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Enteric Biopolymer Composite Coatings to Enhance the Viability of Powdered Probiotics During Preparation, Storage, and Simulated Digestions

Anyi Wang
awang9@vols.utk.edu

To the Graduate Council:

I am submitting herewith a dissertation written by Anyi Wang entitled "Enteric Biopolymer Composite Coatings to Enhance the Viability of Powdered Probiotics During Preparation, Storage, and Simulated Digestions." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science.

Qixin Zhong, Major Professor

We have read this dissertation and recommend its acceptance:

Jun Lin, Elizabeth Fozo, Tao Wu

Accepted for the Council:

Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

Enteric Biopolymer Composite Coatings to Enhance the Viability of Powdered Probiotics During Preparation, Storage, and Simulated Digestions

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ABSTRACT

Due to suppressed metabolisms, powdered probiotics are generally more stable and more convenient for applications than the liquid form, but much work is needed to improve viability of powdered probiotics during processing, storage, and digestions. The goal of this dissertation was to fabricate delivery systems with an enteric biopolymer coating and a core of powdered probiotic ingredients. The principle of preparing powdered probiotics was to directly mix a concentrated cell suspension with hygroscopic food ingredient powders. Amorphous spray-dried lactose (SDL) was first studied to prepared powdered *Lactobacillus salivarius* NRRL B-30514 in chapter 2. A smaller amount of cell suspension resulted in reduced water activity and lower hypertonic stress and therefore greater viable bacterial counts initially and during subsequent 6-month storage. The suspension: lactose ratio remarkably affected the lactose crystallinity and physiological states of *L. salivarius*. In chapter 3, milk protein concentrate (MPC) was mixed with SDL at different mass ratios before mixing with the cell suspension. MPC was suggested to preferentially absorb water in cell suspensions, which inhibited the hydration of SDL and thus lowered the hypertonic pressure to the adhered cells. To further improve probiotics viability, amorphous sucrose prepared by co-spray drying with whey protein isolate (WPI) was studied in chapter 4 to utilize the synergistic protection effects of WPI and sucrose. The WPI-Sucrose-probiotics powders (WSPP) with a higher amount of amorphous sucrose showed higher probiotics viability before and after 30-day storage and heating. In order to deliver powdered probiotics, modified rice protein (MRP)-ammonium shellac (NH_4SL) enteric composite coatings were studied in Chapter 5 and interactions between MRP and NH_4SL were studied. MRP and NH_4SL formed complexes to enable suspension of MRP to form smooth films with improved

mechanical and enteric properties. A higher content of MRP preserved films better at gastric conditions, and the resultant coating significantly improved the viability of enclosed WSPP pellets after 30-day ambient storage, heating at 80 ºC for 20 min, and during simulated gastrointestinal digestions. The novel, simple and cost-effective approaches studied in the present dissertation to prepare powdered probiotic ingredients are significant to manufacturing solid probiotics-containing products.

Key words: powdered probiotics; viability; storage; enteric coating; delivery system

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1 Introduction and literature review

1.1 Abstract

The consumption and popularity of functional food products containing viable probiotics have been rising rapidly. The importance of producing probiotic ingredients with high viability and stability during processing, storage, and gastrointestinal digestions boosted the research and development of powdered probiotic ingredients. In this chapter, evaluation, characteristics, health benefits, and stress susceptibility of probiotics are reviewed. Various drying technologies and media used to prepare powdered probiotics are discussed. Methods used to characterize structural, functional, and microbiological properties of powdered probiotics are then reviewed. Finally, strategies to incorporate powdered probiotics in different food products are reviewed for improving the survival of probiotics during manufacturing, storage, and digestions of food products.

1.2 Introduction

The use of probiotics, such as *Lactobacillus* and *Bifidobacterium*, as food supplements has become popular. Over the last decade, there is rising consumption on functional food products containing probiotic bacteria. The U.S. probiotics market size was estimated to be above USD 35 billion in 2016, with an expectation of 7.4% annual growth rate to 2024 (Ahuja & Mamtani, 2018). Over 500 new products supplemented with probiotics, including dairy products such as yogurts and cheeses and beverages such as fruit juices and coffees, have been launched in the past decades (Markets And Markets, 2017). However, probiotics, especially in liquid preparation, are highly susceptible to environmental conditions, such as pH temperature, oxygen,

and water activity (a_w). Therefore, undesired losses of probiotic viability during processing, storage, and gastrointestinal (GI) digestions is an important issue that must be addressed.

Converting liquid cell suspensions into powdered probiotics ingredients using various drying technologies is commonly applied in the microbiological industry to suppress metabolic processes, thus preserving viability during processing, storage, transportation, and consumption (Ramos et al., 2018; Riaz & Masud, 2013). The viability of powdered probiotics can be influenced by various factors from processing to digestions, such as strain selection, drying medium formulation, powder structure, drying method and conditions, storage conditions, *etc*. Therefore, the susceptibility of probiotics to environmental stresses, formulation of drying matrix, available drying methods, powder characterization, and application of powdered probiotics in different food products is reviewed in this chapter.

1.3 An overview of probiotics

One of the most widely accepted definitions of probiotics is presented by an expert committee organized by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), which is that "Probiotic organisms are live microorganisms that when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2001). To be considered as probiotics, microorganisms should fulfil the criteria of 1) having a demonstrated beneficial effect on the host, 2) being non-pathogenic and non-toxic without significant adverse side effects, 3) surviving through the GI tract, and 4) be compatible with product matrix, processing and storage conditions to maintain an adequate number of viable cells in the products (Harish & Varghese, 2006). This section provides an overview of the evaluation processes, characteristics, and health benefits of probiotics.

1.3.1 Evaluation of probiotics for food use

Every bacterial strain must produce some health benefits to be considered as a potential probiotic. The FAO/WHO guidelines suggest that a potential probiotic strain must be accurately identified and characterized for its functional properties using various *in vitro* and *in vivo* tests, followed by safety evaluation (FAO/WHO, 2002). Only well-defined strains can be incorporated in food or pharmaceutical formulations for human use. This section reviews the sequential steps required to evaluate a bacterial strain as an applicable probiotic for food use, including sources and isolation methodology, identification, characterization, and safety assessment (Figure 1-1).

1.3.1.1 Sources and isolation of probiotics

Fermented foods (yogurt, kefir, kimchi, miso, *etc.*) (Fontana et al., 2013), breast milk (Rajoka et al., 2017), human GI tract (Tan et al., 2018), and fecal samples (Seddik et al., 2017) are good sources of probiotics. To isolate potential probiotic strains, the samples are usually homogenized, diluted, and cultured in selective or elective media prepared by supplementing basal media with various selective agents (Roy, 2001). For example, addition of propionic acid in a Columbia agar lowered pH of the medium to inhibit the growth of *Enterococcus, Staphylococcus*, and *Micrococcus* species in human feces, which was used for the selective isolation of *Bifidobacterium* spp. from human fecal samples (Beerens, 1991). A de Man, Rogosa, and Sharpe (MRS) medium supplemented with vancomycin as a selective antibiotic was successfully used for selective isolation of *Lactobacillus plantarum* from a yoghurt culture (Veselá et al., 2019). After anerobic incubation at proper conditions, the colonies are isolated and transferred to broth or a new agar plate.

1.3.1.2 Identification of probiotics

Accurate identification of bacterial isolates using both phenotypic and genotypic methods is important for selection of potential probiotics (FAO/WHO, 2002). Many phenotypic methods previously used to identify bacterial strains, such as colony and cell morphologies, Gram staining, growth requirements, fermentation types and products, enzymes production, and metabolic activities, are now only used for preliminary screening of isolates (Fontana et al., 2013; Shokryazdan et al., 2017). Alternatively, genotypic methods based on molecular microbiology have been applied to identify the taxonomy (genus, species, and strains) of microbial isolates. 16S rRNA gene sequencing method is one of the most frequently used tools for microbial identification due to its accuracy and capability to specify the belonging of a strain to a species and identify taxonomical relationships among microbial strains (Petti et al., 2005). Several DNA fingerprinting techniques, such as pulsed field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD), and denaturing gradient gel electrophoresis (DGGE), can be combined with gene sequencing for strain typing and identification (FAO/WHO, 2002; Hippe et al., 2011).

1.3.1.3 Characterization of probiotics

The accurate classification and identification of potential probiotic strains are useful to understand the origin, habitat, physiological features, safety and technical applicability of probiotics (Holzapfel et al., 2001). Concurrently, a well-identified strain must be subjected to various *in vitro* assays to characterize its functional properties. According to FAO/WHO (2002), *in vitro* tests commonly used for screening and characterizing probiotic strains include: 1) gastric

acidity and bile acid resistance, 2) bile salt hydrolase activity, 3) adhesion to human intestinal epithelial cells and/or mucus, 4) antimicrobial activity against potential pathogens, and 5) ability to reduce pathogen adhesion to surface. The results of these tests are important to predict the survival, colonization, and hypocholesterolemic, and anti-pathogenic activities of probiotic strains in humans after oral administration (Shokryazdan et al., 2017). Besides these main criteria, additional *in vitro* tests are needed for probiotic strains claimed for specific properties such as antioxidant activity, anticancer effect, and immunomodulation (Aarti et al., 2017; Gut et al., 2019; Shehata et al., 2019). To develop probiotics for human use, *in vitro* tests are not sufficient for describing their efficacy in humans, which require substantiation from *in vivo* animal and finally human trials (FAO/WHO, 2002). The efficacy of probiotic foods compared with placebo being composed of food carrier without the test probiotic strains is measured generally in the form of randomized, double-blind, placebo-controlled human trials or other appropriate designs (FAO/WHO, 2002). The principal outcome of efficacy studies on test probiotics in clinical trials should include transient colonization in intestines, no adverse effects on patients, and one or more health benefits demonstrated by significantly improved health conditions, reduced risk of illness, or faster recovery from diseases (e Silva & Gomes, 2014; FAO/WHO, 2002).

1.3.1.4 Safety evaluation of probiotics

Probiotic strains such as *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* species associated with foods have been considered to be safe for a long history (Dunne et al., 2001). The FAO/WHO guidelines recommend that every potential probiotic strain should be assessed with safety evaluations before considered as generally recognized as safe (GRAS) and applied in

probiotic products (FAO/WHO, 2002). Currently, there is no universal international standard for safety evaluation of probiotics. In the FAO/WHO guidelines, the antibiotic resistance patterns, side-effects during human trials, and toxin production of probiotic strains need to be assessed (FAO/WHO, 2002). The European Food Safety Authority also proposed the "Qualified Presumption of Safety (QPS)" as an approach to study the safety status of bacteria, including taxonomy identification, familiarity study based on scientific literature and *in vitro* and *in vivo* tests, pathogenicity exclusion, and end use definition (EFSA, 2007).

1.3.2. Characteristics of microorganisms used as probiotics

Microorganisms identified as probiotics are commonly classified as lactic acid bacteria (LAB) and non-LAB (Table 1-1). LAB, including genera of *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Enterococcus*, and *Lactococcus*, are bacteria that produce lactic acid as their major fermentation product and most commonly used as probiotics (Venema & Meijerink, 2015). Non-LAB probiotics include the yeast genus *Saccharomyces* and other bacterial genera, such as *Bacillus* and *Escherichia* (Venema & Meijerink, 2015). The section reviews the biological characteristics of these microorganisms used as probiotics.

1.3.2.1 Lactic acid bacteria (LAB)

Lactobacillus is a genus of Gram-positive, non-spore-forming, catalase-negative, facultative anaerobic or microaerophilic, and rod-shaped bacterial species which are able to produce lactic acid as main metabolite of the fermentation (Venema & Meijerink, 2015). Lactobacilli are widespread in fermented foods (e.g. dairy, meat, vegetables, beverages, *etc.*) and digestive systems of humans or animals. More than 106 *Lactobacillus* species have been identified, out of

which 56 species have been reported to have probiotic potential, including *L. acidophilus*, *L. casei*, *L. brevis*, *L. rhamnosus*, *L. plantarum*, and *L. salivarius*, etc (Otieno, 2011). Many species of lactobacilli are GRAS and therefore are most common probiotic bacteria used for food applications and feed production.

The genus *Bifidobacterium* groups are Gram-positive, non-spore-forming, catalase-negative, generally anaerobic, and polymorphic branched rods that produce acetic and lactic acids as their major metabolites (Otieno, 2011). Despite the distinctions between bifidobacteria and LAB that have been reviewed by Sonomoto and Yokota (2011), *Bifidobacterium* is still commonly classified as LAB due to their common metabolism and structural characteristics (Gomand et al., 2019). Bifidobacteria belonging to the phylum *Actinobacteria* are the most predominant microorganisms in the GI tract of humans and therefore most of them are isolated from digestive systems and feces of humans and animals. At present, more than 30 species of bifidobacteria have been identified and eight of them, including *B. breve*, *B. lactis*, *B. longum*, *B. bifidum*, *B. infantis*, *etc.*, have been reported to have probiotic capabilities (Otieno, 2011). Bifidobacteria are another group of awidely used probiotic bacteria and are often mixed with lactobacilli in commercial probiotic products to synergistically confer beneficial effects (Leser et al., 2015).

Other important LAB include genera of *Lactococcus*, *Streptococcus*, and *Enterococcus*. They are typically Gram-positive, catalase-negative, and facultative anaerobes. *Lactococcus lactis* is applied as probiotics in manufacturing dairy products such as cheese and fermented milk (Kimoto-Nira et al., 2007). *Streptococcus thermophilus* is also used as a probiotic strain in the production of yogurt (Otieno, 2011). *Enterococcus* is usually present in Mediterranean ripened cheese and *E. faecium* has been found to have probiotic potential (Nero et al., 2015).

1.3.2.2 Non-lactic acid bacteria

Although LAB are the most widely used probiotics, the probiotic potentials of other bacteria and yeasts have also been confirmed. For example, *Bacillus coagulans* and *Bacillus subtilis* that are endospore-forming, Gram-positive, and facultative anaerobic bacteria have been studied to have probiotic capabilities and applied in pharmaceutical and food developments. These bacteria are metabolically inactive when forming spores that are extremely resistant to harsh treatments such as heating, drying, and freezing (Baccigalupi et al., 2015). Certain *Escherichia coli* strains like *E. coli* Nissle 1917 have also demonstrated clinical and preclinical beneficial effects on the host (Olier, 2015). In addition, certain eukaryotic microorganisms are also used as probiotics due to the ability to withstand the harsh conditions of gut and execute beneficial effects in the host. Among the eukaryotic probiotics, yeasts are the predominant group in which *Saccharomyces cerevisiae* is the only yeast strain commercialized for human uses (Nayak, 2011).

1.3.3 Health-promoting effects of probiotics

In order to confer beneficial effects, administered probiotics must be able to survive through the GI tract, be viable at sufficiently high levels in the intestine, successfully adhere to mucus and/or epithelial cells, and adequately grow or persist by retarding their elimination from the digestive track via intestinal transit (Bertazzoni et al., 2013; Fung et al., 2011). Furthermore, the health-promoting effects of probiotics highly depend on the strain, dose, probiotic formulation, and physiological conditions of the host (individual gastric pH, intestinal motility, composition of intestinal microbiota, administration of antibiotics, *etc.*) (Bertazzoni et al., 2013; Shokryazdan et al., 2017). Generally, probiotics have been reported to alleviate diarrhea and lactose

intolerance symptoms, prevent inflammatory bowel disease (IBD), exhibit immunomodulatory and anticolorectal cancer effects, *etc.*, via various proposed mechanisms (Fung et al., 2011), which are summarized in Table 1-1 and also reviewed in this section.

1.3.3.1 Alleviation of diarrhea

Diarrhea is defined as the increased liquidity of stools typically associated with an increased frequency of bowel movements and an increased fecal weight (De Vrese & Offick, 2010) and is the second leading cause of morbidity among children under the age of 5 (CDC, 2015). There are several types of diarrhea according to their etiology and acute infectious and antibiotic-associated diarrhea (AAD) are the two most common diarrhea diseases (Yan & Polk, 2006). The prevention and treatment of the two most common diarrhea diseases using probiotics are reviewed in this section.

The acute infectious diarrhea is primarily caused by viral and bacterial pathogens, such as rotavirus, *Shigella*, *Salmonella*, *Escherichia coli*, and *Clostridium difficile*, with the rotaviruses infection being the most common cause of acute diarrhea in infants and children (Blush III & Matzo, 2012). Probiotics have been utilized for effective prevention or treatment of acute infectious diarrhea by stimulating the GI immune response, competitively inhibiting the adherence of pathogens to the intestinal epithelium, and producing substances, such as organic acids and antimicrobials, that are inhibitory to pathogens (Fung et al., 2011; Young, 2010). A placebo-controlled and randomized trial of 81 children with infectious diarrhea showed that administration of lyophilized *L. casei* variety *rhamnosus* at a dose of 4×10^8 CFU/day for 7 days enhanced the immunoglobulin A (IgA) response to rotavirus and reduced clinical severity and intestinal inflammatory reaction (Lai et al., 2019). Reducing incidence and frequency of diarrhea,

attenuating clinical severity, and shortening acute infections are the most prominent probiotic effects against acute diarrhea (Homayoni Rad et al., 2016).

Antibiotic use can cause disruption of intestinal microflora and excessive proliferation of *Clostridium difficile* that produces toxins A and B and accelerates colonization of intestinal pathogens, often resulting in AAD and symptoms related to toxin excretion (De Vrese & Offick, 2010; Marteau et al., 2001). AAD varies in incidence but can occur in 25-30% of hospitalized patients exposed to antibiotic administration with 25% of cases caused by *C. difficile* disease (De Vrese & Offick, 2010). Probiotics, including various strains of *Lactobacillus spp*., *Bifidobacterium spp*., *Streptococcus spp*., *Enterococcus faecium*, and yeasts (*Saccharomyces boulardii*) (Fung et al., 2011), may be a suitable option for treating AAD by increasing immune responses, producing proteases to degrade *C. difficile* toxins, and reestablishing the destructed intestinal microbiota (McFarland, 2006; 2009). However, the effectiveness of probiotics is found to be strain-, dose-, and disease-specific. A number of meta-analyses show the efficacy of *L. rhamnosus* GG in prevention and treatment of AAD but not in the treatment of *C. difficile*-associated diarrhea, whereas *S. boulardii* is moderately effective in the prevention of AAD but more efficacious in prevention and treatment of *C. difficile* infections (Hawrelak et al., 2005; Mantegazza et al., 2018; McFarland, 2006; Szajewska et al., 2016). The overall evidence suggests the therapeutic role of probiotics in alleviating AAD, and more clinical trials are needed to determine the suitable strains and dosages for adult, geriatric, and pediatric use.

1.3.3.2 Alleviation of lactose intolerance

Lactose maldigestion results from a lower than normal concentration of lactase in the brush border membrane of the mucosa of the small intestine that leads to incomplete digestion of

lactose (Fung et al., 2011). Fermentation of undigested lactose by the gut bacteria leads to accumulation of microbial metabolites and gases which cause discomfort, bloating, rumbling, and diarrhea (He, Venema, et al., 2008; Honda et al., 2007). Some LAB with the ability to produce β-galactosidase (also call lactase), such as *Lactobacillus rhamnosus* (Agustina et al., 2007), *Bifidobacterium longum* (He, Priebe, et al., 2008), and *Streptococcus thermophilus* (Agustina et al., 2007), are generally supplemented into dairy products to hydrolyze lactose present. Therefore, consumption of fermented dairy products has been shown to efficiently alleviate symptoms of lactose intolerance due to the decreased lactose concentration and increased microbial β-galactosidase content in fermented products, positive effects on colonic microbiota, and reduced sensitivity to symptoms (Oak & Jha, 2019). Furthermore, it is crucial for probiotics to be alive or at least have an intact cell wall to protect β-galactosidase from the acidity of the stomach (Homayoni Rad et al., 2016).

1.3.3.3 Prevention of inflammatory bowel disease (IBD)

IBD is a chronic and recurrent inflammation in the GI tract and refers primarily to Crohn's disease (CD) and ulcerative colitis (UC) (Pintado et al., 2014). CD can occur in any parts along the GI tract but is mainly in the terminal ileum with symptoms like abdominal pain, bloody diarrhea, and malnutrition, while UC is confined to the colon and characterized by mucosal inflammation, erosion, and ulceration (Fung et al., 2011). The exact etiology of IBD remains unclear, but a dysfunctional interaction between the intestinal microbiota and the mucosal immune system is proposed to initiate the disorder (Sullivan & Nord, 2002). The mechanism of action underlying the beneficial effects of probiotics against IBD is not completely understood. But some common mechanisms include stabilizing the human commensal microbiota, inhibiting

pathogen growth and colonization, enhancing intestinal epithelial barrier function, and improving the mucosal immune system (LeBlanc & LeBlanc, 2015; Sartor & Muehlbauer, 2007). For example, a double-blinded, placebo-controlled, and randomized trial of 56 patients with mild to moderate UC showed that administration of freeze-dried *Bifidobacterium longum* 536 at a dose of 2-3×10^{11} CFU/day for 8 weeks resulted in a significant ($P < 0.05$) improvement in clinical symptoms of rectal bleeding, mucosal findings, and stool frequency (Tamaki et al., 2016).

