cherax cainii). Scientific reports, 10(1), 1-13.

Frece, J., Kos, B., Svetec, I.-K., Zgaga, Z., Mr sa, V., & Su skovi c, J. (2005). Importance of s-layer proteins in probiotic activity of *Lactobacillus acidophilus* m92. Journal of Applied Microbiology, 98(2), 285-292.

Goh, Y. J., Azc arate-Peril, M. A., O'Flaherty, S., Durmaz, E., Valence, F., Jardin, J., Lortal, S., & Klaenhammer, T. R. (2009). Development and application of a uppbased counterselective gene replacement system for the study of the s-layer protein slpx of *Lactobacillus acidophilus* ncfm. Applied and environmental microbiology, 75(10), 3093-3105.

Grosu-Tudor, S.-S., Brown, L., Hebert, E. M., Brezeanu, A., Brinzan, A., Fadda, S., Mozzi, F., & Zam r, M. (2016). S-layer production by *Lactobacillus acidophilus* ibb 801 under environmental stress conditions. Applied microbiology and biotechnology, 100(10), 4573-4583.

Gualerzi, C. O., Giuliodori, A. M., & Pon, C. L. (2003). Transcriptional and posttranscriptional control of cold-shock genes. Journal of molecular biology, 331(3), 527-539.

Haffner, F. B., Diab, R., & Pasc, A. (2016). Encapsulation of probiotics: insights into academic and industrial approaches. AIMS Materials Science, 3(1), 114-136.

Her, J.-Y., Kang, T., Hoptowit, R., & Jun, S. (2019). Oscillating magnetic field (omf) based supercooling preservation of fresh-cut honeydew melon. Transactions of the ASABE, 62(3), 779-785.

Huang, W.-C., Pan, C.-H.,Wei, C.-C., & Huang, H.-Y. (2020). *Lactobacillus* plantarum ps128 improves physiological adaptation and performance in triathletes through gut microbiota modulation. Nutrients, 12(8), 2315.

Hubalek, Z. (2003). Protectants used in the cryopreservation of microorganisms. Cryobiology, 46(3), 205-229.

James, C., Reitz, B., & James, S. J. (2015). The freezing characteristics of garlic bulbs (allium sativum l.) frozen conventionally or with the assistance of an oscillating weak magnetic field. Food and bioprocess technology, 8(3), 702-708.

Jha, P. K., Sadot, M., Vino, S. A., Jury, V., Curet-Ploquin, S., Rouaud, O., Havet, M., & Le-Bail, A. (2017). A review on effect of dc voltage on crystallization process in food systems. Innovative Food Science & Emerging Technologies, 42, 204-219.

Khaleghi, M. & Kermanshahi, R. K. (2012). Effect of environmental stresses on s-layer production in *Lactobacillus acidophilus* atcc 4356. Advances in applied biotechnology, 21,1-17.

Khaleghi, M., Kermanshahi, R. K., Yaghoobi, M. M., Zarkesh-Esfahani, S., & Baghizadeh, A. (2010). Assessment of bile salt effects on s-layer production, slp gene expression and, some physicochemical properties of *Lactobacillus acidophilus* atcc 4356. Journal of microbiology and biotechnology, 20(4), 749-756.

Khem, S., Small, D. M., & May, B. K. (2016). The behaviour of whey protein isolate in protecting *Lactobacillus* plantarum. Food chemistry, 190, 717-723.

Kunal Ahuja & Kritika Mamtani. (2021, May). *Probiotics Market Size By Ingredients*

(Lactobacilli, Bifidobacterium, Streptococcus, Bacillus), By End-Use (Human, Animal), By Application (Food and Beverages {Dairy Products, Non-Dairy Products, Cereals, Baked Food, Fermented Meat Products, Dry Food}, Dietary Supplements {Food, Nutritional, Specialty Nutrients, Infant Formula}, Animal Feed) (Report ID: GMI418). https://www.gminsights.com/industry-analysis/probiotics-market

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage t4. nature, 227(5259), 680-685.

Lee, Y. K. & Salminen, S. (2009). Handbook of probiotics and prebiotics. John Wiley & Sons.

Liu, B., Song, J., Yao, Z., & Bennacer, R. (2017). Effects of magnetic field on the phase change cells and the formation of ice crystals in biomaterials: Carrot case. Journal of Thermal Science and Engineering Applications, 9(3).

Lourens-Hattingh, A. & Viljoen, B. C. (2001). Yogurt as probiotic carrier food. International dairy journal, 11(1-2), 1-17.