1.3.3.4 Modulation of immune functions

Probiotic bacteria are claimed to modulate the mucosal and systemic immune responses against the antigens in the host body through nonspecific and specific immunomodulation (Jain et al., 2010). Oral administration of live LAB was found to enhance nonspecific host resistance to bacterial pathogens and thereby facilitate the exclusion of pathogens in the gut (Mandal & Mandal, 2011). *Lactobacillus plantarum* NDC 75017 has been shown to stimulate *in vitro* release of pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-6 and tumor necrosis factor-α (TNF-α), and also activate the production of macrophages and phagocytosis in mice reflecting the stimulation of nonspecific immunity (Jiang et al., 2016). Specific immunomodulatory effects by probiotic bacteria are achieved by modulating the immune responses of host toward harmful antigens (Mandal & Mandal, 2011). An increase in rhesus rotavirus-specific IgA antibodies was detected in Balb/c mice with acute rhesus RV (RRV) diarrhea (Qiao et al., 2002).

1.3.3.5 Prevention of colorectal cancer

Colorectal cancer is one of the most common cause of cancer mortality in the developed countries (LeBlanc & LeBlanc, 2015). Several probiotic strains have demonstrated protective

effects in the prevention or treatment of the early stage of colorectal cancers by beneficially modulating the intestinal microbiota, inactivating carcinogenic compounds, producing antioxidant enzymes, and improving immune response of the host (Fung et al., 2011; Jain et al., 2010; LeBlanc & LeBlanc, 2015). For example, supplementing synbiotic composed of prebiotic oligofructose-maltodextrin-enriched probiotics (*L. acidophilus*, *B. bifidum*, and *B. infantum*) increased fecal counts of *Lactobacillus*, reduced counts of *Pseudomonas*, *Congregibacter*, and *Clostridium*, and decreased tumor incidence and volume in 1,2-dimethylhydrazine dihydrochloride-induced colorectal cancer in mice (Kuugbee et al., 2016). Oral administration of *L. casei* BL23 inhibited the development of azoxymethane-induced colorectal cancer in mice with downregulated colonic IL-22 (a cytokine that promotes proliferation of cancer cells) and upregulated *caspase-7*, *caspase-9*, and *Bik*, which are the genes involved in cancer cell apoptosis (Jacouton et al., 2017).

1.4. Probiotic susceptibility and response to environmental stresses during dehydration, storage, and digestions

To confer beneficial effects, a sufficient number of probiotic bacteria must be viable and functional once reached to the colon. However, the manufacturing process (pasteurization, freezing-drying, high hydrostatic pressure, *etc.*), food matrix composition (sugars or salts concentration, pH, natural antimicrobials, *etc.*), storage conditions (freezing, vacuum packaging, *etc.*), and GI digestions (stomach acid, bile salts, enzymes, *etc.*) can impose various stresses that may influence the physiological properties and health benefit potential of probiotics (Capozzi et al., 2015). The susceptibility to a particular stressor may be strain- and species-dependent and vary considerably (Spano & Massa, 2006). In addition, probiotics are equipped with a wide array

of stress-sensing systems and adaptation mechanisms that protect them from extreme environmental stresses (Mbye et al., 2020). Therefore, this section focuses on reviewing the major environmental stresses encountered by probiotic bacteria during processing, storage, and digestions and their effects on the viability and functionality of probiotics.

1.4.1 Temperature-induced stress

The optimum growth temperature of the majority of probiotics is within the range of 30-40 ºC (Terpou et al., 2019). A sudden downshift or upshift of environmental temperature may impose temperature-induced stresses on living cells to cause physiological changes of cellular structures that could be detrimental to their survival.

Low temperatures (but with values > 0 ºC) are used for fermentation during cheese ripening and refrigerated storage of fermented products to prevent spoilage. Cold temperatures depress basic metabolism by reducing cellular membrane fluidity, enzymatic activity, and efficiency of RNA transcription and protein synthesis (van de Guchte et al., 2002). The cold temperature may arrest probiotic growth but generally is not detrimental to cells. However, the storage of probiotics-containing frozen dairy products (e.g. ice cream and frozen yogurt) at -20 ºC and the preparation of lyophilized probiotic with temperature downshifted even to -196 ºC can impose severe cold stress on living cells (Polo et al., 2017). In these cases, the ice crystals formed in the extracellular/intracellular media can irreversibly damage cellular membranes and cause cell injury or death (Lorv et al., 2014).

Heat stress is commonly encountered by probiotics during food processing, such as thermal dehydration, pasteurization, pelleting, and roasting, with thermal treatments above 50 ºC. Although thermotolerance varies among species and strains, temperatures above 65 ºC are highly

detrimental to probiotics (Gomand et al., 2019). It has been reported that no viable *Lactobacillus acidophilus* and *Bifidobacterium animalis* was detected after inoculated in melted cheese at 70-80 ºC and enumerated immediately (Rodgers, 2007). A reduction of *Lactobacillus salivarius* viability from 6.68 to 1.10 log CFU/mL in skim milk was observed after pasteurization at 72 ºC for 15 s (Zhang et al., 2015). Spray drying of free *Lactobacillus salivarius* in peptone media at an inlet temperature of 165 ºC and an outlet temperature of 90 ºC resulted in a viability reduction of 5.65 log CFU/g (Zhang et al., 2015). Heat stress can result in unfolding and subsequent aggregation of proteins and degradation and destabilization of nucleic acids, which cause the depression of cellular metabolism (Mbye et al., 2020). In addition, high temperatures may increase membrane fluidity to disrupt cellular activities (Ferrando et al., 2016).

1.4.2 Osmotic stress

Bacteria need to maintain a relatively constant positive turgor pressure for active metabolism to occur. However, probiotic cells can be subjected to severe osmotic stress during processing like salting and drying. The drying process can also cause concentration of solutes like salts and sugars in the media, which imposes osmotic pressure on probiotics (Mbye et al., 2020). A sudden removal of extracellular water causes an increase of the environmental osmolarity (hypertonic pressure), which causes a lethal loss of cell turgor pressure and changes the cell volume and intracellular solute concentration (De Angelis & Gobbetti, 2004). The induced osmotic stress can rapidly compromise essential cell functions by inducing the membrane lipids changing from a liquid-crystalline state to a gel state and eventually causing membrane leakage, which may reduce the growth and survival rate of probiotics and affect metabolic activities and cause cell mortality during rehydration (Fonseca et al., 2019).

1.4.3 Oxidative stress

Aerobic conditions can be experienced by probiotics during dehydration and ambient storage as well as in the host after ingestion. Probiotics typically are facultative anerobic microorganisms that grow well anaerobically, in which bifidobacteria are generally strict anaerobic and more sensitive to oxygen than lactobacilli (Fiocco et al., 2020). Oxidation of components in food products produces reactive oxygen species (ROSs), such as superoxide radical anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\bullet$), which play a detrimental role in imposing oxidative stress on probiotic cells in food products (De Angelis & Gobbetti, 2004; Mbye et al., 2020). Generally, the susceptibility of probiotic cells to oxidative stress results from their lack of catalase and superoxide dismutase enzyme activity (Mbye et al., 2020). ROSs are reactive with cellular substances, such as proteins, lipids, and nucleic acids, and thus highly toxic to cells (Miyoshi et al., 2003).

1.4.4 Acid stress

Generation of acidic end products by LAB creates a mild to medium acidic environment (~pH 4.6) of fermented probiotic foods, such as yogurt, pickles, and kimchi (Heunis et al., 2014). At low pH, undissociated lactic acid can passively diffuse through the cell membrane and subsequently dissociate into protons and charged derivatives to reduce the intracellular pH (pH_i), which determines the stationary growth phase of certain LAB (De Angelis & Gobbetti, 2004; Lorca & de Valdez, 2001). Although most LAB can survive at low pH, their acid resistance is strain-specific. For example, *Lactobacillus* can grow and survive at pH 3.7-4.3 (Tripathi & Giri, 2014), while the survival of *Bifidobacterium* decreases below pH 4.6-5.0 (Boylston et al., 2004).

Furthermore, probiotics encounter extreme acid stress in the stomach where HCl is present and the empty stomach pH is commonly found below 2.0 (Minekus et al., 2014). Exposure to stomach acid leads to the intracellular accumulation of protons that consequently lowers the pH_i and alters the transmembrane pH, thus impairing the transmembrane transport processes relying on the proton motive force (Fiocco et al., 2020). The cytosol acidification also reduces the activity of acid-sensitive enzymes and negatively affects protein function, nucleic acid structure, and metabolic routes (Corcoran et al., 2008; van de Guchte et al., 2002).

1.4.5 Bile stress

Bile salts are derivatives of cholic acid and are conjugated to glycine or taurine in the liver (Hofmann, 1999). Their surface active and amphipathic properties play an important role in the dispersion and adsorption of lipids (Russell & Setchell, 1992). The detergent-like bile salts with potent antimicrobial activity can also disassemble the lipid structure of cellular membrane and even trigger DNA oxidative damage (Fiocco et al., 2020). Therefore, probiotic bacteria can encounter detrimental bile stress during passage through a bile-rich environment in the small intestine.

1.5 Formulation of powdered probiotics

Probiotic cells are easier to handle in the powder form than in a suspension or slurry and the viability of powdered probiotics can be quantified, allowing the dosage to be readily controlled (Anal & Singh, 2007). The use of biodegradable biopolymers, including polysaccharides, proteins, and lipids alone or in combinations as carriers to formulate powdered probiotic ingredients has been wildly studied. Cryo-, thermo-, or osmo-protective agents can be

incorporated in the drying media to protect probiotic survival during dehydration and subsequent storage. The following sections review the important biopolymers used to formulate polysaccharide-, protein-, and lipid-based drying matrices and their effectiveness in protecting probiotics during dehydration, storage, and GI digestions.

1.5.1 Polysaccharide-based systems

Polysaccharides are polymers of monosaccharides and most of them have a degree of (DP) in the range 200-3,000 (BeMiller & Huber, 2017). Polysaccharides that have been evaluated or used to prepare powdered probiotics include starch and resistant starch (Muhammad et al., 2017), maltodextrin (Hernández-Carranza et al., 2014), cellulose derivatives (Park et al., 2016), pectin (Huq et al., 2016), various plant and microbial gums (Arepally & Goswami, 2019; Chaikham et al., 2017), alginates (Rajam et al., 2012), and chitosan (Cook et al., 2011; Flores-Belmont et al., 2015). Water-soluble polysaccharides can be directly dissolved in cell suspensions to formulate drying media prior to dehydration. Maltodextrin dissolved at 15% (w/v) in jussara juice added with probiotic *Bifidobacterium animalis spp.* Lactis was used as a thermoprotectant and wall material to result in a reduction of only ~1 log CFU/g after spray drying (Paim et al., 2016). Probiotic powders prepared by spray drying a medium composed of cellulose acetate phthalate as the wall material and *Bifidobacterium*. *lactis* and *Lactobacillus acidophilus* were effective in protecting both bacteria when inoculated in 0.1 M HCl solutions due to the enteric property of cellulose acetate phthalate (Favaro-Trindade & Grosso, 2002). Polysaccharides, such as alginate, κ-carrageenan, and chitosan, with the gelling characteristic are able to form hydrogel beads or microcapsules to entrap probiotic cells to protect bacteria during the subsequent dehydration (Sarao & Arora, 2017). For example, freeze drying of *Lactobacillus bulgaricus* L2 encapsulated

in alginate beads showed ~100% viable cells (Mortazavian et al., 2008). Further adding chitosan into alginate beads before freeze drying was found to improve the survival rate of encapsulated *Lactobacillus acidophilus* when exposed to simulated gastric fluids for 2 h (de Araújo Etchepare et al., 2016). Other matrix types have been developed based on specific properties of polysaccharides to prepare powdered probiotics. As shown in Figure 1-2, amylose was precipitated over the enzyme-treated porous potato starch granules which were filled with *Lactobacillus rhamnosus* before freeze drying to a powder form, and the encapsulated bacteria can survive at least 6 months at ambient conditions (Mattila-Sandholm et al., 2002).

1.5.2 Protein-based systems

Plant proteins like zein (Laelorspoen et al., 2014) and soybean proteins (Aubuchon, 2006) and animal proteins like milk proteins (Heidebach et al., 2010) and gelatin (Zárate & Nader-Macias, 2006) have been studied as matrices for efficient drying of probiotic cultures. Particularly, milk protein ingredients (e.g. caseins, whey proteins, and milk protein concentrates) are widely studied as probiotic carriers due to their nutritive value, cost-effectiveness, acceptability, palatability, and biocompatibility with other food ingredients (Heller, 2001; Sanders & Marco, 2010). Specific or nonspecific interactions between milk protein and probiotic cells in the media followed by adhesion of hydrophobic portions of unfolded proteins to cells during dehydration can lead to bacteria being coated within protein capsules, which has been proposed to be the protective effect of dairy proteins on survival of probiotics during drying (Burgain et al., 2014; Khem et al., 2016; Liu et al., 2017). Khem *et al*. (2016) reported spray drying of *Lactobacillus plantarum* in 10% (w/v) whey protein isolate solution showed a survival rate of about 45% higher than spray-drying in 10% (w/v) lactose solution. Ananta *et al.* (2005)

also reported that a *Lactobacillus rhamnosus* GG survival rate of 60% was achieved when skim milk was used as a spray drying carrier and the storage stability of dried probiotics was decreased at a lower concentration of skim milk in the drying medium.

In addition, dairy proteins could be gelled using enzymatic/chemical cross-linking or heat-controlled sol-gel transition to produce a high-density gel network that can better protect probiotics during drying and storage (Damodaran, 2017; Ramos et al., 2018). For example, the enzyme transglutaminase was added to sodium caseinate and *Lactobacillus* F19 and *Bifidobacterium* Bb12 cell mixture followed by emulsification and heating to induce cross-linking between the glutamine and lysine moieties of casein (Heidebach et al., 2010). After freeze drying, the survival rate of encapsulated *L.* F19 was significantly higher than that of free cells and the storage stability of encapsulated *B.* Bb12 was more than 1 log CFU/g higher compared to free cells after 90-day storage at 25 ºC (Heidebach et al., 2010). Encapsulation of *Lactobacillus rhamnosus* R011 in whey protein gel particles prepared by heat-controlled sol-gel transition offered protection during freeze drying and better probiotic stability than the ungelled treatment during 2-week storage of biscuits containing powdered probiotics and frozen cranberry juice (Reid et al., 2007).

Although buffering capacity of proteins can reduce the impact of stomach acid on the pH within the protein matrix and therefore protect bacteria, hydrolysis of proteins by pepsins may destroy the capsule structure to expose the carried cells to harsh conditions (Vidhyalakshmi et al., 2009). Entrapment of probiotics in the microcapsules of protein-polysaccharide complex coacervates formed through electrostatic interactions between oppositely charged macromolecules can enhance probiotic protection in comparison with free cells during GI

digestions (Devi et al., 2017). Eratte *et al.* (2017) reported that encapsulating *Lactobacillus casei* in whey protein isolate-gum arabic complex coacervates followed by spray drying to produce microcapsules significantly increased the protection of probiotics in the simulated gastric fluid at pH 3.0. Soy protein isolate-carrageenan covalent conjugates formed via the Maillard reaction have also been studied to encapsulate *Bifidobacterium longum* by spray drying, and the encapsulation effectively protected the bacteria during storage, pasteurization, and simulated GI digestions (Mao et al., 2018).

1.5.3 Lipid-based systems

A large number of studies reported encapsulation of probiotics in micro-/nano-emulsions (Marino et al., 2017; Zhang et al., 2015), solid lipid micro-/nano-particles (Kim et al., 2008; Okuro et al., 2013), and high internal phase emulsions (Su et al., 2018). However, dehydration of these lipid-based systems to prepare powdered probiotics has not been studied as extensively as those based on proteins and polysaccharides. Ying *et al.* (2016) suspended *Lactobacillus rhamnosus* GG in sunflower oil, followed by emulsification in a mixture solution of whey protein isolate and resistant starch. After spray drying, the microencapsulated probiotics were found to be more stable than freeze-dried *L. rhamnosus*, maintaining $>10^7$ CFU/g viable cells after 12-month storage at 25 ºC. In a separate study, encapsulation of *Lactobacillus casei* 431 in tuna oil emulsified with whey protein isolate, further cross-linked with gum arabic, maintained significantly higher viability than the treatment without tuna oil after spray or freeze drying (Eratte et al., 2015).

1.6 Methods of producing powdered probiotics

In order to prepare powdered probiotics, a drying process is usually needed to reduce the water content of a probiotic culture into a level where the microbial metabolism is slowed down with the purpose of prolonging storage viability. Currently, several drying technologies have been developed and applied to prepare powdered probiotic ingredients. Prior to selecting one of them, industry should have considered the susceptibility of dehydrated bacterial strains, physicochemical properties of the prepared probiotic powders, processing conditions to incorporate probiotic powders into a food product, properties and storage conditions of the food product, and the balance between costs and benefits (Zuidam & Shimoni, 2010). In this section, we will review the most important drying technologies based on the drying temperatures used, as well as the approaches applied to improve probiotic survival during drying.

1.6.1 High temperature drying methods

1.6.1.1 Spray drying

During spray drying, a liquid feed is atomized into a spray of fine droplets in a drying chamber. The spray contacted with and suspended by hot drying air results in moisture evaporation and the formation of dry particles that are subsequently separated from the hot air and collected as the final product (Barbosa-Cánovas et al., 2005). The spray-dried particles are usually in the form of powders, agglomerates, or granules. The advantages of spray drying include low cost, high productivity, easy and automatic drying operations, continuous and rapid processing, as well as constant powder specifications (Liu et al., 2019). Therefore, spray drying is one of the most commonly used drying technologies in the dairy industry (Schuck et al., 2016) and is becoming

more and more popular in the microbiological industry to produce powdered probiotics. However, the inactivation of bacteria caused by thermal, osmotic, and oxidative stresses during and after dehydration is the main disadvantage of spray drying (Santivarangkna, Kulozik, et al., 2008). Thermal stress is the critical factor that influences the probiotic viability, and the outlet temperature (T_{outlet}) is considered to play a more significant role than the inlet temperature (T_{inlet}) in affecting the viability of spray-dried probiotics (Huang et al., 2017). Zhang *et al.* (2016a) reported that a relatively small change in T_{outlet} from 94-96 ºC to 98-100 ºC causes a significant reduction of *Lactobacillus salivarius* viability from 4.54 to 3.55 log CFU/g. In addition, a loss of bound water at the cell surface during dehydration can induce osmotic stresses, leading to a detrimental transition of the cell membrane from the lamellar to the gel phase (Huang et al., 2017). Furthermore, the shear force during the atomization process could also cause probiotic inactivation, as reported by Riveros *et al.* (2009) that lowering the spray nozzle pressure from 0.15 to 0.1 MPa resulted in an increased viability of *Lactobacillus acidophilus* by ~2 log CFU/g.

1.6.1.2 Fluidized bed drying

Fluidized bed drying is another method to prepare powdered probiotics using high drying temperatures. Probiotics are usually needed to be encapsulated in a wet solid form using a supporting material such as whey proteins (Schell & Beermann, 2014) or alginate beads (Cook et al., 2014), prior to suspending in the upward-moving hot air flow to evaporate moisture (Barbosa-Cánovas et al., 2005). Fluidized bed drying has milder drying conditions than spray drying and requires a lower cost and energy than freeze drying (Liu et al., 2019). More importantly, it is easy to scale up and can prepare multi-coating layers to protect the probiotics-containing core materials by spraying a solution of biopolymers with different functions on the

surface of bioactive cores (Martín et al., 2015). Azim *et al.* (2012) prepared probiotics powders by spray coating a mixture of stearic acid, palmitic acid, and vegetable wax on freeze-dried *L. reuteri* C10, showing <1 log CFU/g reduction during fluidized bed drying and maintained satisfactory stability during storage for up to 70 days. Reversely, a cell suspension can also be sprayed and dried on carriers using fluidized bed drying to prepare powdered probiotics. For example, by spraying a *L. paracasei* suspension supplemented with trehalose and maltodextrin on inert carrier microcrystalline cellulose using a fluidized bed system, probiotic powders were prepared with more than 9 log CFU/g of viable cell counts (Semyonov et al., 2012). The disadvantages of this technology are the difficulty to master and being relatively time-consuming which are prone to inactivate probiotic bacteria (Liu et al., 2019).

1.6.2 Low temperature drying methods

1.6.2.1 Freeze drying

The typical freeze-drying process is composed of three steps, namely freezing, primary drying, and secondary drying (Barbosa-Cánovas et al., 2005). Initially, a liquid mixture of probiotic suspensions and cryoprotectants is frozen under atmospheric conditions and extracellular ice crystals are formed and separated from the residual sample. In the subsequent primary drying step, the frozen solvent that is unbound to cells is sublimated under high vacuum, and the bound water is removed via desorption in the secondary drying (Aschenbrenner et al., 2015; Barbosa-Cánovas et al., 2005). Freeze-dried products are dry, light, and porous, and have good reconstitution properties to regain their original shape and texture after rehydration, making freeze drying a popular method of producing dried food products with high quality (Barbosa-Cánovas et al., 2005).

Compared to spray drying, freeze drying process is milder and thus more protective to maintain high probiotic viability. Therefore, freeze drying has been used as the standard process to produce dry probiotics for decades (Liu et al., 2019). However, the high production costs due to the slow drying rate and use of vacuum are the major disadvantages of freeze drying (Barbosa-Cánovas et al., 2005). In addition, the formation of ice crystals during freezing can cause mechanical and osmotic stresses to damage cell membrane integrity, and the removal of bound water in the subsequent desorption phase can destabilize cellular substances like phospholipids and proteins to cause additional viability losses (Aschenbrenner et al., 2015; Liu et al., 2019).

1.6.2.2 Spray freeze drying

Spray freeze drying is a drying technology that combines features of both spray and freeze drying. A drying medium containing probiotic cells is atomized into a cold liquid vapor phase (e.g. liquid nitrogen) to produce frozen droplets, which are subsequently dried with the help of a freeze dryer (Martín et al., 2015). Spray freeze drying has various benefits such as low temperature, good scalability, and ability to produce particles with controlled size, good flowability, low hygroscopicity, and large specific surface area (Rajam & Anandharamakrishnan, 2015; Sarao & Arora, 2017). *Lactobacillus plantarum* powders using whey protein as the carrier prepared by spray freeze drying showed more spherical shape with numerous fine pores and 20% higher cell viability than the spray-dried powders, which agrees with the advantages of spray freeze drying (Dolly et al., 2011). Using fat matrices as the carriers can further protect probiotics during GI digestions. Spray freeze drying of *Lactobacillus acidophilus* LA3 and *Bifidobacterium animalis* subsp. *lactis* BLC1 in molten vegetable fat emulsified with gelatin and gum arabic prepared solid lipid microcapsules that maintained a significantly higher probiotic survival rate

(>75%) than free cells under simulated GI conditions (Silva et al., 2018). Similar results were also reported by Pedroso *et al.* (2013) where solid lipid microparticles prepared by spray freeze drying of *Bifidobacterium animalis* subsp. *lactis* and *Lactobacillus acidophilus* in cocoa butter were effective in protecting the probiotics against simulated gastric conditions and 90-day storage at 20 ºC. However, low encapsulation efficiency, high energy input, and long duration of processing are the main disadvantages of spray freeze drying method to limit its widespread application in the food industry (Sarao & Arora, 2017).

1.6.2.3 Other low temperature drying methods

Several drying methods performed at relative low temperature (e.g. room temperature) have been studied at laboratory scale studies. Electrospinning has been introduced as a novel method to incorporate probiotics into nanofibers through the action of an external electric field imposed on a polymer solution (Martín et al., 2015). Škrlec *et al.* (2019) developed composite poly(ethylene oxide)/lyoprotectant nanofibers loaded with *Lactobacillus plantarum* ATCC 8014 through electrospinning and found a high loading of probiotic cells of 7.6×10^{11} CFU/g which remained stable during 24-week storage at 4 ºC. Supercritical technology is another novel method to prepare probiotic powders by immobilizing probiotics in interpolymer complexes formed in supercritical CO_2 as the solvent prior to gasifying the supercritical CO_2 through depressurizing to obtain dried microcapsules (Liu et al., 2019). Thantsha *et al.* (2014) used polyvinylpyrrolidone and viny lacetate-co-crotonic acid, both of which can be plasticized in supercritical CO_2 to form an interpolymer complex though hydrogen bonding, to encapsulate *Bifidobacterium lactis* Bb12 and *Bifidobacterium longum* Bb46. The prepared probiotic powders with the a_w of 0.25-0.43 showed more than 6 log CFU/g viability after 12-week storage at 30 ºC.

Other drying methods have also been reported. For example, the gum arabic solution was used as a carrier for electrospray-assisted drying of *Lactobacillus plantarum* which remained more than 96% viability after dehydration (Zaeim et al., 2018).

1.6.3 Approaches to improve probiotic survival during drying

In order to obtain probiotic powders with higher bacteria viability and better powder quality, different approaches from formulating the drying media to the subsequent drying process are reviewed in this section. It is worth noting that various approaches are usually combined to optimize a specific drying strategy in realistic applications based on the properties of selected probiotic strains, powder formulation and quality, drying methods and devices, *etc*.

1.6.3.1 Stress adaption of probiotics

Triggering the stress adaption of probiotics prior to drying is an effective strategy to improve their survivability during drying where heat, osmotic, and oxidative stresses usually occur. When probiotic bacteria are exposed to a sub-lethal level of a given stress (e.g. osmotic stress or high temperature), the cellular stress-response system can be induced as an adaptation phenomenon leading to greater tolerance to even higher doses of the stress during the subsequent drying (Watson & Preedy, 2015). Zhang *et al.* (2016a) reported that the viability reduction of spray-dried *Lactobacillus salivarius* was significantly decreased after heat adaption of the probiotics-containing drying media at 50 ºC for 15 min. The authors proposed that the heat adaptation may induce an increase of saturated and straight-chain fatty acids to maintain the fluidity of the membrane (De Angelis & Gobbetti, 2004) as well as the production of heat shock proteins to promote the correct folding of nascent polypeptides and regulate transcription and

translation of proteins (Russell & Fukunaga, 1990). In addition to heat adaptation, exposing bacteria to sublethal osmotic stress has also been reported to have positive effects on probiotic survival during drying. Nag and Das (2013) presented the survival of *Lactobacillus casei* after fluidized bed drying and during storage at 25°C for 52 weeks was significantly improved after incubation in media at a hyperosmotic condition (0.6 M NaCl) till the early stationary phase.

1.6.3.2 Addition of protectants

Addition of thermo- or cryo-protectants into the drying media is one of the most commonly used strategies to avoid undesired cellular damage during various drying processes, especially applicable for the freeze drying. It was reported that only 4% of *Lactobacillus delbrueckii* was viable after freeze drying using water as the solvent (Sheu et al., 1993). Generally, the protectants can be divided into high molecular weight and low molecular weight agents.

The high molecular weight protectants mainly include proteins (e.g. milk proteins and gelatin), polysaccharides (e.g. maltodextrin and inulin), and lipids (e.g. low melting point fats) (Liu et al., 2019; Martín et al., 2015). As described in the Section 1.4, many of these biopolymers are commonly used to formulate the drying media where they may attach on the surface of probiotic cells to form a viscous coating to prevent cellular damage during freeze or spry drying (Liu et al., 2019). Specifically, the low melting point fat has the ability to absorb thermal energy during the solid-to-liquid phase transition when the T_{inlet} is higher than its melting point and was recently used as a thermo-protectant to improve *Lactobacillus zeae* LB1 viability from 15% to 63% when it was added to sodium caseinate during spray drying (Liu et al., 2015).

The low molecular weight protectants commonly refer to disaccharides, such as trehalose, xylose, glucose, sucrose, maltose, and lactose, as well as polyols, such as mannitol and sorbitol

(Aschenbrenner et al., 2015; Santivarangkna, Kulozik, et al., 2008). Miao *et al*. (2008) reported that trehalose and the combination of lactose and maltose were the most effective cryoprotective additives to protect the viability of freeze-dried *Lactobacillu. paracasei*. The addition of trehalose or lactose also resulted in approximately three to four times higher survival rates of spray-dried *Lactobacillus rhamnosus* than the control treatment without protectants (Broeckx et al., 2017). The water replacement and formation of a glassy matrix are commonly accepted as the protection mechanisms of disaccharides and polyols (Aschenbrenner et al., 2015). The water replacement hypothesis suggests that hydrogen bonds initially formed between the polar headgroups of phospholipids at the surface of cellular bilayers and water are lost during dehydration but are replaced by protectants with hydroxyl groups, thus preventing the transition of cell membrane into a gel phase (Santivarangkna, Higl, et al., 2008). The protective effects of forming a glassy matrix can be explained based on the fact that disaccharides and polyols have a relatively high glass transition temperature (T_g) and can be easily vitrified into a glass state with a high viscosity to retard metabolic activities and biomolecular reactions to protect bacterial survival (Aschenbrenner et al., 2015).

1.6.3.3 Encapsulation of probiotics prior to drying

Although some review papers define probiotics contained in dried microcapsules as microencapsulated probiotics (Dianawati et al., 2016a; Liu et al., 2019; Riaz & Masud, 2013), this section specifically reviews the strategies to fabricate encapsulation systems for probiotic in the media prior to drying processes. Structures like electrostatic complexes/coacervates (Zhao et al., 2018), hydrogel beads (Rajam et al., 2012), emulsions (Marino et al., 2017), solid lipid nanoparticles (Okuro et al., 2013), *etc.*, have been developed to entrap and protect probiotic cells

from environmental stresses during drying and the subsequent storage. Reversely, without encapsulation, a lot of free cells are present on the droplet surface and thus may be inactivated during drying and storage. For instance, the viability of *Lactobacillus plantarum* encapsulated in gelatin/gum complex coacervates was significantly higher than that of *L. plantarum* in gelatin solution after spray drying and during subsequent 50-day storage at 25 ºC (Zhao et al., 2018). Su *et al.* (2018) also found that encapsulation of *Lactobacillus plantarum* in high internal phase emulsions stabilized with whey protein isolate microgels showed a significantly increased viability after pasteurization at 63 °C for 30 min compared to free probiotics cells suspended in grape seed oil.

1.6.3.4 Optimization of drying parameters

Adjustment of drying parameters is an effective and applicable strategy for improving the survival of probiotic bacteria during high temperature drying methods, in which the spray drying process has been most extensively studied and optimized. Spray drying conditions including T_{inlet}/T_{outlet}, flow rate, atomizing air pressure, and residence time directly impact the heat and mass transfer between air and droplets, which plays an important role in changing droplet size, moisture content, and probiotic viability (Fu et al., 2018). It has been proposed that at the initial stage of drying, evaporation of water at the droplet surface can quickly counteract the heat convection from hot air, so that the droplet temperature is stabilized at the wet-bulb temperature (usually below 40 ºC) which is not detrimental to the probiotic viability (Huang et al., 2017). However, afterwards, once the water content is reduced to a relatively low level, the droplet temperature starts to rise toward the T_{outlet} depending on the resident time (Huang et al., 2017). Therefore, T_{outlet} is considered to be the principal factor that affects the post-drying viability of

spray-dried probiotics. Normally, reducing the T_{inlet} can result in a lower T_{outlet} and thus a higher survival rate of probiotics during spray drying. Ghandi *et al.* (2012) reported that the viability of spray-dried *Lactococcus lactis* increased from 0.1 to 14.7% when the T_{inlet}/T_{outlet} decreased from 200ºC/65ºC to 130ºC /38ºC. At a constant T_{inlet}, feed rate is another factor that can strongly affect the variation of T_{outlet} and thus the survival rate of spray-dried probiotics. Zhang *et al.* (2016b) found that at a constant T_{inlet} of 170 ºC, the T_{outlet} was decreased from 98-100 °C to 70-72 °C by increasing the feed rate, resulting in an increase of *L. salivarius* viability by 2.4 log CFU/g. Under the same $T_{inlet}/T_{outlet,}$ increasing the atomizing air pressure could induce a higher shear stress on cells and reduce the droplet size to increase the exposure of probiotic to hot air, both of which lead to a lower survival rate of dried probiotics (Zhou et al., 2004). The residence time of probiotics exposed in the drying chamber is also a critical factor influencing the probiotic viability during spray drying: the shorter the residence time, the higher the bacterial survival after spray drying (Fu et al., 2018). The residence time is mainly controlled by the aspirator setting value, and it has been reported that increasing the aspirator power level from 0 to 12 can increase the survival rate of spray-dried *Bifidobacterium* cells from 11.3% to 29.6% (O'Riordan et al., 2001).

However, apart from bacterial viability after spray drying, the quality of spray-dried powders should also be considered for application convenience and probiotic stability during storage. For instance, although low T_{outlet} is favorable for the probiotic survival, it can lead to a high moisture content of spray-dried powders which is unfavorable for prolonged storage of probiotics (Zhang et al., 2016a). In addition, spray-drying involves multiple factors and conditions that are helpful to maintain probiotic survival when used individually often cannot be

simultaneously achieved in a spray drying step, exemplified by the elongated residence time when lower drying temperature (Fu et al., 2018). Therefore, optimization of drying parameters needs to be comprehensively considered based on different devices and specific requirements for realistic applications.

1.7 Characterization of powdered probiotics

Probiotic powders possess many structural, physical, functional, and microbiological properties, including particle morphology, particle size, flowability, rehydration ability, a_w, bacterial viability, biophysical states of bacteria, *etc*. Several properties of significance to quality, stability, and application of powdered probiotics are reviewed below.

1.7.1 Structural properties

1.7.1.1 Morphology

The particle morphology can strongly influence the physical and functional properties of powders, such as flowability, rehydration, sticking, and caking (Bhandari et al., 2013). Microscopy methods including light microscopy, confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM) are important tools for surface and morphological observations, in which SEM is the most commonly used method in studying the morphology of probiotic powders. SEM visualizes particle structures by detecting the backscattered or secondary electrons emitted from the surface of the specimen being shot by a focused electron beam (Amelinckx et al., 2008). Due to the narrowness of the excitation beam, the SEM images have

high-resolution, high-magnification, and high depth-of-field features that are suitable for bacterial observation (Bergmans et al., 2005). *Lactobacillus* F19 was found to be randomly distributed in the transglutaminase-induced casein gels after freeze drying (Figure 1-3A) (Heidebach et al., 2010). The core-shell structure with entrapped *Lactobacillus acidophilus* prepared by electrospraying a *L. acidophilus*-containing alginate core solution into a zein shell solution followed by drying was clearly imaged using SEM (Figure 1-3B-D) (Laelorspoen et al., 2014). However, due to the low contrast and low conductivity between the background and samples, powders are usually needed to be coated with a conductive carbon or gold, which in turn could cause the creation of artifacts. The high vacuum conditions during SEM operation may also alter the native structures of powders (Bhandari et al., 2013). Other microscopy technologies like TEM, CLSM, and AFM are currently used to image probiotic microcapsules in liquid preparations (Falsafi et al., 2020), and more studies are required to develop their functions in probiotic powder analysis.

1.7.1.2 Particle size

Particle size is an important parameter determining the appearance, flowability, density, and rehydration properties of powders, and is influenced by the composition of drying matrix, the type of drying equipment, and processing conditions (Abdalla et al., 2017). The particle size of powders can be measured using direct (sieving, microscope counting, and electrozone particle counting) and indirect methods (sedimentation and laser diffraction - LD) (Schuck et al., 2012). Microscopy, especially SEM, is the most commonly used technology for characterizing particle size, size distribution, and morphology, because it allows direct observation of particles ranging from nanometer to millimeter scale. However, under-representative sampling and statistical

errors associated with preferential particle orientation and large particle agglomeration can result in underestimated particle size results (Shekunov et al., 2007). Therefore, in current studies analyzing particle size of probiotic powders, SEM is usually used to estimate particle size or combined with other technology like LD. LD measures the light diffraction pattern caused by passing a standard He-Ne laser light (λ = 632.8 nm) through a dispersion of powder, and the diffraction pattern is then treated by light scattering theory to calculate the particle size distribution (Hackley et al., 2004). LD can be used to measure dry powders, powders dispersed in aqueous or non-aqueous dispersants, and aerosols with a size range of about 0.1-3,000 μm (Jillavenkatesa et al., 2001). Compared to microscopy, LD has shorter analytical time, higher precision, better reproducibility, lower cost, and wider measurement range, and thus is becoming the essential technology of powder particle size analysis in the food and pharmaceutical industries (Fitzpatrick, 2013; Shekunov et al., 2007). Depending on different drying methods, spray-dried particles are usually spherical with diameters ranging from 10 to 250 μm, and larger agglomerates with a size ranging from 50 to 5000 μm are common in fluidized bed drying preparations (Chandran et al., 1990).

1.7.2 Physical and functional properties

1.7.2.1 Flowability

The flowability refers to the ability of a powder to move among neighboring particles or along the container wall surface, and plays an important role in influencing handling, packaging, and storage of powders (Schuck et al., 2012). The major forces involved in resisting powder flow are internal friction and cohesion (Barbosa-Cánovas et al., 2005). The former is the Coulomb

frictional resistance between particles, while the latter refers to the inter-particle attraction that can resist flow (Barbosa-Cánovas et al., 2005). The flowability of a powder can also be affected by the shape, size distribution, and surface composition of particles (Barbosa-Cánovas et al., 2005). Generally, large agglomerates show better flowability than fine particles, and an increased amount of water or fat on the particle surface can lead to a lower flowability by increasing the contact area between particles (Fitzpatrick, 2013).

Calculating the Hausner ratio (eq. 1) and Carr index (eq. 2) using the following equations operated by pouring a certain mass of powder into a graduated cylinder followed by tapping the cylinder for a specific number of taps according to (USP, 2012) is widely used to compare the flowability of probiotic powders. Stummer *et al.* (2012) reported that the Hausner ratio and Carr index of fluidized-bed dried *Enterococcus faecium* in skim milk were significantly higher than that of freeze-dried cells, indicating the superior flowability of the former likely due to the more spherical particle shape. Arepally and Goswami (2019) also found spray-dried *Lactobacillus acidophilus* formulated with a higher concentration of gum arabic showed better flowability due to larger particle size and lower moisture content, with a fair flowability found in the 7.5% and 10% gum rabic treatments.

$$\text{Hausner Ratio} = \frac{\text{Tapped bulk density (g/cm}^3\text{)}}{\text{Loose bulk density (g/cm}^3\text{)}} \qquad (1)$$

$$\text{Carr index (\%)} = \frac{\text{Tapped bulk density (g/cm}^3\text{)} - \text{Loose bulk density (g/cm}^3\text{)}}{\text{Tapped bulk density (g/cm}^3\text{)}} \times 100 \quad (2)$$

1.7.2.2 Rehydration ability

No matter powdered probiotic ingredients are used to prepare solid or liquid probiotic products, probiotics need to be released from the microcapsules after rehydrating in water before

conferring beneficial functions. Therefore, the rehydration ability is an important functional property for probiotic powders and is characterized by three parameters, namely wettability, dispersibility, and solubility (Barbosa-Cánovas et al., 2005).

Wettability reflects the immersion ability of powder particles to overcome surface tension at the interface between solid and liquid at a certain temperature and is often measured as the time taken for the powder to be completely wetted and penetrate the surface of still water (Schuck et al., 2012). Dispersibility is defined as the ability of a powder to break up into increasingly smaller particles with gentle mixing, and is expressed as the amount of dry matter (% w/w) that can pass through a sieve with a mesh size of 200 mm after mixing the powder in water for 15 s with a spatula (Schuck et al., 2012). Solubility corresponds to the total solubilization of a powder to obtain a solution or stable suspension, and the solubility index is defined as the sediment volume (mL) after adding a powder into 100 mL of water with high speed mixing for 90 s at 25 ºC followed by standing for 15 min and centrifugation at 160 *g* for 5 min (Tamime, 2009).

Several strategies can be used to improve the powder rehydration properties, such as increasing the hydrophilicity of particle surface, using freeze-drying to prepare powders with high porosity, increasing particle size, and optimizing rehydration conditions (Jeantet et al., 2010; Selomulya & Fang, 2013). For example, spouted bed drying of *Lactobacillus casei*-fermented orange juice with 15% (w/w) of maltodextrin as the drying agent showed fast rehydration time than that with gum arabic at the same concentration (Alves et al., 2016). However, highly rehydratable powders can easily absorb moisture during storage, which is unfavorable for prolonged probiotic storage stability. In addition, Kosank *et al.* (1992) found that

dried bacteria undergoing rapid rehydration can experience an instantaneously increased osmotic stress, resulting in less cellular viability compared to slowly rehydrated bacteria.