Mattila-Sandholm, T., Myllärinen, P., Crittenden, R., Mogensen, G., Fonden, R., & Saarela, M. (2002). Technological challenges for future probiotic foods. International Dairy Journal, 12(2-3), 173-182.

Mazloom, Z., Youse nejad, A., & Dabbaghmanesh, M. H. (2013). Effect of probiotics on lipid profile, glycemic control, insulin action, oxidative stress, and in ammatory markers in patients with type 2 diabetes: a clinical trial. Iranian journal of medical sciences, 38(1), 38.

Meng, X., Stanton, C., Fitzgerald, G., Daly, C., & Ross, R. (2008). Anhydrobiotics: The challenges of drying probiotic cultures. Food Chemistry, 106(4), 1406-1416.

Naito, M., Hirai, S., Mihara, M., Terayama, H., Hatayama, N., Hayashi, S., Matsushita, M., & Itoh, M. (2012). Effect of a magnetic field on drosophila under supercooled conditions. PloS one, 7(12), e51902.

Nireesha, G., Divya, L., Sowmya, C., Venkateshan, N., Lavakumar, V., et al. (2013). Lyophilization/freeze drying-an review. International journal of novel trends in pharmaceutical sciences, 3(4), 87-98.

Otero, L., P erez-Mateos, M., Rodr guez, A. C., & Sanz, P. D. (2017). Electromagnetic freezing: Effects of weak oscillating magnetic fields on crab sticks. Journal of Food Engineering, 200, 87-94.

Ouwehand, A. C. & Salminen, S. J. (1998). The health effects of cultured milk products with viable and non-viable bacteria. International Dairy Journal, 8(9), 749-758.

Palomino, M. M., Waehner, P. M., Martin, J. F., Ojeda, P., Malone, L., Rivas, C. S., Acosta, M. P., Allievi, M. C., & Ruzal, S. M. (2016). Influence of osmotic stress on the profile and gene expression of surface layer proteins in *Lactobacillus acidophilus* atcc 4356. Applied microbiology and biotechnology, 100(19), 8475-8484.

Phadtare, S. et al. (2004). Recent developments in bacterial cold-shock response. Current issues in molecular biology, 6(2), 125-136.

Purnell, G., James, C., & James, S. J. (2017). The effects of applying oscillating magnetic fields during the freezing of apple and potato. Food and Bioprocess Technology, 10(12), 2113-2122.

Santivarangkna, C., Kulozik, U., & Foerst, P. (2006). Effect of carbohydrates on the survival of

Lactobacillus helveticus during vacuum drying. Letters in applied microbiology, 42(3), 271-276.

Schwenger, E. M., Tejani, A. M., & Loewen, P. S. (2015). Probiotics for preventing urinary tract infections in adults and children. Cochrane Database of Systematic Reviews, (12).

Semikhina, L. & Kiselev, V. (1988). Effect of weak magnetic fields on the properties of water and ice. Soviet Physics Journal, 31(5), 351-354.

Semyonov, D., Ramon, O., Kaplun, Z., Levin-Brener, L., Gurevich, N., & Shimoni, E. (2010). Microencapsulation of *Lactobacillus* paracasei by spray freeze drying. Food Research International, 43(1), 193-202.

So, S. S., Wan, M. L., & El-Nezami, H. (2017). Probiotics-mediated suppression of cancer. Current opinion in oncology, 29(1), 62-72.

Teitelbaum, J. E. & Walker, W. A. (2002). Nutritional impact of pre-and probiotics as protective gastrointestinal organisms. Annual review of nutrition, 22(1), 107-138.

Wang, Y.-C., Yu, R.-C., & Chou, C.-C. (2004). Viability of lactic acid bacteria and *bifidobacteria* in fermented soymilk after drying, subsequent rehydration and storage. International journal of food microbiology, 93(2), 209-217.

Wong, S., Jamous, A., O'Driscoll, J., Sekhar, R., Weldon, M., Yau, C. Y., Hirani, S. P., Grimble, G., & Forbes, A. (2014). A *Lactobacillus* casei shirota probiotic drink reduces antibiotic-associated diarrhoea in patients with spinal cord injuries: a randomised controlled trial. British Journal of Nutrition, 111(4), 672-678.

Zhan, X., Zhu, Z., & Sun, D.-W. (2019). Effects of extremely low frequency electromagnetic field on the freezing processes of two liquid systems. Lwt, 103, 212-221.