1.7.2.3 A_w

a_w is defined as the ratio of the partial pressure of water vapor of a food system (P_p, Pa) to the partial pressure of the vapor pressure of pure water (P_w, Pa) under the same temperature and total pressure (Schuck et al., 2012):

$$a_w = \frac{P_p}{P_w} \quad (3)$$

Determination of a_w of a food powder can be done by directly measuring the water vapor pressure using a water activity meter. a_w estimates the thermodynamically available water for various biological or physiochemical reactions, thus making it a more important parameter than moisture content to understand the probiotic survivability and powder quality after dehydration and storage (Syamaladevi et al., 2016). High a_w (> 0.85) can support the growth of microorganism by activating microbial metabolism and facilitating moisture migration within the powder matrix, which is unfavorable for prolonged storage of probiotic powders (Maltini et al., 2003). Conversely, a low a_w (0.10-0.25) is generally recommended for effectively improving the long-term storage stability of dry foods containing live probiotics (Teijeiro et al., 2018). However, too low a_w (<0.10) may cause the oxidative and osmotic stresses that result in viability reduction (Vesterlund et al., 2012). a_w of probiotic powders is highly related to the drying method, drying parameters, and storage conditions. For example, Zhang *et al.* (2016a) found that decreasing the T_{outlet} from 98-100 ºC to 70-72 ºC resulted in an increase of a_w of spray-dried *Lactobacillus salivarius* from 0.14 to 0.25, corresponding to a higher probiotic viability initially

which, however, was dramatically reduced by 3.5 log CFU/g after storing in a desiccator at 21 ºC for 2 weeks.

1.7.3 Microbiological properties

In order to provide health benefits, an adequate amount of viable cells must survive through dehydration and storage before reaching the colon. Powdered probiotics need to be properly rehydrated and diluted before enumeration. Solid level, rehydration medium, and pH are important factors that need to be considered during rehydration (Champagne et al., 2011). Rehydration media should have buffering capacity, and therefore solutions containing peptone, NaCl, or phosphate salts with pH close to the optimum pH for microbial growth are commonly used (Abe et al., 2009). Subsequently, suitable homogenization methods, such as manual shaking, vortexing, and blending, may be required to facilitate the rehydration of probiotic powders to obtain homogenous cell suspensions (Champagne et al., 2011), and the rehydration time and homogenization methods should be optimized based on microbial properties, drying matrix composition, and immobilization form of probiotic cells. Probiotics microencapsulated in alginate beads, emulsions, or complexes prior to drying may require longer rehydration time, high shear speed, pH adjustment, or addition of surfactants to release encapsulated bacteria (Zhang et al., 2015). The prepared cell suspensions can be subjected to the following assessments on the microbiological properties.

1.7.3.1 Probiotic viability counts

Culture-dependent colony counts is still the “gold standard” for viability counts (Champagne et al., 2011). Therefore this section focuses on the standard plate count methodology. MRS agar

is the most widely used base plating medium for pure cultures of LAB. For mixed cultures, selective or differential culture media are required for detection and enumeration of specific probiotic species. For example, bifidobacterial-selective media can be designed by supplementing various selective agents that lower the redox potential such as cysteine, ascorbic acid, and sodium sulphite, or bifidobacterial-resistant antibiotics like kanamycin and mupirocin (Rasic, 1990). However, the main disadvantage of these selective media is that they may impose additional stresses and thus underestimate the counts of target bacteria (Champagne et al., 2011). The cell suspension can be spread or poured on agar plates followed by incubation usually at 37 ^{o}C under anaerobic environment using anaerobic jars or oxygen-less cabinet incubators for about 24-48 h.

Recently, alternative culture-independent methods, such as microscopic counts (e.g. CLSM and fluorescent microscopy), nucleic acid amplification techniques (e.g. real time-quantitative polymerase chain reaction (PCR) and reverse transcriptase PCR), and cell sorting techniques (e.g. flow cytometry), have been used to accurately enumerate probiotic strains (Davis, 2014). These modern molecular tools offer the potential to enumerate not only culturable but also stresses, injured, or viable but non-culturable (VBNC) bacteria which are discussed below.

1.7.3.2 Biophysical states of probiotics

As a response to environmental stresses, such as starvation, osmotic pressure, thermal change, and radiation, during dehydration and storage, bacteria may enter a VBNC state. In this state, bacteria typically fail to form colonies on conventional culture media but show other characteristics that can be measured to indicate the maintained cell viability, for example, cellular integrity and metabolic activities e.g., respiration, gene transcription, and protein

synthesis (Oliver, 2005; Pinto et al., 2015). To date, several methods for determining viable cell counts have been developed, including membrane integrity, metabolic activity, membrane potential, intracellular enzymatic activity, and global gene expression (Oliver, 2005; Pinto et al., 2015). Evaluation of cellular integrity and metabolic activities using fluorescence stains followed by comparing with the plate-counting results has been extensively used to detect VBNC cells. The membrane integrity can be measured using a LIVE/DEAD® *Bac*Light™ bacteria viability kit containing green-fluorescent SYTO® 9 to stain all cells and red-fluorescent fluorescent propidium iodide (PI) only to penetrate and label bacterial cells with a damaged membrane (Anonymous, 2004). The cellular metabolic activity can be indicated by using a redox indicator, 5-cyano-2,3-di-(p-tolyl)tetrazolium chloride (CTC), that, once absorbed by viable cells, is reduced into an insoluble and red-fluorescent formazan via bacterial respiration (Anonymous, 2005). Due to the discriminatory power of fluorescent staining methods, the results can be analyzed using fluorescent microscopy, quantitative measurements with a fluorescence microplate reader, and flow cytometer/fluorometer (Ramamurthy et al., 2014).

1.8 Application of powdered probiotics in food products

It is generally accepted that a food matrix labeled as "contains live cultures" should have a minimum of 10^6-10^7 CFU/g viable probiotic bacteria (Bertazzoni et al., 2013). Powdered probiotics can be incorporated in liquid or solid food products for consumption and ingestion. Therefore, powdered probiotics incorporated into different forms of food matrix as affected by manufacturing process, storage, and digestions are discussed in this section.

1.8.1 In liquid preparations

Liquid probiotic products including yogurt, beverages, and ice creams. Cheese is considered as a liquid probiotic product in this review because probiotics and rennet are usually added to milk to facilitate the coagulation of caseins followed by heating, pressing, and ripening, and therefore probiotics are no longer in the powdered form (Boylston et al., 2004). The probiotic survival during manufacturing, storage, and digestions of liquid probiotic products is discussed below.

1.8.1.1 Manufacturing processes

Probiotics in the powdered form are usually used as starter cultures in fermentation processes or directly supplemented into the final products especially when probiotics are encapsulated in microcapsules. In the latter case, the impact of particle size on the sensory properties of final products needs to be assessed. It has been reported that particulates larger than 10 μm in dairy products can cause sandy texture (Heidebach et al., 2012; Walstra et al., 2005). The processing steps involved in preparing liquid probiotic products, such as rehydration, heating, pumping/blending, pH change, ripening, salting, and freezing, may impose various environmental stresses and lead to substantial viability loss (Champagne et al., 2005). Adding antioxidants, yeast extracts, prebiotics, and preservatives into the food matrix as well as optimizing the process conditions by using vacuum or nitrogen flushing, applying sublethal stresses, and modifying fermentation parameters can be used to improve probiotic viability during manufacturing (Farnworth & Champagne, 2010).

1.8.1.2 Storage conditions

Free probiotics in liquid preparations are very susceptible to environmental stresses related to temperature, a_w, oxygen, and pH during storage, and thus have a short shelf-life. Therefore, lowering storage temperature is a predominate method to prolong probiotic survivability during storing liquid probiotic products due to the depressed microbial metabolism. Yogurt and beverages usually require refrigerated storage (3-5 ºC) and probiotic viability in fermented milks seems stable for up to 4 weeks at 5 ºC (Makinen et al., 2012). Ice creams and other frozen dairy desserts generally require freezing storage (-20 ºC). According to Homayouni *et al*. (2008), *Lactobacillus casei* and *Bifidobacterium lactis* in ice creams maintained 10^8-10^9 CFU/g after storing at -20 ºC for three months. However, although ice creams are considered as a desirable probiotics carrier, repeated freezing and thawing that possibly occur during ice cream storage and consumption could cause cellular damage and cell death (Flach et al., 2018).

1.8.1.3 Digestion conditions

The harsh environment in the GI tract is considered to be an even tougher challenge for probiotics compared to surviving processing and storage conditions. Free probiotics without encapsulation are susceptible to the low gastric pH, enzymes, and bile salts, but it has also been suggested that dairy matrices may have a buffering capacity to protect ingested bacteria during transit through the upper GI tract (Würth et al., 2015). Probiotics encapsulated in microcapsules are more resistant to stresses during digestions. Ortakci and Sert (2012) reported that after incubating yogurt containing *Lactobacillus acidophilus* for 2 h in simulated gastric juice,

Lactobacillus acidophilus ATCC 4356 encapsulated in calcium alginate showed a viability reduction of only 3 log CFU/g, while no viable free *L. acidophilus* was detected.

1.8.2 In solid preparations

Compared to liquid probiotic preparations that usually result in high a_w and need cold chain transportation, solid probiotic products like chocolates, oats, cereals, protein and snack bars, and probiotic pellets are becoming more and more popular because of their application convenience, long shelf-life, and low cost (Flach et al., 2018). The probiotic survival during manufacturing, storage, and digestions of solid probiotic products are reviewed below.

1.8.2.1 Manufacturing processes

In solid preparations, powdered probiotics can be directly used as an ingredient to manufacture final products. The processing steps involved in preparing solid probiotic products, such as grinding, blending, shearing, compression, extrusion, pelleting, baking, roasting, and pasteurization, commonly impose thermal and mechanical shocks on probiotics (Dianawati et al., 2016b; Gomand et al., 2019). Due to the depressed metabolic activities and protection from matrix materials, powdered probiotics in solid preparations generally display better resistance to these stresses compared to those in liquid preparations. For example, freeze-dried *Lactobacillus acidophilus* LA-2 mixed with soy flour, soy protein isolate, and non-fat dry milk were heated to 85 ºC for 15 s and then blended at high speed for 5 min to prepare a soy protein bar in which more than 8 log CFU/g viable *L. acidophilus* were detected after preparation (Chen & Mustapha, 2012). In contrast, the viability of spray-dried *Lactobacillus salivarius* loaded in skim milk

powders after suspension in phosphate buffer at pH 7.0 was reduced from 6.7 to 1.1 log CFU/mL after heating at 72 ºC for 15 s (Zhang et al., 2015).

1.8.2.2 Storage conditions

Powdered probiotics generally exhibit better storage stability than free probiotics in liquid media under same storage conditions. Therefore, solid products containing powdered probiotics can be stored under ambient conditions. Chen and Mustapha (2012) reported less than 2 log CFU/g viability loss of freeze-dried *Lactobacillus acidophilus* LA-2 supplemented in soy protein bars after storage at room temperature for 12 weeks. Saarela *et al.* (2006) also found that freeze-dried *Lactobacillus rhamnosus* E800 and E522 incorporated in chocolate-coated breakfast cereals maintained more than 7 log CFU/g viable cells after storing at 20 ºC for 12 weeks. Conversely, the viability of spray-dried *Lactobacillus salivarius* suspended in phosphate buffer at pH 7.0 was reduced from 7.2 to 3.9 log CFU/mL after 20-day storage at 4 ºC (Zhang et al., 2015). Other storage conditions like low a_w and low oxygen content are also crucial to prolong probiotic stability.

1.8.2.3 Digestion conditions

Probiotic powders are typically mixed or blended with many other food ingredients to process solid probiotic products. Therefore, probiotic cells are possibly embedded or immobilized as the core material in a food matrix, which may slower the diffusion of living cells into gastric acids and retard permeation of the acidic fluid into the cells (Heidebach et al., 2012). Succi *et al.* (2017) found the survival of freeze-dried *Lactobacillus paracasei* F19 and *Lactobacillus rhamnosus* GG individually incorporated into dark chocolate during simulated GI

digestions is strain-dependent, but overall more than 5 log CFU/g viable cells were maintained after digestions. In addition, additional enteric coating on probiotic pellets/tablets has been suggested to further improve probiotic resistance to GI conditions. Chan and Zhang (2002) developed a sodium alginate-hydroxypropyl cellulose composite coating on tablets prepared by compressing freeze-dried *Lactobacillus acidophilus*. The coated cells showed a 10^4-10^5-fold increase in cell survival compared with free cells under acidic gastric conditions. The authors suggested that the formation of a hydrogel barrier by the compacted sodium alginate layer retarded the permeation of the acidic fluid into the cells.

1.9 Hypothesis and overview of dissertation research

The overall hypothesis of this dissertation is that enteric composite coatings can be prepared from food biopolymers to protect probiotics in the enclosed pellet during preparation, storage, and simulated digestion. In the present study, probiotics pellets are prepared by direct compression of powdered probiotics. To increase the scalability without sophisticated equipment, the working hypothesis is that directly mixing a concentrated cell suspension (composed of 70-80% water) and dairy ingredient powders can be used to prepare powdered probiotics to enhance probiotic viability during storage and thermal treatment. The dairy ingredient powders prepared for the current research are spray-dried lactose (SDL), milk protein concentrate (MPC), and spray-dried whey protein isolate (WPI)/sucrose mixture powders (WSP). All these ingredients have been reported to function as protectants in drying media to protect survival of probiotics during dehydration (Chávez & Ledeboer, 2007; Ramos et al., 2018; Zhu et al., 2016). Furthermore, as amorphous SDL is metastable and hygroscopic, water is rapidly absorbed and lower the T_g and eventually becomes the chemically bound form after inducing

irreversible lactose crystallization (Lai & Schmidt, 1990; Price & Young, 2004). Dehydrated MPC is also hydroscopic and can bind with water initially on the polar groups, and additionally water layers can form progressively on the initial water layer (Kinsella & Fox, 1986; McSweeney & Fox, 2009). Amorphous sucrose can be stabilized by WPI during spray drying (Adhikari et al., 2009), and the prepared WSP, by utilizing water sorption properties of sucrose and WPI, may synergistically protect probiotics during powder preparation and storage when compared to sucrose or WPI alone.

To test the hypothesis, the viability, storage stability, physical properties of lactose-probiotics powders and physiological states of probiotic *Lactobacillus salivarius* NRRL B-30514 as affected by different lactose: water molar ratios were characterized in Chapter 2. Chapter 3 was conducted to study different mass ratios of MPC and SDL on physical properties of powdered *L. salivarius* to the significance in bacterial survival. In Chapter 4, WSP with different WPI:sucrose mass ratios was studied for the protection of powdered probiotics during storage and heating. WPI was studied to stabilize amorphous sucrose after spray drying, before mixing with *L. salivarius* suspensions. The WPI/sucrose-probiotics powders (WSPP) with the highest viability and thermal stability were subsequently used in Chapter 5 to evaluate the potential of modified rice protein (MRP)-ammonium shellac (NH_4SL) composite coatings in improving the viability of WSPP in millimeter-sized pellets during storage, thermal treatment, and simulated GI digestions. The working hypothesis in Chapter 5 is that enteric properties of shellac-based coating can be improved by incorporating MRP with the unique pH-dependent solubility (Wang et al., 2015). The possibility of preparing homogenous coating suspensions by stabilizing MRP in alkaline aqueous ethanol solutions of NH_4SL and physical, mechanical, and enteric delivery

properties of films casted from MRP-NH_4SL coating suspensions formulated with various MRP concentrations were studied to understand coating properties.

1.10 Acknowledgements

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Appendix

Table 1-1 Microorganisms used as probiotics and their reported health effects in human clinical trials (Adapted from (Ouwehand et al., 2002) with modification).

Classification	Genus	Species	Example strains	Health benefits
Lactic acid bacteria (LAB)	*Lactobacillus*	*acidophilus*	La5	Reduction of antibiotic-associated diarrhea
		casei	Shirota	Shortening of rotavirus diarrhea; immunomodulation
		plantarum	299v	Relief of irritable bowel disease syndrome
		rhamnosus	GG	Shortening of rotavirus diarrhea; immunomodulation; relief of inflammatory bowel disease; prevention of allergy
		salivarius	UCC118	Reduction of inflammatory bowel disease symptoms
	Bifidobacterium	*breve*		Reduction of irritable bowel disease symptoms
		lactis	Bb12	Treatment of allergy; shortening of rotavirus diarrhea; reduction of travellers diarrhea incidence
	Lactococcus	*lactis*		Improved mucosal vaccination
	Enterococcus	*durans*	LAB18s	Antipathogenic activity
	Streptococcus	*thermophilus*		Immunomodulation
Non-LAB	*Bacillus*	*subtilis*	2335	Treatment of acute enteric infections
	Escherichia	*coli*	Nissle 1917	Fewer relapses of inflammatory bowel disease
	Saccharomyces	*cerevisiae*	boulardii	Fewer relapses of inflammatory bowel disease

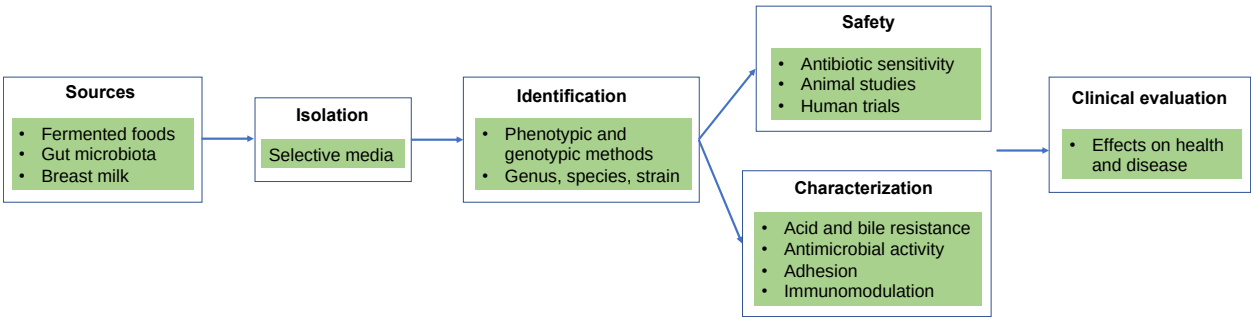

Figure 1-1 Flow chart describing the sequential steps required for qualifying a bacteria strain as a novel probiotic (Adapted from (Fontana et al., 2013) with modification).

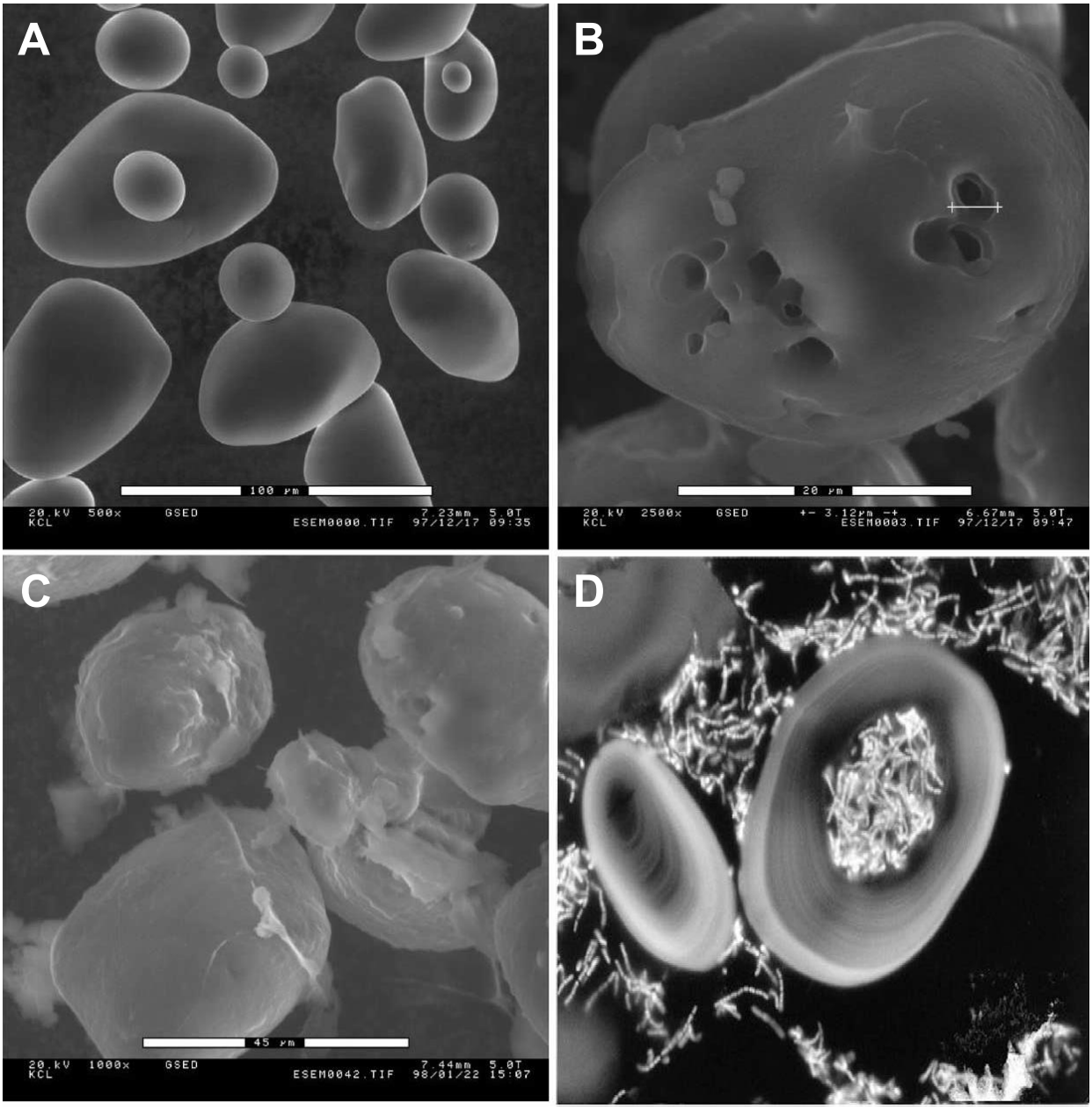

Figure 1-2 Scanning electron micrographs of potato starch granules (A), hydrolyzed potato starch granules with pores on the surface (B), amylose-coated potato starch granules (C), and the cross-section of *Lactobacillus rhamnosus*-entrapped potato starch granules (D) (Adapted from (Mattila-Sandholm et al., 2002)).

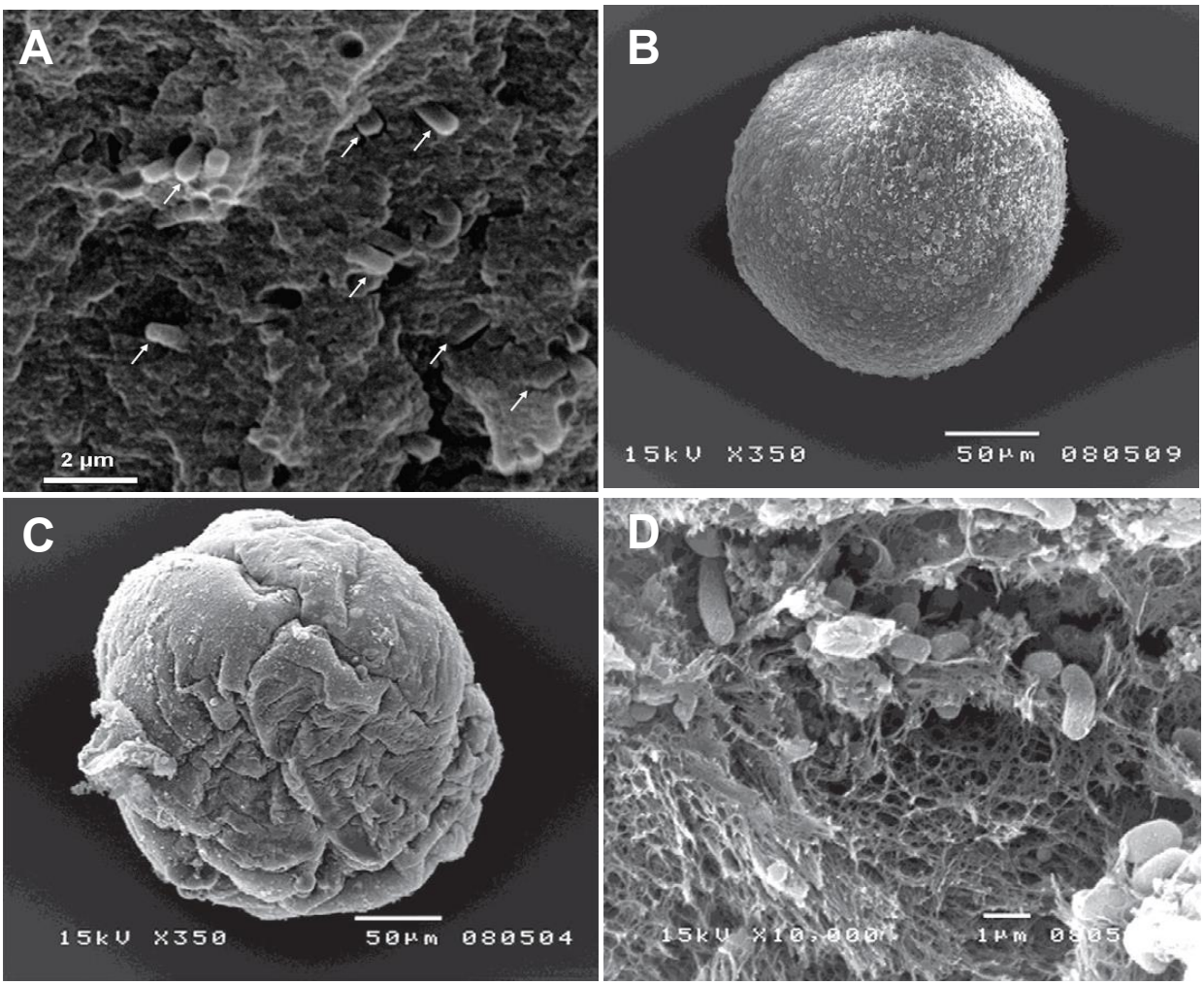

Figure 1-3 Scanning electron micrographs of the cross-section of transglutaminase-induced casein gels (A). Arrows in A highlight the encapsulated Lactobacillus F19 randomly distributed in the gel network (Adapted from Heidebach et al. 2012 (Heidebach et al., 2012)). SEM images of alginate-zein core-shell microcapsules without (B) and with (C) the shell layer, and the cross-section (D) of microcapsules with encapsulated *Lactobacillus acidophilus.* (Adapted from (Laelorspoen et al., 2014)).

Chapter 2 Probiotic powders prepared by mixing suspension of *Lactobacillus salivarius* NRRL B-30514 and spray-dried lactose: physical and microbiological properties

A version of this chapter was originally published by Anyi Wang, Jun Lin, and Qixin Zhong:

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My primary contributions to this paper include sample preparation, data collection and analysis, results interpretation and writing.

2.1 Abstract

Preparation of powdered probiotics is important for storage and application. In this work, a novel method to prepare powdered probiotic ingredients was studied by mixing a *Lactobacillus salivarius* NRRL B-30514 suspension with amorphous spray-dried lactose at suspension: lactose (v:w) ratios (SLR) of 1:5, 1:15, and 1:25. The simple procedure resulted in lactose-probiotics powders (LPPs) with greater probiotic viability initially and during subsequent 6-month storage at a smaller SLR. In LPPs with SLRs of 1:5 and 1:15, X-ray diffraction spectroscopy and scanning electron microscopy results indicated the formation of lactose crystals, and *Bac*Light® assay suggested the significantly lowered membrane integrity of probiotics due to hypertonic pressure of lactose dissolved in excessive water. A viable but non-culturable state of *L. salivarius* in LPPs may exist based on the *Bac*Light® and CTC reduction assays. The present study may provide a novel approach to prepare powdered probiotic ingredients.

Keywords: probiotics, dehydration, spray-dried lactose, viability, storage

2.2 Introduction

Probiotics are live microorganisms that confer health benefits on a host when administered in adequate amounts (FAO/WHO, 2001). The beneficial effects of probiotics in humans include anti-pathogenic action within the human gut flora, enhanced immune responses to fight tumors, and alleviated intestinal barrier dysfunctions such as symptoms of inflammatory bowel diseases and diarrhea (Ash & Mueller, 2016; Homayoni Rad et al., 2016). The rising consumption and variety of functional food products containing probiotic bacteria call for technologies to fortify foods with an adequate number of viable cells to confer specific health benefits of any probiotic product (Ramos et al., 2018). It is generally accepted that a minimum number of viable probiotic bacteria within a food matrix should reach 10^6-10^7 CFU/g in order to be labeled as "contains live cultures" (Bertazzoni et al., 2013). This requires feasible probiotic ingredients convenient for production. As probiotics in liquid preparations face environmental stresses related to pH, temperature, oxygen, and water activity (a_w), the powdered probiotic ingredients may be more suitable to obtain stability during production, storage, transportation, and consumption (Fu et al., 2018; Liu et al., 2017).

Currently, industrial preparation of powdered probiotics is commonly done with spray or freeze drying (Dianawati et al., 2016; Liu et al., 2017). In freeze drying, a sample is frozen, followed by subsequent sublimation of water (Broeckx et al., 2016). Whereas, in spray drying, a sample is dehydrated by evaporation of water using hot air. However, structural and functional damages and further cell mortality induced by thermal stresses and water removal are still the critical limitations of these conventional dehydration methods (Hlaing et al., 2017; Iaconelli et al., 2015). Temperature-induced shocks, heating or freezing, lead to damages of cellular

structures and defunctionalization of cellular substances such as proteins, lipids and nucleic acids (Fiocco et al., 2019; van de Guchte et al., 2002). In addition, the osmotic stress resulting from the removal of water bound to cells can force a phase transition of membrane lipids from a liquid crystalline state to a gel state, which ultimately gives rise to membrane leakage and cell mortality during rehydration (Fonseca et al., 2019). Other stresses due to oxidation and acidity during dehydration can also cause cellular injuries and the loss of probiotic viability (Franca et al., 2007; Liu et al., 2017).

Therefore, approaches such as optimization of drying parameters, addition of thermal or cryo-protectants, and development of novel drying methods have been widely studied to reduce loss of probiotic viability after dehydration (Liu et al., 2017). For example, reducing the outlet temperature of spray drying from 100 to 70 °C led to a 2.4 log CFU/g increase of *Lactobacillus salivarius* NRRL B-30514 viability, and achieving a sufficiently low a_w is critical to maintain viability during storage (Zhang et al., 2016). Carbohydrates (e.g. trehalose, glucose, and lactose) and proteins (e.g. whey protein and casein) were reported to be good probiotic protectants during spray or freeze drying (Chen et al., 2017). However, along with the improved bacterial survival, undesired consequences can occur, such as lowered powder yield by reducing drying temperature and increased material costs of unconventional protectant ingredients (Liu et al., 2017).

The hypothesis of the present work is that mixing amorphous lactose and a concentrated cell suspension can be used to form chemically bound water to prepare probiotic powders with low a_w and therefore good viability during storage. A concentrated cell suspension is composed of ~70-80% water, and the amount of amorphous lactose is expected to influence the physical state

of lactose and consequently the viability and biophysical states of probiotics. Metastable amorphous disaccharides, such as trehalose, sucrose, and lactose, are very hygroscopic. With exposure to high relative humidity (RH > 50%), the absorbed water acts as a plasticizer to facilitate the molecular mobility of disaccharides and induce an irreversible transition from amorphous to stable crystalline structures (Afrassiabian et al., 2019). Lactose is an economical carbon source recovered from dairy by-products (whey), and amorphous lactose can be prepared by spray drying a lactose solution (Shi & Zhong, 2015). Lactose also functions as an efficient protectant during dehydration of probiotics, because its hydroxyl groups interact with the phosphate head groups at the surface of cellular bilayers to replace hydrogen bonds initially formed with water that is lost during dehydration (Vaessen et al., 2019). However, to date, this straightforward hypothesis has not been tested.

The first objective of this work was to study the viability and storage stability of powdered *L. salivarius* NRRL B-30514 after mixing a concentrated cell suspension with spray-dried lactose at various ratios. The second objective was to characterize physical properties of lactose-probiotics powders (LPPs) and physiological states of the bacteria as affected by different lactose: water molar ratios. *L. salivarius* NRRL B-30514 was chosen as a model probiotic strain because it has been identified as a probiotic bacterium (Messaoudi et al., 2013) and applied in our previous encapsulation and spray drying studies (Zhang et al., 2015; 2016). This is the first study producing powdered bacterial ingredients by mixing a cell suspension with an amorphous water-binding carbohydrate at ambient conditions. The simple procedures eliminate the need of sophisticated equipment and the thermal deactivation of probiotics, which may be developed into

a novel approach to prepare powdered probiotic ingredients. The powder, however, may not be used to serve lactose-intolerant consumers.

2.3 Materials and methods

2.3.1 Materials

α-Lactose monohydrate recovered from bovine milk was a kind gift from Leprino Foods (Denver, CO, USA). de Man, Rogosa, and Sharpe (MRS) broth medium and agar (dehydrated) were from Oxoid Ltd (Altrincham, Cheshire, England). Unless stated otherwise, other chemicals were purchased from either Sigma-Aldrich Corp. (St. Louis, MO, USA) or Thermo Fisher Scientific, Inc. (Pittsburgh, PA, USA).

2.3.2 Preparation of spray-dried lactose

Lactose was dissolved in deionized (DI) water at 10 g/100 mL. The solution was then spray dried as reported previously (Zhang et al., 2015) with minor modifications. A Buchi-B290 Mini Spray dryer (BÜCHI Corporation, Flawil, St. Gallen, Switzerland) was used at an inlet temperature of 170 °C, an outlet temperature of 95-100 °C, a pump rate of 15%, and an aspirator setting of 100% (38 m^3/h). The spray-dried lactose powder was immediately collected and stored in a desiccator at ambient conditions before further use.

2.3.3 Bacterial strain and culture preparation

All glassware, centrifuge tubes, pipette tips, and solutions used in this study were sterilized at 121 °C for 15 min. Frozen stock culture (20 µL) of *L. salivarius* NRRL B-30514 (Department

of Animal Science at the University of Tennessee, Knoxville, TN, USA) in MRS broth with 33.33% v/v glycerol was recovered in 5 mL fresh MRS broth at 37 °C for 18 h under anaerobic conditions. Anaerobic conditions were achieved using an anaerobic jar and GasPak™ EZ anaerobe container system sachets with indicators (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The recovered *L. salivarius* was then incubated in 100 mL MRS broth using above conditions to reach the late-exponential growth phase. Cells were subsequently harvested by centrifugation at 4500 *g* for 30 min (Sorvall ST 16R, Thermo Scientific Company, Waltham, MA, USA) at 4 °C and washed twice with a phosphate-buffered saline (PBS, pH 7.4), with centrifugation at above conditions in each step. The washed pellets were diluted with 250 µL PBS to a final cell concentration of about 10^{10} CFU/mL.

2.3.4 Preparation of lactose-probiotics powders (LPPs)

The *L. salivarius* suspension (10^{10} CFU/mL) was mixed with spray-dried lactose powder at suspension: lactose (v:w) ratios (SLRs) of 1:5, 1:15, and 1:25, using a protocol at ambient conditions, with steps of a food blender (Osterizer galaxie, Oster Inc., Fort Lauderdale, FL, USA) for 20 s, a mortar for 5 min, and a coffee grinder (Hamilton beach, Hamilton Inc., Glen Allen, VA, USA) for 20 s to improve powder homogeneity. The LPPs at day 0 were sampled within 30 min after mixing. The remainder powders were sealed in zip-lock bags that were placed in desiccators and stored at room temperature (RT) or 4 °C for up to 6 months. Three independent replications were conducted for each formulation.

The mass yield of LPPs was calculated using Eq. (1). To evaluate homogeneity of bacterial distribution, LPPs in each zip-lock bag were randomly sampled for 3 locations to calculate the coefficient of variation (CV) from 9 total enumeration results.

$$\text{Mass yield (\%)} = \frac{\text{Mass of LPPs in ziplock bag (g)}}{\text{Mass of lactose powder (g)+Mass of cell suspension (g)}} \times 100 \quad (1)$$

where the mass of added cell suspension was calculated using a density of 1.03 g/mL estimated gravimetrically.

2.3.5 Calculation of theoretical lactose:water molar ratio of LPPs

The theoretical lactose:water molar ratio of LPPs was calculated from Eq. (2).

$$\text{Lactose: water molar ratio} = \frac{\text{Mass of lactose (g)}/342.3\ (\text{g}\cdot\text{mol}^{-1})}{(\text{Mass of cell suspension (g)} \times \text{water content (\%wb)})/18.0\ (\text{g}\cdot\text{mol}^{-1})} \quad (2)$$

where the mass of cell suspension was calculated using a density of 1.03 g/mL estimated gravimetrically.

In order to measure the water content of cell suspension, about 0.3 mL cell suspension was weighed and dried at 100 °C in a model Precision 6958 convection oven (Thermo Scientific, Inc., Waltham, MA, USA) for 24 h as reported previously (Shi & Zhong, 2015). The wet-basis (wb) water content of *L. salivarius* suspension was calculated from Eq. (3). Two independent replicates (n = 2) were measured twice each.

$$\text{Water content (\%wb)} = \frac{\text{Mass before drying (g)} - \text{Mass after drying (g)}}{\text{Mass before drying (g)}} \times 100 \quad (3)$$

2.3.6 Enumeration of L. salivarius

Bacteria were enumerated using the spread plating method. A cell suspension was serially diluted in PBS and then plated on MRS agar. The plates were anaerobically incubated at 37°C for 24 h before counting colonies. For powdered *L. salivarius*, 0.100 g of a LPP sample was suspended in 10.0 mL PBS, followed by enumeration as the cell suspension.

2.3.7 Viability of powdered L. salivarius during storage

Viable cells in LPPs after storage in desiccators at RT or 4 ºC in a walk-in cooler for 10, 20, 30, 90, and 180 days were enumerated with the method presented in section 2.6.

2.3.8 Water activity measurement

The a_w of spray-dried lactose and LPPs stored at RT or 4 °C in a walk-in cooler for 0, 10, 20, 30, 90, and 180 days was measured using a model Aqualab Series 3 meter (Decagon Devices Inc., Pullman, WA, USA).

2.3.9 Physical and biological properties of lactose-probiotics powders

Fresh LPPs prepared with different SLRs were placed in a desiccator for 12 h at RT before following characterizations. Twelve hours were observed to be sufficient for lactose in LPPs to complete crystallization because no significant changes of crystallinity ($P > 0.05$) were observed with prolonged storage time (data not shown).

2.3.9.1 Scanning electron microscopy (SEM)

The morphology of spray-dried lactose and LPPs was characterized using SEM. Powders were glued onto an adhesive tape mounted on a specimen stub and then coated with gold to avoid charging in the microscope. Imaging was performed with a LEO 1525 SEM microscope (SEM/FIB Zeiss Auriga, Oberkochen, Germany) at 15-20 K times of magnifications.

2.3.9.2 X-ray diffraction spectroscopy (XRD)

X-ray diffraction patterns of powders were characterized using a model Empyrean 2 diffractometer (PANalytical Inc., Westborough, WA, USA) with Ni-filtered Cu-Kα radiation (45 kV, 40 mA). The measurement conditions included a 2θ scanning range of 5-35°, a step size of 0.013°, and a scanning speed of 0.05°/s. The evaluation of the data was conducted with X`Pert HighScore® software (PANalytical Inc., Westborough, WA, USA). The crystallinity of lactose was evaluated using the profile fitted area under the peak at 2θ of 12.4°, because it is the characteristic peak of recrystallized lactose, mainly α-lactose monohydrate (Fu et al., 2019). Crystallinity of LPPs was determined using the calibration curve, based on the profile fitted areas under 2θ of 12.4° for spray-dried lactose (0% crystallinity) and α-lactose monohydrate (100% crystallinity) mixtures of different mass ratios (Fix & Steffens, 2004).

2.3.9.3 LIVE/DEAD® BacLight™ assay for bacterial membrane integrity

A LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit L7012 (Life Technologies Corp., Eugene, OR, USA) was used to evaluate bacterial membrane integrity (Oliver, 2005). The standard curve for analyzing relative viability of *L. salivarius* suspensions in a Synergy 2 multi-mode reader (BioTek Instruments Inc., Winooski, VT, USA) was established according to the Fluorescence Microplate Readers protocol of Molecular Probes (Anonymous, 2004). For *L. salivarius* in powdered samples, 0.100 g of LPPs prepared at a SLR of 1:5 or 1.00 g of LPPs prepared at SLRs of 1:15 and 1:25 was suspended in 10.0 mL of 0.85% NaCl solution (~pH 6.0) to a cell concentration of about 1×10^7 CFU/mL, followed by centrifugation at 4500 *g* for 10 min at 4°C and resuspension of pellets in 10.0 mL of 0.85% NaCl to minimize the hypertonic

pressure of dissolved lactose. The bacteria were then stained and evaluated following the protocol (Anonymous, 2004).