Chapter 4

CONCLUSIONS AND FUTURE WORK

This research aimed to evaluate OMF-assisted supercooling pretreatment in viability enhancement of freeze-dried *L. acidophilus*. The results showed the protective effect of SLP for freeze-dried *L. acidophilus*, and OMF-assisted supercooling pretreatment promoted the expression of SLP. After freeze-drying, 78.83% of *L. acidophilus* cells were survived with supercooling pretreatment, while the viability of control group cells was lower than 60%. This is caused from the different pretreatments, in the OMF (10 mT, 30 Hz)-assisted supercooling pretreatment group, *L. acidophilus* samples were successfully preserved at -7 °C for 12 hours, whereas control samples were spontaneously nucleated within 2 hours. During the 12 hours supercooling pretreatment, *L. acidophilus* cells produce SLP and gradually acclimate to the lower temperature. Thus, a more intense SLP band in SDS-PAGE and thicker surface layers in TEM were observed, which further proved the effect of supercooling pretreatment. Fig 4.1 shows the process differences between this project and in the industry, compared with the industry process, this project less dependent to cryoprotectants, minimized free damage with higher cell viability, and improved production yield. In general, this project showed the potential of supercooling pretreatment on the viability enhancement of freeze-dried probiotics, providing a novel solution of cryoprotectants chosen during the freezing process.

To further apply supercooling pretreatment to enhance the viability of freeze-dried probiotics, the system can be perfected from the packaging and supercooling system optimization. Viability maintaining during storage is one of the key factors to evaluate the freeze-dried probiotics. To date, various encapsulation technologies have been explored to retain the viability during storage. Hongpattarakere et al. (2013) utilized a vacuum-packed aluminum foil laminated

polyethylene sachet, that maintained the high viability of freeze-dried *L. plantarum* after 8-week storage period. Heidebach et al. (2010) investigated casein-based microcapsules on the viability enhancing of probiotics; they found that after 3 months of storage, the viability of *Lactobacillus* F19 can still meet the minimum demanded for therapeutic effect of probiotics. Wang et al. (2016) suggested that inulin-sodium alginate skim milk encapsulation can effectively maintain the viability of freeze-dried *L. plantarum* for 7 weeks. Therefore, the viability of probiotics can be maintained at an effective amount with appropriate packaging, and packaging in this project needs to be further improved.

Table 4.1 Mainly process differences between this project and in the industry.

Supercooling pretreatment	Industry process
• 12-hour pretreatment	• No pretreatment
• No cryoprotectant	• Cryoprotectant/antioxidation mix
• Open-space packaging	• Pouch, airtight, foil package
• 0-day, 80% viability, decrease to 20% within 14-day	• 0-day, 70% viability, maintain several months

In this study, OMF-assisted supercooling successfully maintained supercooling state, improved SLP production, and enhanced the viability of *L. acidophilus*. However, some mechanisms about supercooling and OMF should be explored more. Supercooling shows a promising future in inhibiting ice crystallization and extending the shelf life of foods (Stonehouse & Evans 2015). However, the supercooling state can be quickly shifted to the next state by various factors, and ice crystallization introduced randomly makes it hard to achieve technical stability and reproducible results (James et al., 2011; Stonehouse & Evans 2015; Fukuma et al. 2012). MF

also effectively affects the formation of ice crystallization in some research (James et al. 2015); nevertheless, the underlying mechanisms of ice nucleation are still unclear; it is at high risk to industrialize MF, either for ice nucleation control or supercooling state extension. Moreover, the spontaneous and stochastic nature builds barriers for researchers to investigate the process of ice nucleation. In summary, a wider range of MF should be investigated, and a more specific theoretical model should be optimized in the future (Dalvi-Isfahan et al. 2017; Otero et al. 2017; Hartmann et al. 2011).

In summary, supercooling technology boosts the natural defenses of probiotics, providing a novel solution of cryoprotectants chosen during the freezing process. The high viability after freeze-drying shows there is a massive potential of supercooling technology in enhancing the viability of freeze-dried probiotics.

REFERENCES

Fukuma, Y., Yamane, A., Itoh, T., Tsukamasa, Y., & Ando, M. (2012). Application of supercooling to long-term storage of sh meat. Fisheries science, 78(2), 451-461.

Hartmann, S., Niedermeier, D., Voigtländer, J., Clauss, T., Shaw, R., Wex, H., Kiselev, A., & Stratmann, F. (2011). Homogeneous and heterogeneous ice nucleation at lacis: operating principle and theoretical studies. Atmospheric Chemistry and Physics, 11(4), 1753-1767.

Heidebach, T., Först,, P., & Kulozik, U. (2010). In uence of casein-based microencapsulation on freeze-drying and storage of probiotic cells. Journal of food engineering, 98(3), 309-316.

Hongpattarakere, T., Rattanaubon, P., & Buntin, N. (2013). Improvement of freezedried *Lactobacillus plantarum* survival using water extracts and crudefibers from food crops. Food and bioprocess technology, 6(8), 1885-1896.