2.3.9.4 CTC reduction assay for bacterial respiratory activity

A redox probe 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Polyscience Inc., Warrington, PA, USA) was used to evaluate the bacterial respiratory activity (Oliver, 2005). Bacterial suspensions were prepared using the same procedures in section 2.9.3, except that PBS was used to replace the 0.85% NaCl solution. Subsequently, 0.100 mL of 50 mM CTC working solution was added in 1.00 mL *L. salivarius* suspension and gently vortexed. After incubation in a model I24 incubator shaker (New Brunswick Scientific Co., Enfield, CT, USA) with a cover for 2 h at 37 °C and 150 rpm, the bacteria were semi-quantitatively analyzed using a MACSQuant® Analyzer 10 flow cytometer (Miltenyi Biotec Inc., Auburn, CA, USA). Viable *L. salivarius* cells (1×10^7 CFU/mL) without staining were used as the control.

2.3.10 Statistical analysis

Means and standard deviations (SD) were calculated from three independent LPPs replicates. One-way analysis of variance (ANOVA) was preformed using SAS (version 9.4, SAS Institute, Cary, NC, USA). Fisher's least significant-difference (LSD) test was used to compare differences of means at a significance level of 0.05.

2.4 Results and discussion

2.4.1 Mass yield and homogeneity of LPPs

The mass yield of LPPs prepared with different SLRs was all greater than 93%, and the CV of bacterial distribution in LPPs was all less than 5% (Table 2-1). The data indicate that most mass was collected after the adopted procedures and the developed protocol was efficient to prepare powders with evenly distributed *L. salivarius* (Nielsen, 2010).

2.4.2 Crystallinity of LPPs

The crystallinity of lactose in LPPs was studied using XRD, with diffractograms shown in Figure 2-1. No crystalline peak was observed in spray-dried lactose, indicating the amorphous nature of lactose after spray drying. After mixing spray-dried lactose with cell suspensions, all LPPs showed crystalline structures. The crystallinity of lactose in LPPs estimated from XRD is summarized in Table 2-1. LPPs prepared with SLRs of 1:5 and 1:15 showed significantly higher ($P < 0.05$) crystallinity than that with a SLR of 1:25. As shown in Table 2-1, the lactose: water molar ratio in the LPPs prepared with SLRs of 1:5 and 1:15 is over 1, which favors the formation of lactose monohydrate and therefore crystallization (Schuck et al., 2012). The incomplete crystallization (~86%) of these two LPPs is in agreement with another study (Shetty et al., 2018), because lactose recrystallization is initiated on the powder particle surface and the crystallized shell might impede the absorption of sufficient water for the crystallization of inner amorphous lactose.

2.4.3 Viability of L. salivarius in LPPs after preparation

The viability of *L. salivarius* in LPPs after preparation (day 0) is shown in Table 2-2. The simple mixing procedures resulted in higher viable bacterial counts in LPPs prepared with a smaller SLR. At day 0, the viability of *L. salivarius* in LPPs prepared with a SLR of 1:5 showed more than 0.5 log CFU/g lower than the other two SLRs, which may be due to the hyperosmotic pressure of lactose dissolved in excessive water. It was demonstrated that sugar stress was less detrimental, but a sudden increase of the hypertonic pressure imposed by high sugar concentrations did result in a detrimental change of cellular volume and membrane integrity (De Angelis & Gobbetti, 2004). In addition, lactose in LPPs prepared with a SLR of 1:25 was mostly in the glassy state (low crystallinity %; Table 2-1) and the high viscosity of amorphous lactose can restrict molecular motility and interaction and thus improve bacterial viability (García, 2018).

2.4.4 Viability of L. salivarius in LPPs during storage

The viability of *L. salivarius* in LPPs during storage at 4 °C and RT in a desiccator was determined for up to 180 days (Table 2-2). The viable *L. salivarius* in LPPs prepared with a SLR of 1:5 reduced to a level below the detection limit after 10-day storage at both 4°C and RT. In contrast, *L. salivarius* in LPPs prepared with SLRs of 1:15 and 1:25 had much improved stability, showing respective reductions of only 0.49 and 0.56 log CFU/g after 180-day storage at 4 °C, but becoming undetectable after 90-day storage at RT.

Differences in the storage stability of probiotics in powders can be correlated to a_w (Liu et al., 2017). The freshly prepared LPPs with a SLR of 1:5 had a_w of 0.88 (Table 2-3), indicating

water mobility is high enough to activate bacterial metabolism and thus leads to probiotic mortality due to high hyperosmotic pressure of dissolved lactose (Tripathi & Giri, 2014). For LPPs prepared with the other two SLRs, the significantly lower ($P < 0.05$) a_w limits free water molecules available for cellular metabolic activities and therefore maintains probiotic viability during long-term storage. The a_w of these two treatments, around 0.3 (Table 2-3), is only slightly higher than the recommended a_w range (0.001-0.25) used for long-term storage of dry foods containing live probiotics (Teijeiro et al., 2018), and the minor difference can be contributed to different probiotic strains and dehydration mechanisms.

In addition, the survival of *L. salivarius* in LPPs prepared with SLRs of 1:15 and 1:25 became undetectable on days 90 and 180, respectively, after storage at RT but showed insignificant ($P > 0.05$) changes when stored at 4 °C (Table 2-2). Peredo *et al* (2016) also reported that the viability of *L. plantarum* (Lp33) encapsulated using potato starch as a prebiotic was more than 1 log CFU/g higher after 30 days when stored at 4 ºC than at 22 ºC. The lower temperature favoring the probiotic stability during storage mainly results from the lowered metabolic activities of bacteria at a decreased temperature (Albadran et al., 2015). Data in Table 2-2 and Table 2-3 suggest that a_w is a critical parameter determining short-term viability of probiotics in LPPs and is to be combined with storage temperature to obtain long-term storage viability.

2.4.5 Morphology of LPPs

SEM images of spray-dried lactose and LPPs are shown in Figure 2-2. Spray-dried lactose had a spherical shape with a diameter between 1 and 10 μm and displayed a smooth and intact surface, as reported previously (Shi & Zhong, 2015). In contrast, SEM micrographs of LPPs

prepared with 1:5 and 1:15 SLRs clearly showed crystalline structures, which are similar to α-lactose monohydrate crystals (Pawar et al., 2018). The morphology of LPPs prepared with a 1:25 SLR ratio was similar to that of spray-dried lactose (Figure 2-2D). The SEM data in Figure 2-2 further confirmed the XRD results about lactose crystallinity as affected by SLRs (Figure 2-1; Table 2-1). Furthermore, the small and convex bulges with a size of about 600-700 nm on the surface of LPPs prepared with an SLR ratio of 1:25 (Figure 2-2D), in reference to the smooth surface of spray-dried lactose (Figure 2-2A), can be speculated as the adhering *L. salivarius* (Khem et al., 2016).

The visual appearance of LPPs is shown in Figure 2-3. The LPPs prepared with a SLR of 1:25 was similar to that of spray-dried lactose with macroscopic clumps (Figure 2-3A,B). A high extent of amorphous lactose can quickly absorb moisture from the environment to cause sticking of particles to form agglomerates (Shi & Zhong, 2015). Formation of lactose crystals (Figure 2-2B,C) and structural rearrangements of LPPs prepared with SLRs of 1:5 and 1:15 agreed with macroscopic caking (Figure 2-3C,D).

2.4.6 Biophysical states of L. salivarius in LPPs

In order to elucidate the biophysical states of *L. salivarius* in LPPs, cellular membrane integrity and metabolic activity were characterized and compared with plate-counting enumeration results. The LIVE/DEAD® *Bac*Light™ assay kit is composed of two nucleic acid stains, one of which is green-fluorescent SYTO® 9, and the other is red-fluorescent propidium iodide (PI). The SYTO 9 generally stains all cells, while PI can only penetrate and label bacterial cells with a damaged membrane, causing a fluorescence reduction of SYTO 9 stain (Anonymous, 2004). Based on this principle, LIVE/DEAD® *Bac*Light™ assay kit has been

widely used to study cellular membrane integrity and viable cell counts (Kumar & Ghosh, 2019). As shown in Figure 2-4, the counts of viable *L. salivarius* in LPPs detected by *Bac*Light™ demonstrated a similar trend with those by plate-counting enumeration, verifying the higher viable bacterial counts in LPPs prepared at a smaller SLR. In addition, *Bac*Light™ assay suggested significantly lower ($P < 0.05$) membrane integrity of *L. salivarius* in LPPs prepared at a SLR of 1:5 (10.0%) than at SLRs of 1:15 (31.4%) and 1:25 (39.9%), which can be attributed to the effect of hypertonic pressure of dissolved lactose damaging cytoplasmic membrane as discussed in section 3.3. Another interesting phenomenon was that the percentages of live *L. salivarius* detected by *Bac*Light™ was higher than those by the direct enumeration, indicating possible existence of a viable but non-culturable (VBNC) state of *L. salivarius* in LPPs.

Bacteria in the VBNC state typically fail to grow on conventional culture media but have other measurable characteristics such as cellular integrity and metabolic activities to indicate cells are still alive (Ayrapetyan & Oliver, 2016). To verify the occurrence of the VBNC state of *L. salivarius* in LPPs, the CTC reduction assessment was used to detect the respiratory activity of *L. salivarius* in LPPs. The assay is based on the principle that CTC, a commonly used redox indicator, can be absorbed by viable cells and reduced via bacterial respiring into an insoluble and red-fluorescent formazan (Anonymous, 2005). The viable bacterial counts detected in the CTC assay showed a similar trend as the *Bac*Light™ assay and were significantly higher ($P < 0.05$) than those enumerated by the pour plate method (Figure 2-4). Therefore, *L. salivarius* after being mixed with spray-dried lactose can exist at the VBNC state to result in lower viable cell counts in conventional plating assays. In addition, the percentages of live bacteria detected in the CTC assay were significantly lower ($P < 0.05$) than those detected in the *Bac*Light™ assay,

suggesting a portion of dormant *L. salivarius* in LPPs which had intact cellular membranes but suppressed metabolic activities (Pinto et al., 2015).

Conditions inducing the VBNC state of bacteria are highly dependent on specific bacterial strains and have been studied mostly for pathogenic bacteria, such as *Escherichia coli* O157: H7 (Zhang et al., 2018) and *Listeria monocytogenes* (Robben et al., 2018), and occasionally for probiotic strains, such as *L. rhamnosus* (Chiron et al., 2018) and *Bacillus coagulans* (Majeed et al., 2018). Cells usually enter the VBNC state as a response to environmental shocks, such as starvation, thermal change, osmotic pressure, and radiation (Rowan et al., 2015). Similarly, the hypertonic shock or low a_w induced during mixing a cell suspension with spray-dried lactose powder in the present study can induce the VBNC state of *L. salivarius*. The VBNC state of powdered *L. salivarius* has the promising significance to maintain viability during storage and possibly regain physiological functions after consumption.

2.5 Conclusion

In summary, powdered *L. salivarius* can be prepared by simply mixing a cell suspension with spray-dried lactose, and the amount of cell suspension influenced physical properties of lactose in LPPs and the viability of *L. salivarius*. A smaller SLR resulted in a lower hypertonic stress and therefore greater viable bacterial counts initially and during subsequent storage. Lowering the storage temperature from RT to 4 °C further improved the survivability of *L. salivarius*, with the 4 °C treatments showing insignificant changes during 180-day storage. The hypertonic stress and reduced a_w during the mixing procedure appeared to have induced the VBNC state of *L. salivarius* in LPPs, with the mechanisms and possible physiological functions to be studied. Nevertheless, the presented mixing protocol consisting of simple procedures and

equipment may be significant to preparing probiotic ingredients to facilitate the development of functional foods. Future studies, however, are needed to explore the viability of probiotics after reconstitution, including the possibility of recovering from the VBNC state, *in vitro* and *in vivo*.

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Appendix

Table 2-1 Mass yield, homogeneity of bacteria (in coefficient of variation), theoretical lactose: water molar ratio, and crystallinity % estimated in X-ray diffraction spectroscopy of lactose-probiotics powders prepared by mixing a *L. salivarius* cell suspension with spray-dried lactose at different volume: weight ratios

Cell suspension: lactose (v:w)	Mass yield (%) *	Coefficient of variation (%)	Lactose: water molar ratio	Crystallinity % *,**
1:5	93.74±2.14[b]	4.89	1:3.4	86.82±0.34[a]
1:15	97.56±1.02[a]	1.77	1:1.1	86.11±0.20[a]
1:25	98.89±0.87[a]	1.48	1:0.7	9.40±1.07[b]

* Numbers are mean ± SD (n = 3). Different superscript letters indicate significant differences in the mean of all samples ($P < 0.05$).

** Fresh lactose-probiotics powders were placed in a desiccator for 12 h at RT before XRD measurement.

Table 2-2 Viable cell counts of powders prepared by mixing different cell suspension: spray-dried lactose (v:w) ratios during 180-day storage at 4 °C or room temperature (RT, ~21 °C) in desiccators.

Cell suspension: lactose (v:w)		Viable cell count (Log CFU/g) *					
		Day 0	Day 10	Day 20	Day 30	Day 90	Day 180
1:5	4°C	6.83±0.33^{ab}	<DL**	<DL	<DL	<DL	<DL
	RT		<DL	<DL	<DL	<DL	<DL
1:15	4°C	7.36±0.13^{ab}	7.56±0.27^{a}	7.29±0.48^{ab}	6.93±0.71^{ab}	6.89±0.27^{ab}	6.87±0.31^{ab}
	RT		7.54±0.42^{ab}	7.13±0.54^{ab}	6.74±0.60^{b}	<DL	<DL
1:25	4°C	7.45±0.11^{ab}	7.57±0.26^{a}	7.44±0.35^{ab}	7.09±0.50^{ab}	6.95±0.13^{ab}	6.89±0.27^{ab}
	RT		7.50±0.20^{ab}	7.13±0.31^{ab}	6.79±0.44^{ab}	<DL	<DL

* Numbers are mean ± SD (n = 3). Means with different superscript letters indicate significant differences of all treatments ($P < 0.05$).

** Below the detection limit (DL) of 3.00 log CFU/g.

Table 2-3 Water activity of powders prepared by mixing different cell suspension: spray-dried lactose (v:w) ratios during 180-day storage at 4 °C or room temperature (RT, ~21 °C) in desiccators.

Cell suspension: lactose (v:w)		Water activity *					
		Day 0	Day 10	Day 20	Day 30	Day 90	Day 180
1:5	4°C	0.88±0.03^{a}	0.88±0.04^{a}	0.44±0.05^{b}	0.36±0.23^{bc}	0.34±0.08^{bc}	0.33±0.04^{bc}
	RT		0.43±0.09^{b}	0.37±0.07^{bc}	0.32±0.19^{bc}	0.32±0.10^{bc}	0.30±0.06^{bc}
1:15	4°C	0.32±0.08^{bc}	0.33±0.10^{bc}	0.38±0.08^{bc}	0.34±0.13^{bc}	0.33±0.07^{bc}	0.33±0.02^{bc}
	RT		0.27±0.05^{bc}	0.27±0.13^{bc}	0.28±0.12^{bc}	0.33±0.05^{bc}	0.30±0.05^{bc}
1:25	4°C	0.24±0.08^{c}	0.26±0.08^{bc}	0.33±0.06^{bc}	0.31±0.11^{bc}	0.34±0.10^{bc}	0.32±0.05^{bc}
	RT		0.22±0.04^{c}	0.24±0.09^{c}	0.24±0.06^{c}	0.33±0.00^{bc}	0.33±0.02^{bc}

* Numbers are mean ± SD (n = 3). Means with different superscript letters indicate significant differences of all treatments ($P < 0.05$).

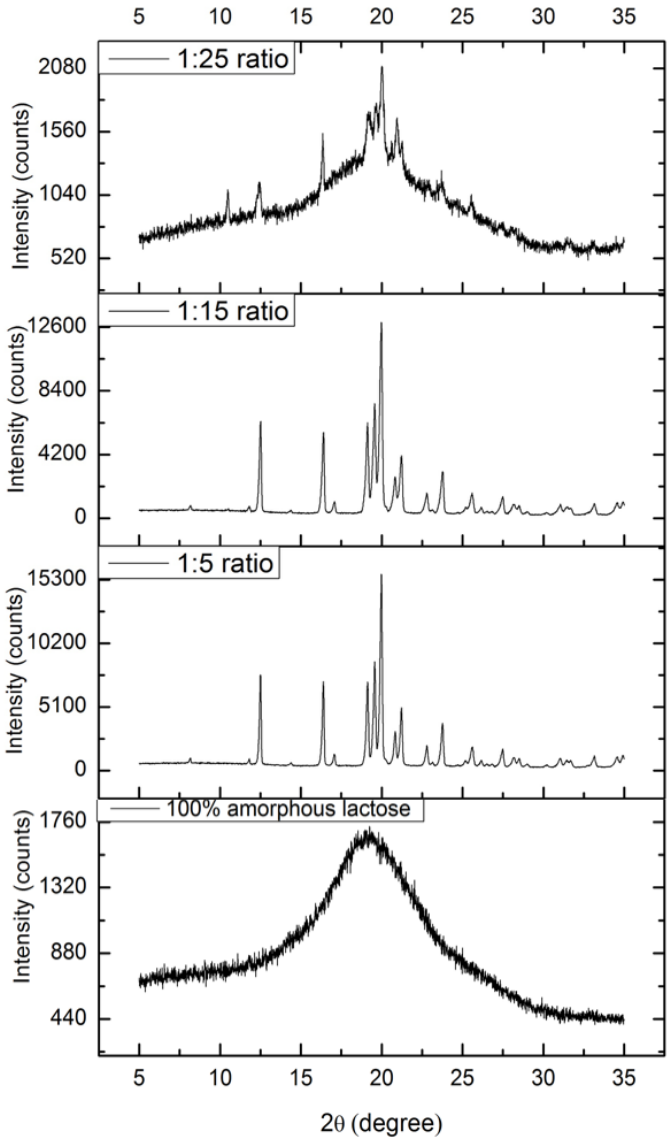

Figure 2-1 X-ray diffractograms of spray-dried lactose and powders prepared with cell suspension: lactose (v:w) ratios of 1:5, 1:15, and 1:25.

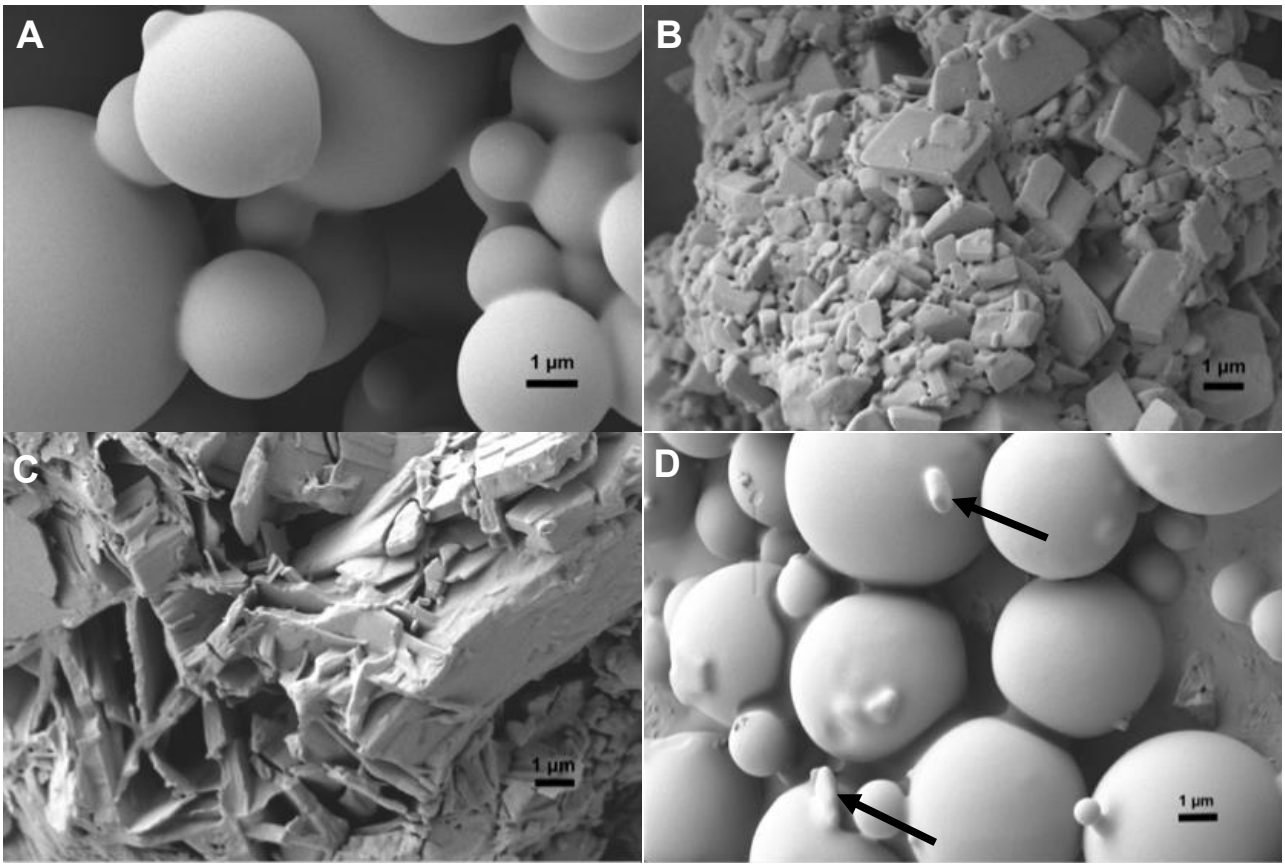

Figure 2-2 Scanning electron micrographs of freshly prepared spray-dried lactose (A) and powders prepared with cell suspension: lactose (v:w) ratios of 1:5 (B), 1:15 (C), and 1:25 (D). Arrows in D highlight possible *L. salivarius* cells. Scale bar = 1 µm.