James, C., Hanser, P., & James, S. J. (2011). Super-cooling phenomena in fruits, vegetables and seafoods. In 11th International Congress on Engineering and Food (ICEF 2011), Athens, Greece (pp. 22-26).

James, C., Reitz, B., & James, S. J. (2015). The freezing characteristics of garlic bulbs (allium sativum l.) frozen conventionally or with the assistance of an oscillating weak magnetic field. Food and bioprocess technology, 8(3), 702-708.

Otero, L., P erez-Mateos, M., Rodr guez, A. C., & Sanz, P. D. (2017). Electromagnetic freezing: Effects of weak oscillating magnetic fields on crab sticks. Journal of Food Engineering, 200, 87-94.

Stonehouse, G. & Evans, J. (2015). The use of supercooling for fresh foods: A review. Journal of

Food Engineering, 148, 74-79.

Wang, L., Yu, X., Xu, H., Aguilar, Z. P., & Wei, H. (2016). Effect of skim milk coated inulin-alginate encapsulation beads on viability and gene expression of *Lactobacillus plantarum* during freeze-drying. LWT-food Science and Technology, 68, 8-13.

Appendix A

Table A.1 Cell wall thickness analysis after different pretreatment. (F = 109.73, df = 154, P < 0.05). Cells without any treatment, No pretreatment, supercooling at -7 °C, SC (-7 °C), freezing at -18 °C, F (-18 °C), refrigerator at 7 °C, R (7 °C), freezing at -7 °C, F (-7 °C).

Treatment condition	Surface layer thickness (nm)	Increase rate (%)
No pretreatment	6.23 ± 1.24 c	-
SC (-7 °C)	15.01 ± 3.23 a	140.93
F (-7 °C)	8.94 ± 1.54 b	43.50
F (-18 °C)	7.19 ± 1.22 c	15.41
R (7 °C)	9.06 ± 0.96 b	45.43

Table A.2 Cell wall thickness analysis of different pretreatment *L. acidophilus* after 7-day stored at -18 °C. (F = 29.65, df = 171, P < 0.05). Cells without any treatment, No pretreatment, supercooling at -7 °C, SC (-7 °C), freezing at -18 °C, F (-18 °C), refrigerator at 7 °C, R (7 °C), freezing at -7 °C, F (-7 °C).

Treatment condition	Surface layer thickness (nm)	Increase rate (%)
No pretreatment	6.23 ± 1.24 c	-
SC (-7 °C)	8.17 ± 1.55 b	31.14
F (-7 °C)	7.15 ± 1.83 bc	14.77
F (-18 °C)	9.33 ± 1.28 a	49.76
R (7 °C)	9.52 ± 1.73 a	52.81

Table A.3 Cell wall thickness analysis of different pretreatment *L. acidophilus* after 14-day stored at -18 °C. (F = 18.36, df = 241, P < 0.05). Cells without any treatment, No pretreatment, supercooling at -7 °C, SC (-7 °C), freezing at -18 °C, F (-18 °C), refrigerator at 7 °C, R (7 °C), freezing at -7 °C, F (-7 °C).

Treatment condition	Surface layer thickness (nm)	Increase rate (%)
No pretreatment	6.23 ± 1.24 b	-
SC (-7 °C)	8.71 ± 1.88 a	39.81
F (-7 °C)	7.08 ± 1.81 b	13.64
F (-18 °C)	7.06 ± 1.20 b	13.32
R (7 °C)	8.26 ± 1.42 a	32.58

Table A.4 Cell wall thickness analysis of different pretreatment *L. acidophilus* after 7-day stored at room temperature (25 °C). (F = 30.87, df = 124, P < 0.05). Cells without any treatment, No pretreatment, supercooling at -7 °C, SC (-7 °C), freezing at -18 °C, F (-18 °C), refrigerator at 7 °C, R (7 °C), freezing at -7 °C, F (-7 °C).

Treatment condition	Surface layer thickness (nm)	Increase rate (%)
No pretreatment	6.23 ± 1.24 b	-
SC (-7 °C)	7.30 ± 1.61 b	17.17
F (-7 °C)	6.07 ± 0.85 b	-2.57
F (-18 °C)	9.41 ± 1.70 a	51.04
R (7 °C)	6.70 ± 1.25 b	7.54

Table A.5 Cell wall thickness analysis of different pretreatment *L. acidophilus* after 14-day stored at room temperature (25 °C). ($F = 3.24$, $df = 244$, $P < 0.05$). Cells without any treatment, No pretreatment, supercooling at -7 °C, SC (-7 °C), freezing at -18 °C, F (-18 °C), refrigerator at 7 °C, R (7 °C), freezing at -7 °C, F (-7 °C).