Figure 2-3 Appearance of freshly prepared spray-dried lactose (A) and powders prepared with cell suspension: lactose (v:w) ratios of 1:25 (B), 1:15 (C), and 1:5 (D).

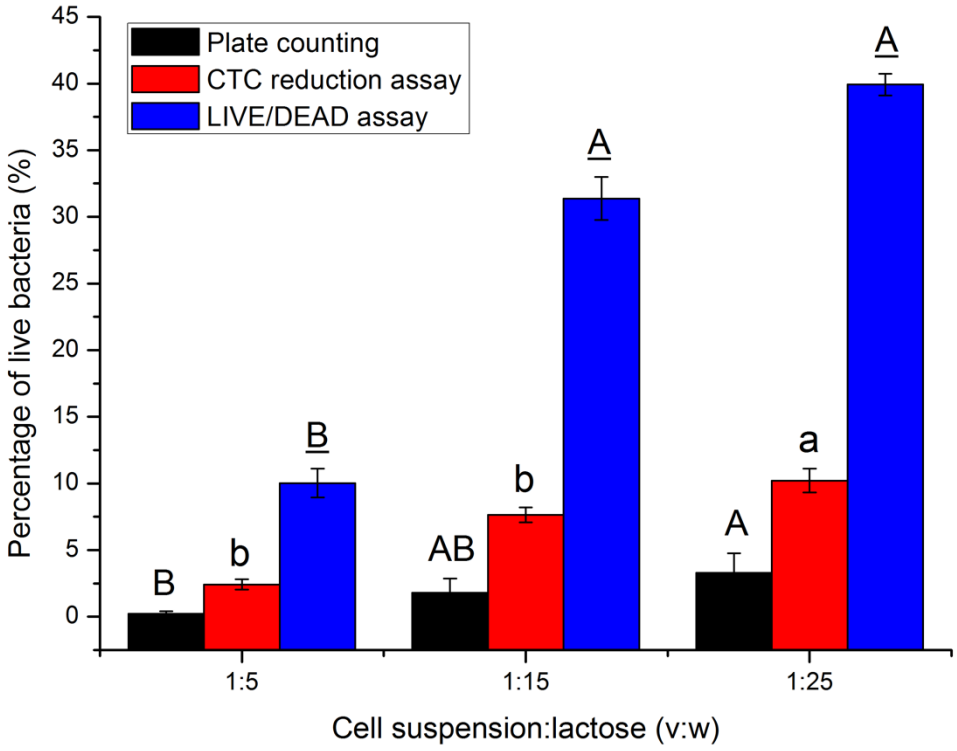

Figure 2-4 Percentages of viable *L. salivarius* in the powders prepared with cell suspension: lactose (v:w) ratios of 1:5, 1:15, and 1:25 as determined using plate counting, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) reduction, and LIVE/DEAD BacLight™ assays. Fresh powders were placed in a desiccator for 12 h at RT before the determinations. Fresh cell suspensions without addition of probiotics powder were used to obtain measurements corresponding to 100% live bacteria. Error bars are SD (n = 3). Different letters above bars with the same color indicate significant differences ($P < 0.05$) among different treatments assessed with the same method.

Chapter 3 Physical and microbiological properties of powdered *Lactobacillus salivarius* NRRL B-30514 as affected by relative amounts of dairy proteins and lactose

A version of this chapter was originally published by Anyi Wang, Jun Lin, and Qixin Zhong:

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My primary contributions to this paper include sample preparation, data collection and analysis, results interpretation and writing.

3.1 Abstract

The objective of this study was to characterize physical and microbiological properties of powders prepared by mixing *Lactobacillus salivarius* NRRL B-30514 suspensions with skim milk powder (SMP), spray-dried lactose (SDL), milk protein concentrate (MPC), or MPC/SDL at a mass ratio of 1:2, 1:1 or 2:1 to understand the relative significance of proteins and lactose in bacterial survival. The probiotic viability and storage stability were significantly improved with the increase of dairy protein content. Based on water sorption isotherms and X-ray diffraction spectroscopy, MPC was suggested to preferentially absorb water in cell suspensions, which inhibited the hydration of SDL and therefore lowered the hypertonic pressure to the adhered cells. The LIVE/DEAD® *Bac*Light™ and CTC reduction assays detected higher membrane integrity and respiratory activity of bacteria for treatments with more proteins. Findings from the current study indicated the more significant role of milk proteins than lactose protecting bacteria during dehydration.

Keywords: probiotics powder, survival, protectants, dairy ingredients, biophysical states

3.2 Introduction

Functional foods fortified with probiotics have shown rising consumption and popularity over the last decade (Heidebach et al., 2012). Supplementing an adequate amount of beneficial probiotics in a food matrix (10^6~10^7 CFU/g) may improve intestinal microbial balance, alleviate lactose intolerance, and enhance immunological and digestive functions of the host (FAO/WHO, 2001; Sanders & Marco, 2010). Dairy products, such as yogurt and yokult, are the most popular food carriers of probiotics (Dianawati et al., 2016). However, high susceptibility of probiotics in liquid preparations to environmental stresses, such as pH, temperature, and water activity (a_w), leads to a short shelf life and requires costly refrigerated transportation and storage (Zhang et al., 2016). Therefore, production of powdered probiotic ingredients is necessary for prolonged storage and enhanced application convenience.

Spray drying and freeze drying are the most commonly applied dehydration methods to produce powdered probiotics in the microbiological industry (Meng et al., 2008), but each method has some critical shortcomings. The stress due to heating or freezing during dehydration can affect cellular activities and deactivate functional proteins, lipids, and nucleic acids (De Angelis & Gobbetti, 2004; Peighambardoust et al., 2011). In addition, evaporation in spray drying and sublimation in freeze drying can remove a large quantity of inter- and intracellular water, causing osmotic stress, cellular membrane leakage, and consequently cell mortality (Huang et al., 2017; Iaconelli et al., 2015). Therefore, in order to reduce the loss of probiotics viability, addition of protectants prior to dehydration has been widely studied as one of the most effective approaches.

Dairy ingredients, such as lactose, skim milk powder (SMP), and milk protein concentrate (MPC, SMP minus lactose), are commonly incorporated in the media during dehydration of probiotics, because of their nutritive value, cost-effectiveness, acceptability, palatability, *etc.* (Heller, 2001; Sanders & Marco, 2010). More importantly, the major components in dairy ingredients, lactose and milk proteins, can protect probiotics during spray and freeze drying. The hydroxyl groups of lactose can interact with the phosphate head groups at the cellular surface to replace hydrogen bonds initially formed with water that is lost during dehydration (Santivarangkna et al., 2008). Milk proteins can coat on the cell membrane as a film during drying to prevent cellular damage (Liu et al., 2017). Interestingly, the mixture of milk proteins and lactose was reported with more significant protection effectiveness on the survival of spray-dried *Bifidobacterium lactis* BB12 than individual components (Chávez & Ledeboer, 2007).

Spray-dried lactose (SDL) and milk protein powders are hygroscopic but have different water sorption properties. For amorphous SDL, water can be rapidly absorbed as a plasticizer to lower the glass transition temperature and thus induce irreversible lactose crystallization (Lai & Schmidt, 1990; Price & Young, 2004). For dehydrated milk proteins, water is initially bound to their polar groups, followed by progressive formation of additional water layers (Kinsella & Fox, 1986; McSweeney & Fox, 2009). The water sorption properties of lactose and milk proteins have been mostly studied to improve the quality of dairy powders (Shrestha, Howes, Adhikari, & Bhandari, 2007; Shrestha, Howes, Adhikari, Wood, et al., 2007). However, the hygroscopicity of dehydrated dairy ingredients has never been utilized to produce powdered probiotics. Therefore, the hypotheses of the present study are that the protective effects and water sorption properties of dairy ingredient powders can be used to prepare powdered probiotics by directly mixing

dehydrated dairy powders and a concentrated cell suspension (composed of 70-80% water), and the different water sorption characteristics of milk proteins and lactose can influence physical and microbiological properties of the prepared probiotic powders.

The specific objective of this study was to characterize physical and microbiological properties of powders prepared by mixing *Lactobacillus salivarius* NRRL B-30514 suspensions with dairy ingredient powders with different mass ratios of protein and lactose to understand their relative significance in bacterial survival. In addition to SMP, SDL, and MPC, MPC was blended with SDL at mass ratios of 2:1, 1:1, and 1:2 before mixing with the cell suspension. The model strain, *L. salivarius* NRRL B-30514, has been identified as a probiotic bacterium (Messaoudi et al., 2013) and used in our previous encapsulation and spray drying studies (Zhang et al., 2015; 2016). Unlike the conventional dehydration methods, the present study utilizes the hygroscopicity of dehydrated dairy ingredients to develop a simple and low-cost method to produce probiotic powders without thermal treatments. The findings from the current study are significant to manufacturing functional foods utilizing probiotics.

3.3 Materials and methods

3.3.1. Materials

Carnation® non-fat milk powder (34.78% protein, 52.17% lactose, as is basis) was a product of Nestlé USA (Solon, OH, USA). MPC (81.82% protein, less than 1% lactose, as is basis) was from 138 Foods, Inc. (Claremont, CA, USA). Bovine α-lactose monohydrate was kindly supplied by Leprino Foods (Denver, CO, USA). de Man, Rogosa, and Sharpe (MRS) broth medium and agar (dehydrated) were from Oxoid Ltd (Altrincham, Cheshire, England). Unless

noted, other chemicals were products of either Sigma-Aldrich Corp. (St. Louis, MO, USA) or Thermo Fisher Scientific, Inc. (Pittsburgh, PA, USA).

3.3.2. Preparation of bacterial suspensions

The *L. salivarius* NRRL B-30514 strain was obtained from Department of Animal Science at the University of Tennessee (Knoxville, TN, USA). Prior to experiments, all glassware, centrifuge tubes, media, and solutions were sterilized by autoclaving at 121 °C for 15 min. Twenty microliters of stock *L. salivarius* was inoculated in 5 mL MRS broth that was anaerobically incubated at 37 °C for 18 h and successively transferred into 100 mL MRS broth with the same incubation conditions to obtain *L. salivarius* cultures at the late-exponential phase. Anaerobic conditions were achieved using an anaerobic jar and GasPak™ EZ anaerobe container system sachets with indicator (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Cell pellets collected by centrifugation at 4500 *g* for 30 min (Sorvall ST 16R, Thermo Scientific Company, Waltham, MA, USA) at 4 °C were washed twice after suspension in phosphate-buffered saline (PBS, pH 7.4) and centrifugation under the same conditions. The washed cells were resuspended in 250 µL PBS at a concentration of about 10^{10} CFU/mL, stored at 4 °C, and used in the same day in further experiments.

3.3.3. Preparation of dairy ingredient powders

Lactose solution was prepared to a solids content of 10% (w/v) in deionized (DI) water and vigorously stirred at room temperature (RT, ~21 °C) for 1 h before feeding into a lab-scale spray drier (Buchi-B290 Mini Spray dryer, BÜCHI Corporation, Flawil, St. Gallen, Switzerland). The spray drying conditions were applied as previously described with minor modifications (Zhang et

al., 2015). The inlet temperature was 170 °C, the outlet temperature was kept at 95-100 °C, the pump rate was 15%, and the aspirator setting was 100% (38 m^3/h). The SDL (a_w = 0.140) was collected for RT storage in a desiccator before further use. SMP (a_w = 0.210) and MPC (a_w = 0.240) were used as received and also stored in a RT desiccator.

Six dairy ingredient powders were prepared for further experiments, including SMP, SDL, MPC, and MPC/SDL prepared by manually mixing MPC and SDL at mass ratios of 1:2, 1:1, and 2:1. To simplify description, nMPC/mSDL is used to code the mixtures hereafter, with n and m representing numbers (1 or 2) in the mass ratio.

3.3.4. Preparation of powdered L. salivarius

The concentrated *L. salivarius* suspension was dropped on each dairy powder at a volume (mL):mass (g) ratio of 1:25, which was determined to be optimum in maintaining bacterial viability in preliminary experiments. The initial cell counts in probiotics powders were estimated to be ~8.60 log CFU/g. The probiotics powder was then prepared first by blending using a food blender (Osterizer galaxie, Oster Inc., Fort Lauderdale, FL, USA) for 20 s, followed by grinding using a mortar for 5 min and a coffee grinder (Hamilton beach, Hamilton Inc., Glen Allen, VA, USA) for 20 s to improve powder homogeneity. Samples prepared with each dairy powder in each zip-lock bag of three independent replications were randomly sampled for 3 locations to calculate the coefficient of variation (CV) from 9 total enumeration results. The developed protocol was efficient to prepare powders with evenly distributed *L. salivarius* because the CV of bacterial distribution was determined to be less than 5% in preliminary experiments. The probiotics powders sealed in zip-lock bags were stored in desiccators at RT or in a 4 °C walk-in

cooler for up to 6 months. Samples tested on day 0 were collected within 30 min after probiotics powders were prepared.

3.3.5. Enumeration of L. salivarius

The spread plating method was used to enumerate bacteria. The *L. salivarius* suspension was serially diluted in PBS and plated on MRS agar for anaerobic incubation at 37 °C for 24 h. To enumerate *L. salivarius* in a powder sample on day 0 and during subsequent storage in desiccators at RT or 4 ºC for10, 20, 30, 90, and 180 days, 0.1 g of powder was vigorously vortexed with 10 mL PBS for 2 min to prepare a suspension for enumeration.

3.3.6. Water sorption isotherms of dairy ingredient powders

Water sorption isotherms of MPC, SDL, SMP, and MPC/SDL mixtures were determined at 25 ºC using a literature method (Labuza et al., 1985) with some modifications. Dairy powders were dehydrated in a Baxter TempCon N7595-1 vacuum oven (Baxter International Inc., Deerfield, IL, USA) at 40 ºC for 12 h. After drying, duplicate samples (~0.5 g) were weighed in an AquaLab sample cup (Decagon Devices Inc., Pullman, WA, USA) that was placed in a desiccator containing a saturated salt slurry to obtain a_w of 0.112 (LiCl), 0.227 (CH_3COOK), 0.341 ($MgCl_2$), 0.434 (K_2CO_3), 0.507 ($Mg(NO_3)_2$), 0.611 ($NaNO_2$), 0.758 (NaCl), 0.845 (KCl), and 0.927 (KNO_3). The sample mass was measured periodically until reaching hygroscopic equilibrium that was concluded when the sample mass became constant (± 0.001 g). The equilibrium moisture content (g H_2O/100 g solid) of each sample was gravimetrically determined as a function of a_w.

3.3.7. X-ray diffraction spectroscopy (XRD)

The powder containing *L. salivarius* was placed in a desiccator at RT for 12 h before XRD measurement. Twelve hours were observed to be sufficient for lactose to complete crystallization in preliminary experiments. The XRD spectra were acquired with a model Empyrean 2 diffractometer (PANalytical Inc., Westborough, WA, USA) with Ni-filtered Cu-Kα radiation (45 kV, 40 mA). The 2θ scanning range was 5-35°, the step size was 0.013°, and the scanning speed was 0.05°/s. The spectral analysis was conducted with X`Pert HighScore® software (PANalytical Inc., Westborough, WA, USA).

A diffraction pattern with absence of the characteristic diffraction peak of α-lactose monohydrate crystals at 2θ of 12.4° (Jouppila et al., 1998) indicated the complete amorphous structure of lactose. The crystallinity of lactose in probiotics powders was determined using the profile fitted area corresponding to the characteristic peak of α-lactose monohydrate using the literature correlation method (Fix & Steffens, 2004). The correlation curve was previously established based on the profile fitted areas at 2θ of 12.4° for mixtures containing different mass ratios of SDL (0% crystallinity) and α-lactose monohydrate (100% crystallinity).

3.3.8 Calculation of theoretical lactose:water molar ratio and yield of powdered probiotics

The theoretical lactose:water molar ratio of powdered probiotics was calculated from Eq. (1).

$$\text{Lactose: water molar ratio} = \frac{\text{Mass of lactose (g)}/342.3\ (\text{g}\cdot\text{mol}^{-1})}{(\text{Mass of cell suspension (g)} \times \text{water content (\%wb)})/18.0\ (\text{g}\cdot\text{mol}^{-1})} \quad (1)$$

where the mass of cell suspension was calculated using a density of 1.03 g/mL estimated gravimetrically; the wet-basis (wb) water content of *L. salivarius* suspension was determined to

be 87.63% after measuring the mass difference before and after drying about 0.3 mL of the cell suspension in a convection oven (model Precision 6958, Thermo Scientific, Inc., Waltham, MA, USA) at 100 °C for 24 h (Shi & Zhong, 2015) (n = 2).

The mass yield of probiotics powder was calculated according to Eq (2).

$$\text{Mass yield (\%)} = \frac{\text{Mass of probiotics powder (g)}}{\text{Mass of dairy powder (g)+Mass of cell suspension (g)}} \times 100 \quad (2)$$

3.3.9. Water activity of powders

The a_w of a powder sample was determined using a model Aqualab Series 3 meter (Decagon Devices Inc., Pullman, WA, USA).

3.3.10. Microbiological properties of powdered L. salivarius

Freshly prepared probiotics powders were placed in a desiccator for 12 h at RT before following characterizations.

3.3.10.1. LIVE/DEAD® BacLight™ assay for bacterial membrane integrity

The membrane integrity of powdered *L. salivarius* was evaluated using a LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit L7012 (Life Technologies Corp., Eugene, OR, USA). The *Bac*Light™ assay was conducted following the Fluorescence Microplate Readers protocol issued by Molecular Probes (Anonymous, 2004). Briefly, the standard curve was established using a Synergy 2 multi-mode reader (BioTek Instruments Inc., Winooski, VT, USA) to determine relative viability of *L. salivarius*. For the powdered *L. salivarius* prepared with SDL, a 0.1 g sample was suspended in 10.0 mL of 0.85% NaCl solution (~pH 6.0), followed by centrifugation at 4500 *g* for 10 min at 4 °C. The resulting cell pellets were resuspended in 1 mL of 0.85% NaCl

solution to adjust the optical density at 670 nm (OD_{670}) to about 0.3 using a SmartSpec Plus spectrophotometer (Bio-Rad Laboratories Inc., Hercules, CA, USA), corresponding to a bacterial concentration of about 1×10^7 CFU/mL. For the powdered *L. salivarius* prepared with other dairy ingredient powders, a suspension with 0.1 g sample in 10.0 mL of 0.85% NaCl solution was dissolved with 0.1 g trisodium citrate by vortexing to dissociate casein micelles that interference OD_{670}, followed by centrifugation and resuspension as above. The viability of *L. salivarius* was not significantly influenced ($P > 0.05$) by the addition of trisodium citrate (data not shown). The resuspended bacteria were then stained and evaluated following the protocol (Anonymous, 2004).

3.3.10.2. CTC reduction assay for bacterial respiratory activity

To analyze the bacterial respiratory activity, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Polyscience Inc., Warrington, PA, USA) was utilized as a redox probe following the protocol issued by Molecular Probes (Anonymous, 2005) with some modifications. For the SDL treatment, *L. salivarius* suspension was prepared by processing samples with the same procedures as in section 2.11.1, except that 0.85% NaCl was replaced by PBS. For other treatments, in order to minimize the interference caused by undissolved milk proteins in flow cytometry assay, suspensions with 0.1 g powdered *L. salivarius* sample in 10.0 mL PBS were centrifuged at 200 *g* for 2 min at 4 °C to precipitate undissolved protein particles while keeping cells suspended. The resulting supernatant was also dissolved with 0.1 g trisodium citrate to dissociate casein micelles. After centrifugation at 4500 *g* for 10 min at 4 ºC, the bacterial pellets were resuspended in 1.0 mL PBS to adjust the *L. salivarius* population to about 1×10^7 CFU/mL. Subsequently, 1 mL of the prepared *L. salivarius* suspension was gently vortexed with 100 µL of

50 mM CTC working solution, followed by incubation without light in a model I24 incubator shaker (New Brunswick Scientific Co., Enfield, CT, USA) for 2 h at 37 °C with agitation at 150 rpm. The stained bacteria were analyzed using an Attune acoustic focusing cytometer (Applied Biosystems, Foster City, CA, USA). A suspension with 1×10^7 CFU/mL viable *L. salivarius* cells without CTC stain was used as a control.