Treatment condition	Surface layer thickness (nm)	Increase rate (%)
No pretreatment	6.23 ± 1.24 b	-
SC (-7 ℃)	6.90 ± 1.49 ab	10.75
F (-7 ℃)	6.64 ± 1.38 ab	6.58
F (-18 ℃)	7.37 ± 1.57 a	18.30
R (7 ℃)	6.97 ± 1.65 ab	11.88

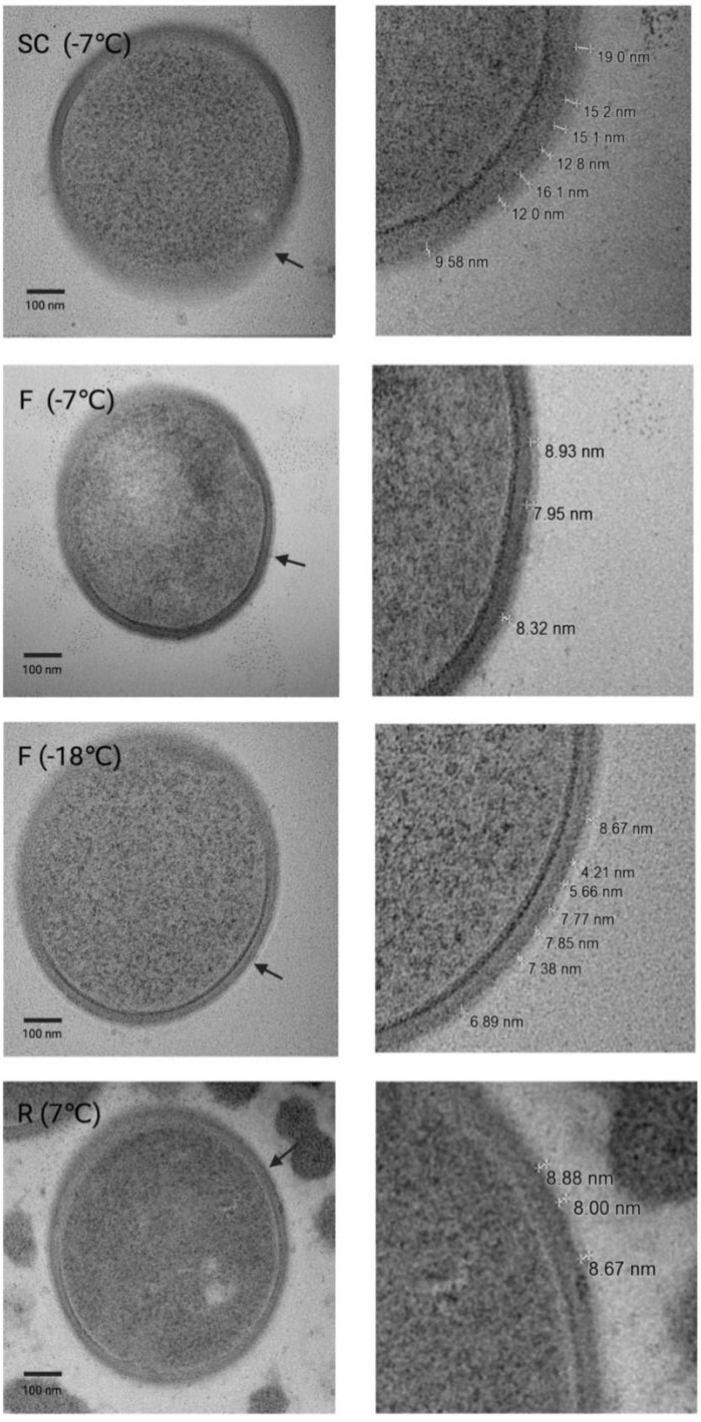


Figure A.1 TEM images of *L. acidophilus* cells after pretreatment. Cells with -7 °C supercooling pretreatment, SC (-7 °C); -7 °C frozen pretreatment, F (-7 °C); -18 °C frozen pretreatment, F (-18 °C); 7 °C refrigerated pretreatment, R (7 °C). The layer at the surface of the cell wall represents SLP (black arrow), scale bars indicate 100 nm, images on the right show the close view of the arrow.

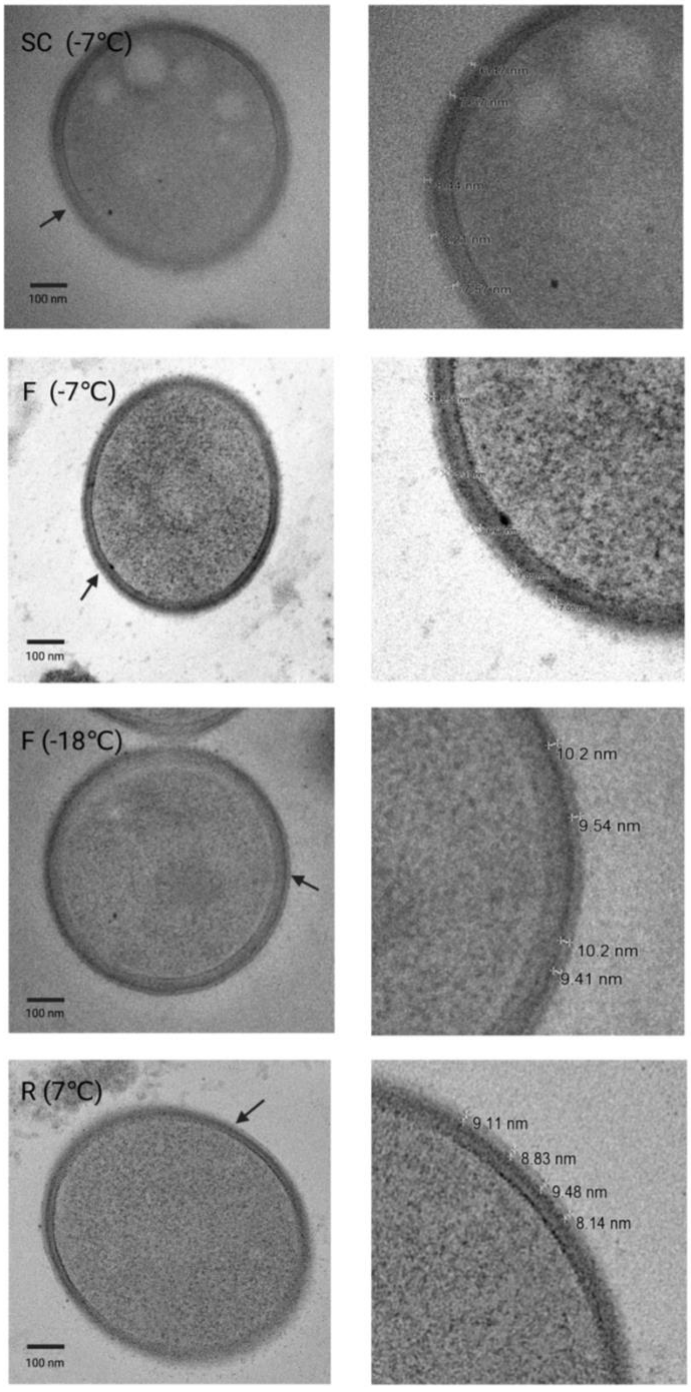


Figure A.2 TEM images of *L. acidophilus* cells after 7-day store at -18 °C. Cells with -7 °C supercooling pretreatment, SC (-7 °C); -7 °C frozen pretreatment, F (-7 °C); -18 °C frozen pretreatment, F (-18 °C); 7 °C refrigerated pretreatment, R (7 °C). The layer at the surface of the cell wall represents SLP (black arrow), scale bars indicate 100 nm, images on the right show the close view of the arrow.

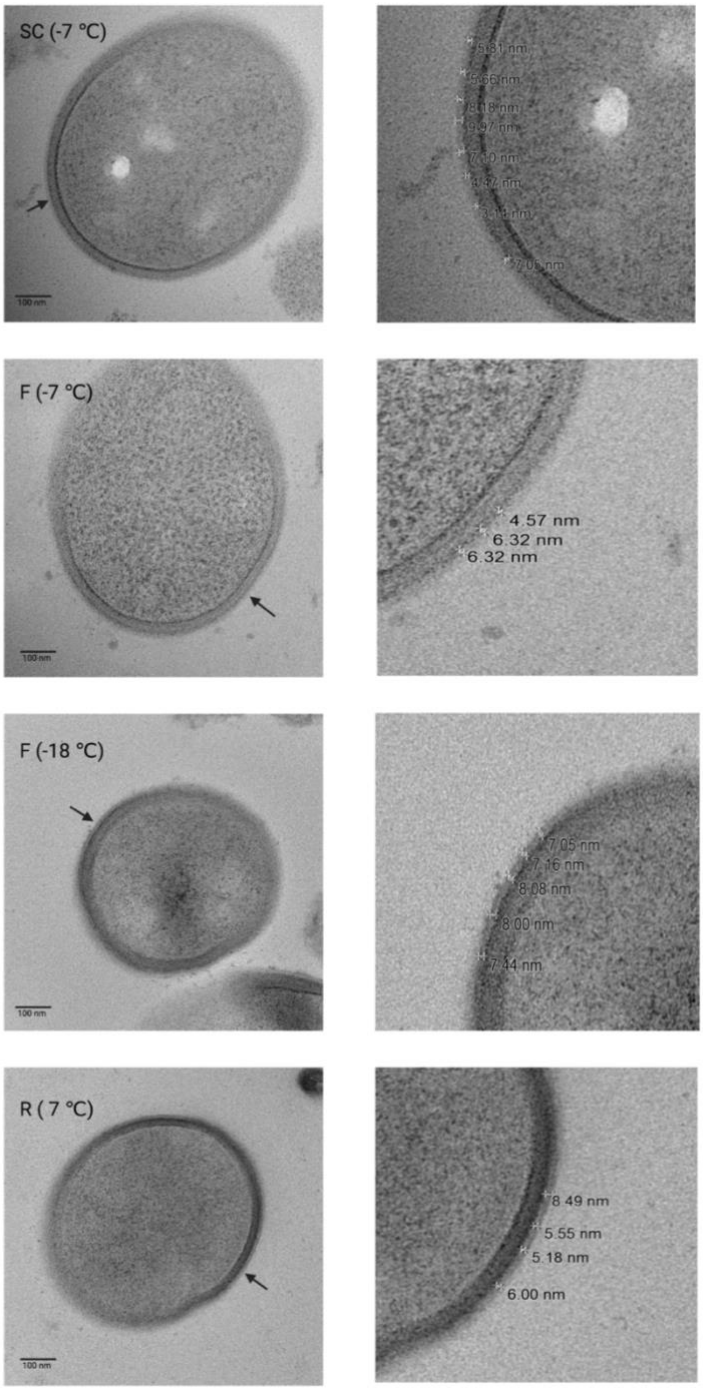


Figure A.3 TEM images of *L. acidophilus* cells after 7-day store at room temperature (25 °C). Cells with -7 °C supercooling pretreatment, SC (-7 °C); -7 °C frozen pretreatment, F (-7 °C); -18 °C frozen pretreatment, F (-18 °C); 7 °C refrigerated pretreatment, R (7 °C). The layer at the surface of the cell wall represents SLP (black arrow), scale bars indicate 100 nm, images on the right show the close view of the arrow.

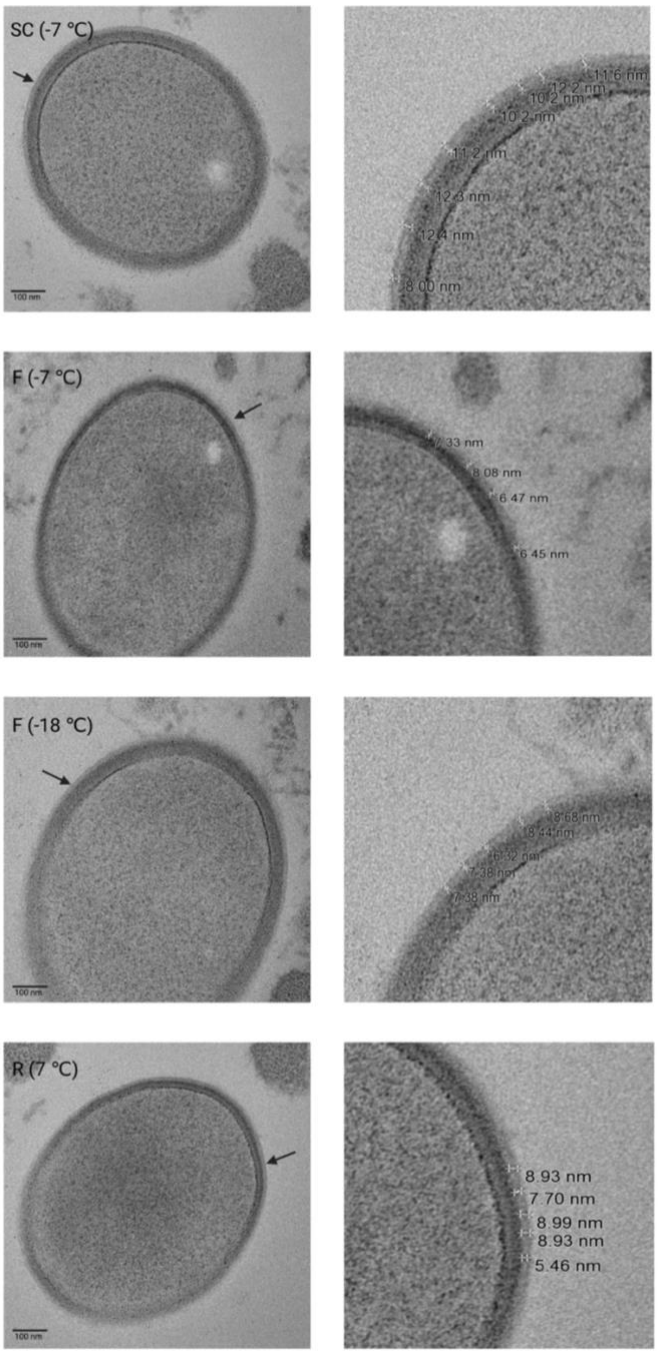


Figure A.4 TEM images of *L. acidophilus* cells after 14-day store at -18 °C. Cells with -7 °C supercooling pretreatment, SC (-7 °C); -7 °C frozen pretreatment, F (-7 °C); -18 °C frozen pretreatment, F (-18 °C); 7 °C refrigerated pretreatment, R (7 °C). The layer at the surface of the cell wall represents SLP (black arrow), scale bars indicate 100 nm, images on the right show the close view of the arrow.

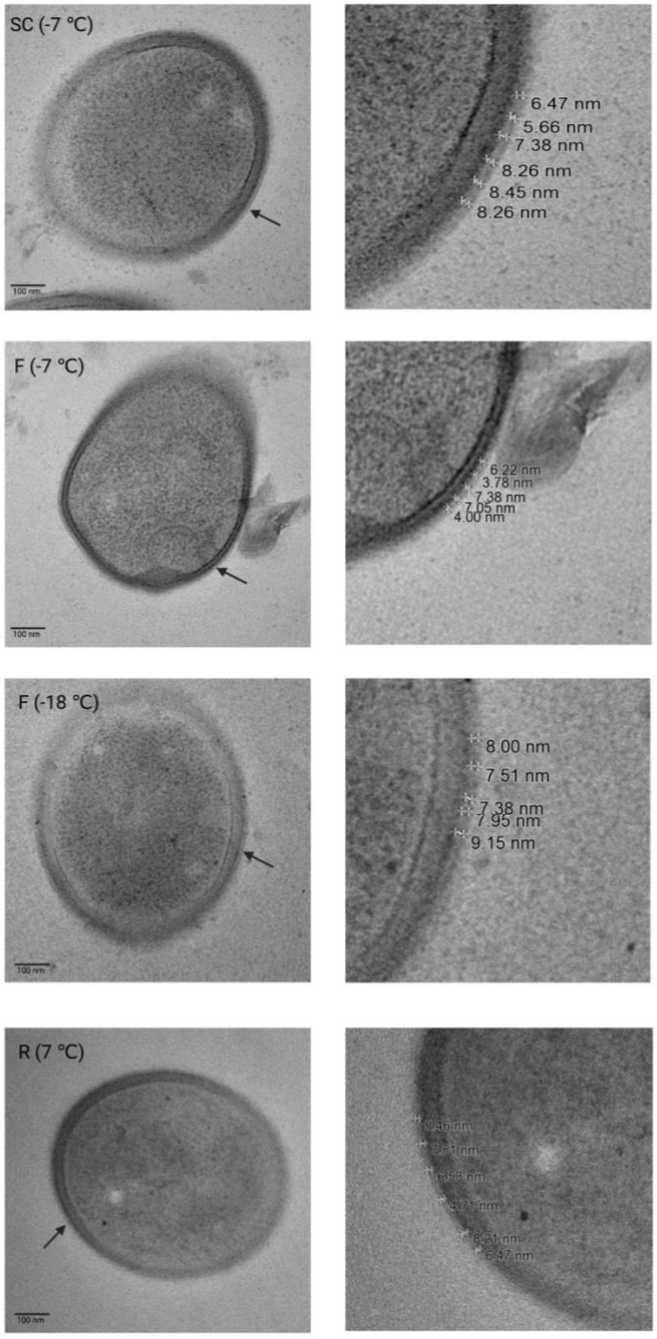


Figure A.5 TEM images of *L. acidophilus* cells after 14-day store at room temperature (25 °C). Cells with -7 °C supercooling pretreatment, SC (-7 °C); -7 °C frozen pretreatment, F (-7 °C); -18 °C frozen pretreatment, F (-18 °C); 7 °C refrigerated pretreatment, R (7 °C). The layer at the surface of the cell wall represents SLP (black arrow), scale bars indicate 100 nm, images on the right show the close view of the arrow.