3.3.11. Statistical analysis

Unless noted otherwise, the mean and standard deviation (SD) were calculated from three independent replicates. The one-way analysis of variance (ANOVA) was conducted using the SAS version 9.4 software (SAS Institute, Cary, NC, USA). Differences between treatment mean values were analyzed using the Fisher's least significant-difference (LSD) at a significance level of 0.05.

3.4 Results and discussion

3.4.1 Viability of powdered L. salivarius prepared with dairy ingredients

The viability of powdered *L. salivarius* prepared with each dairy ingredient powder on day 0 is shown in Table 3-1. The SMP and SDL treatments had a bacterial count of 8.22 and 7.45 log CFU/g, respectively, suggesting the more significant role of protein protecting bacteria during dehydration. This was further verified for MPC/SDL mixture treatments that showed the increase of cell viability from 7.67 to 8.45 log CFU/g when MPC:SDL mass ratio was increased from 1:2 to 1:0.

During storage in desiccators for up to 180 days (Table 3-1), the survival of powdered *L. salivarius* in all treatments remained stable when stored at 4 ºC, contrasting with significantly ($P < 0.05$) decreasing to be eventually undetectable at RT. The more significant protective effects of protein on bacterial survival were also supported by the storage stability of powdered *L. salivarius*. The MPC treatment showed about 1 log CFU/g higher than the SDL treatment during 180-day storage at 4 ºC. Furthermore, powdered *L. salivarius* in treatments with MPC had >7.00 log CFU/g viable cells on day 90 of RT storage, contrasting with the SMP and SDL treatments having undetectable cells at the detection limit of 3.0 log CFU/g.

The more effective protection of dairy proteins than lactose on probiotic survival was also observed after spray drying 300 mL of a suspension with ~2×10^7 CFU/mL *L. salivarius* and 15 g dairy powder at a constant inlet temperature of 165 ºC and outlet temperatures of 96-100 and 70-75 ºC. The total cell counts (~6×10^9 CFU) and mass of dairy powder (15 g) in the suspension were equivalent to those by directly mixing 0.6 mL of ~1×10^{10} CFU/mL cell suspension with 15 g dairy powder at a volume (mL): mass (g) ratio of 1:25. As shown in Table 3-2, spray-dried SMP and MPC treatments respectively had ~1.5 and ~2 log CFU/g higher viable cells than the SDL treatment. Ghandi *et al.* (2012) also reported that the survival rate of spray-dried *Lactococcus lactis* increased from 4.0% when suspended in 10% (w/w) lactose to 10.3% when suspended in 10% (w/w) lactose/sodium caseinate mixture at a mass ratio of 3:1. Furthermore, when compared to spray-drying treatments, the probiotics powders prepared using the present method with the same dairy ingredient generally showed a higher mass yield (Eq. 2, where the mass of cells, not cell suspension, was used for spray drying treatments) and *L. salivarius* viability (Table 3-2). Therefore, the protective effect of dairy proteins on bacterial survival

during dehydration allowed the preparation of powdered probiotics with simple procedures and high efficiency in the present study to obtain a higher population of viable cells than spray drying.

The protective effect of dairy proteins on survival of probiotics during drying has been proposed for possible specific interactions between bacterial cells and milk protein components in liquid media (Burgain et al., 2014), followed by adhesion of hydrophobic portions of unfolded proteins to bacteria during drying (Khem et al., 2016), resulting in cells being coated within protein capsules (Liu et al., 2017). However, unlike spray and freeze drying, probiotic cells in this study were surrounded by dairy powders which absorbed surrounding water to dehydrate the cells. Therefore, evaluation of water binding properties of dairy ingredient powders may help to understand the relative significance of milk proteins and lactose on survival of *L. salivarius*.

3.4.2 Water binding properties of dehydrated dairy ingredients

3.4.2.1 Water sorption isotherms

In order to characterize the hygroscopicity of dehydrated dairy powders, the water sorption isotherms of SMP, MPC, SDL, and MPC/SDL mixtures were determined (Figure 3-1). The moisture content of SDL continuously increased up to a_w of 0.43 and then dramatically decreased because of the occurrence of lactose crystallization (Lai & Schmidt, 1990). Lactose crystallization was notably inhibited in MPC/SDL mixtures. Specifically, crystallization took place at a higher a_w in 2MPC/1SDL and 1MPC/1SDL ($a_w > 0.51$) than 1MPC/2SDL and SDL ($a_w > 0.43$), and the moisture content of MPC/SDL mixtures after crystallization remained higher than that of SDL.

The delayed lactose crystallization with the existence of MPC is in agreement with several studies (Hogan & O'Callaghan, 2010; Kockel et al., 2002), possibly due to the higher affinity of proteins to bind with water than lactose and the hindered mobility of lactose by proteins (Haque & Roos, 2004). The water sorption isotherm of SMP (composed of MPC and SDL at a mass ratio of approximately 2:3) showed a same trend as 2MPC/1SDL where lactose started to crystallize at a_w of 0.51, because the powder particle shell consisting of mostly proteins can impede the absorption of sufficient water for the crystallization of inner amorphous lactose (Price & Young, 2004). As the primary component absorbing water, the numerous polar groups of proteins can strongly and rapidly absorb water at a_w between 0 and 0.34 via hydrogen bonding. Water uptake of MPC then increased mostly linearly at a smaller rate at an a_w range from 0.34 to 0.76 (Figure 3-1) where water molecules progressively adsorb on the preexisting water layers. The formed multilayered water can be available to initiate the hydration of SDL, which is the next component to absorb water (Kinsella & Fox, 1986).

In addition, the molar mass of milk proteins (~30,000 g/mol) is about one hundred times greater than that of lactose (342.3 g/mol). Therefore, hydration of SDL by absorbing the surrounding water of adhered *L. salivarius* cells can lead to a significantly higher molar concentration of solutes than that of MPC at the same cell suspension:powder (v:m) ratio. The increased solute concentration around bacterial cells gives rise to a higher hypertonic pressure (De Angelis & Gobbetti, 2004) and consequently compromised survival of *L. salivarius* in treatments with a higher content of lactose (Table 3-1).

3.4.2.2. Crystallinity of powdered probiotics

The crystallinity of lactose in powdered *L. salivarius* was studied using XRD, with diffractograms shown in Figure 3-2. After mixing dairy powders with cell suspensions, the SDL, 1MPC/1SDL, and 1MPC/2SDL treatments showed crystalline structures. The lactose crystallinity in powdered *L. salivarius* estimated from XRD is summarized in Table 3-3. The water:lactose molar ratios in the SMP and MPC/SDL treatments were over 1, which would favor the formation of lactose monohydrate and therefore lactose crystals (Lai & Schmidt, 1990). However, according to XRD results, no crystalline lactose was observed in the SMP and 2MPC/1SDL treatments, and 1MPC/1SDL and 1MPC/2SDL treatments showed significantly lower ($P < 0.05$) crystallinity than the SDL treatment. The XRD results further verified the previous discussion that MPC can primarily absorb the water in cell suspensions to delay or eliminate lactose crystallization in the powdered *L. salivarius*.

3.4.2.3. Water activity of powdered probiotics

As shown in Table 3-4, the a_w of powdered *L. salivarius* after preparation at RT was all low enough (≤ 0.4) to suppress bacterial metabolism and thus maintain the viability of probiotics during storage (Tripathi & Giri, 2014). Initially, the a_w of SMP and MPC/SDL treatments was around 0.4, corresponding to the linear water sorption region of MPC (Figure 3-1), indicating the water in cell suspensions might be predominately bound as multilayers on proteins and partially form hydrogen bonds with lactose. In addition, lactose in probiotics powders prepared with SDL, 1MPC/1SDL, and 1MPC/2SDL crystallized at a lower a_w (~0.4) than that (~0.5) observed in the corresponding water sorption isotherms (Figure 3-1), probably due to the nucleation of hydrated

lactose facilitated by grinding and the moisture exchange with the environment during sample preparation. During 6-month storage, higher a_w of samples stored at 4ºC than RT was probably caused by the moisture exchange with the environment when the desiccator and zip-lock bags were opened in the walk-in cooler with a relative humidity of ~80%.

3.4.3. Biophysical properties of powdered L. salivarius

To better understand why milk proteins are better than lactose preserving the viability of *L. salivarius* after preparation of powders (Table 3-1), membrane integrity and metabolic activity were characterized as biophysical properties of powdered *L. salivarius*.

3.4.3.1. Bacterial membrane integrity

In the LIVE/DEAD® *Bac*Light™ assay kit, the SYTO® 9 generally labels all cells as fluorescent green, while propidium iodide only penetrates cells with damaged membranes and stains them as fluorescent red, causing a reduction in the SYTO® 9 fluorescence (Anonymous, 2004). Based on their different cell permeability, the LIVE/DEAD® *Bac*Light™ assay kit has been extensively used to evaluate the cytoplasmic membrane integrity (Pinto et al., 2015). According to Figure 3-3, the viable cell counts of *L. salivarius* detected by *Bac*Light™ and plate-counting enumeration showed a similar trend among all treatments, and the MPC treatment maintained significantly higher ($P < 0.05$) viable *L. salivarius* with intact membranes than the SDL treatment. The better effectiveness of milk proteins than lactose preserving cellular membrane integrity supports the discussion in section 3.2 that milk proteins preferentially absorb water in *L. salivarius* suspensions and generates milder osmotic shocks to cause the reduced

damage of cytoplasmic membranes of adhered cells, leading to the improved viability (Table 3-1).

3.4.3.2. Bacterial metabolic activity

Metabolic activity is another important biophysical state indicator of bacteria (Chávez & Ledeboer, 2007). The redox dye CTC was used in this study to detect metabolically active *L. salivarius* after mixing with different dairy ingredient powders, because CTC can be absorbed and reduced by the respiratory enzyme of living cells into an insoluble and red-fluorescent formazan (Gasol & Del Giorgio, 2000). The viable bacterial counts detected in the CTC assay also showed a similar trend as the direct enumeration (Figure 3-3), indicating the metabolic activity of powdered *L. salivarius* can be maintained better with the increased amount of MPC in the powder during mixing with the cell suspension. This phenomenon can be explained in the context of the membrane integrity. As reported by Korber *et al.* (1996), an intact cell membrane, as a selective barrier between cells and the environment, can protect cytoplasmic materials, retain cell turgor, and thus maintain cellular metabolic functions. Therefore, the more significant role of milk proteins than lactose preserving the viability of *L. salivarius* (Table 3-1) is supported by complementary membrane integrity and cellular metabolic activity assay results (Fig. 3).

In addition, SDL appeared to have induced the powdered *L. salivarius* into a viable but nonculturable (VBNC) state to a greater extent than MPC, because more viable cells were detected in both the *Bac*Light™ and CTC assays than the plate-counting results in 1MPC/1SDL, 1MPC/2SDL, and SDL treatments (Figure 3-3). When responding to an environmental shock inducted by factors such as starvation, thermal change, osmotic pressure, and radiation, cells can adapt to the VBNC state (Oliver, 2000). In this study, the environmental shock results from the

hypertonic shock of hydrated compounds during mixing the cell suspension with dairy powders. However, this hypothesis and the mechanism causing the VBNC state of *L. salivarius* are to be studied in the future.

3.5 Conclusions

Powdered *L. salivarius* with a high level of viability and stability was prepared by simply mixing a cell suspension with dairy ingredient powders, and milk proteins were more efficient than lactose on maintaining probiotic viability initially and during subsequent storage. During preparation of powdered *L. salivarius*, a higher amount of proteins in dairy powders delayed the hydration of SDL by predominately absorbing the water in cell suspensions, resulting in a lower hypertonic stress on adhered *L. salivarius*. The better ability of proteins than lactose protecting bacterial viability after powder preparation was further supported by the stronger protective effects of MPC than SDL preserving the membrane integrity and metabolic activity of *L. salivarius*. The protocol developed in the present study also demonstrated the higher powder yield and bacterial survival than spray drying. The present study utilizing dehydrated dairy powders to prepare powdered probiotic ingredients with simple and cost-effective procedures may be significant to the development of relevant functional foods.

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Appendix

Table 3-1 Viable cell counts of powders *L. salivarius* prepared by mixing cell suspensions with skim milk powder (SMP), spray-dried lactose (SDL), milk protein concentrate (MPC), or MPC and SDL at a mass ratio of 2:1 (2MPC/1SDL), 1:1 (1MPC/1SDL), or 1:2 (1MPC/2SDL) at a volume (mL):mass (g) ratio of 1:25 during 180-day storage at 4 ºC or room temperature (RT, ~21 ºC) in desiccators. The powders at day 0 were sampled within 30 min after mixing.

Treatment		Viable cell count (Log CFU/g) *					
		Day 0	Day 10	Day 20	Day 30	Day 90	Day 180
SMP	4°C	$8.22\pm0.22^{a-e}$	$8.02\pm0.26^{a-i}$	$8.09\pm0.08^{a-h}$	$7.90\pm0.09^{a-k}$	$7.76\pm0.25^{a-m}$	$7.59\pm0.08^{d-p}$
	RT		$7.55\pm0.19^{e-p}$	$7.34\pm0.10^{h-q}$	$7.21\pm0.07^{k-q}$	<DL[#]	<DL
SDL	4°C	$7.45\pm0.11^{f-q}$	$7.57\pm0.26^{e-p}$	$7.44\pm0.35^{f-q}$	$7.09\pm0.50^{m-q}$	$6.95\pm0.13^{o-q}$	$6.89\pm0.27^{p-q}$
	RT		$7.50\pm0.20^{f-q}$	$7.13\pm0.31^{l-q}$	6.79 ± 0.44^{q}	<DL	<DL
MPC	4°C	8.45 ± 0.09^{a}	8.46 ± 0.15^{ab}	8.32 ± 0.15^{abc}	$8.23\pm0.16^{a-e}$	$8.15\pm0.01^{a-f}$	$8.03\pm0.20^{a-i}$
	RT		$8.09\pm0.10^{a-h}$	$7.98\pm0.26^{a-j}$	$7.79\pm0.18^{a-m}$	$7.01\pm0.00^{n-q}$	<DL
2MPC /1SDL	4°C	$8.10\pm0.04^{a-h}$	$8.21\pm0.09^{a-e}$	$8.28\pm0.01^{a-d}$	$8.02\pm0.03^{a-i}$	$7.81\pm0.02^{a-l}$	$7.30\pm0.02^{j-q}$
	RT		$7.60\pm0.02^{d-o}$	$8.12\pm0.02^{a-g}$	$7.78\pm0.03^{a-m}$	$7.33\pm0.04^{i-q}$	<DL
1MPC /1SDL	4°C	$7.65\pm0.12^{c-o}$	$7.83\pm0.04^{a-l}$	$7.65\pm0.11^{c-o}$	$7.74\pm0.03^{b-m}$	$7.65\pm0.03^{c-o}$	$7.30\pm0.02^{j-q}$
	RT		$7.56\pm0.03^{e-p}$	$7.69\pm0.06^{c-n}$	$7.78\pm0.03^{a-m}$	$7.42\pm0.01^{g-q}$	<DL
1MPC /2SDL	4°C	$7.67\pm0.15^{c-n}$	$7.91\pm0.25^{a-k}$	$7.59\pm0.03^{d-p}$	$7.88\pm0.01^{a-k}$	$7.69\pm0.18^{c-n}$	$7.43\pm0.23^{g-q}$
	RT		$7.91\pm0.01^{a-k}$	$7.83\pm0.10^{a-l}$	$7.59\pm0.08^{d-p}$	$7.24\pm0.01^{k-q}$	<DL

* Numbers are mean ± SD (n = 3). Means with different superscript letters indicate significant differences of all treatments ($P < 0.05$).

[#] Below the detection limit (DL) of 3.00 log CFU/g.

Table 3-2 Mass yield, water activity, and viable cell counts of powders prepared by mixing a concentrated suspension with ~1×10^{10} CFU/mL *L. salivarius* and skim milk powder (SMP), spray-dried lactose (SDL), or milk protein concentrate (MPC) at a volume (mL):mass (g) ratio of 1:25, in comparison to spray drying suspensions containing ~2×10^{7} CFU/mL *L. salivarius* and 5% (w/v) SMP, SDL, or MPC [*].

Treatment		Mass yield (%)	Water activity	Viable cell count (Log CFU/g)
SDL	Direct mixing	98.89±0.87^{a}	0.24±0.08^{bc}	7.45±0.11^{d}
	Spray drying at T_{out} of 96-100 ºC	65.00±3.74^{b}	0.19±0.01^{bc}	5.24±0.01^{g}
	Spray drying at T_{out} of 70-75 ºC	38.30±1.72^{c}	0.24±0.05^{bc}	6.42±0.20^{f}
MPC	Direct mixing	99.10±0.89^{a}	0.44±0.01^{a}	8.45±0.09^{ab}
	Spray drying at T_{out} of 96-100 ºC	40.37±2.56^{c}	0.20±0.02^{bc}	7.07±0.16^{e}
	Spray drying at T_{out} of 70-75 ºC	18.25±2.97^{e}	0.29±0.04^{b}	8.54±0.06^{a}
SMP	Direct mixing	96.62±1.27^{a}	0.42±0.08^{a}	8.22±0.22^{bc}
	Spray drying at T_{out} of 96-100 ºC	59.70±2.37^{b}	0.18±0.02^{c}	6.89±0.06^{e}
	Spray drying at T_{out} of 70-75 ºC	30.2±3.26^{d}	0.28±0.03^{bc}	7.94±0.13^{c}

* Numbers are mean ± SD (n = 3). Means with different superscript letters indicate significant differences of all treatments ($P < 0.05$).

Table 3-3 Lactose:water molar ratio and crystallinity% estimated in X-ray diffraction spectroscopy of powdered *L. salivarius* prepared by mixing a cell suspension with a dairy powder at a volume (mL):mass (g) ratio of 1:25. [#]

Dairy powder composition	Lactose:water molar ratio	Crystallinity%*
SMP	1:1.3	0
SDL	1:0.7	9.40±1.07[a]
MPC	0:1.0	0
2MPC/1SDL	1:2.1	0
1MPC/1SDL	1:1.3	0.98±0.09[b]
1MPC/2SDL	1:1.0	1.03±0.33[b]

[#] The dairy powder was skim milk powder (SMP), spray-dried lactose (SDL), milk protein concentrate (MPC), or MPC and SDL at a mass ratio of 2:1, 1:1, or 1:2. The powdered *L. salivarius* was placed in a desiccator for 12 h at room temperature (~21 ºC) before the XRD measurement.

* Numbers are mean ± SD (n = 3). Different superscript letters indicate significant differences in the mean of all samples ($P < 0.05$).

Table 3-4 Water activity of powders *L. salivarius* prepared by mixing cell suspensions with skim milk powder (SMP), spray-dried lactose (SDL), milk protein concentrate (MPC), or MPC and SDL at a mass ratio of 2:1 (2MPC/1SDL), 1:1 (1MPC/1SDL), or 1:2 (1MPC/2SDL) at a volume (mL):mass (g) ratio of 1:25 during 180-day storage at 4 ºC or room temperature (RT, ~21 ºC) in desiccators.

Treatment		Water activity*					
		Day 0	Day 10	Day 20	Day 30	Day 90	Day 180
SMP	4°C	0.42 ± 0.08^{ab}	$0.36\pm0.02^{a-h}$	$0.38\pm0.03^{a-h}$	$0.41\pm0.00^{a-k}$	$0.39\pm0.01^{a-e}$	$0.37\pm0.01^{a-g}$
	RT		$0.32\pm0.00^{a-i}$	$0.28\pm0.01^{a-k}$	$0.24\pm0.01^{c-k}$	$0.23\pm0.00^{d-k}$	$0.22\pm0.01^{e-k}$
SDL	4°C	$0.24\pm0.08^{c-k}$	$0.26\pm0.08^{b-k}$	$0.33\pm0.06^{a-i}$	$0.31\pm0.11^{a-j}$	$0.34\pm0.10^{a-i}$	$0.32\pm0.05^{a-i}$
	RT		$0.22\pm0.04^{e-k}$	$0.24\pm0.09^{c-k}$	$0.24\pm0.06^{c-k}$	$0.33\pm0.00^{a-i}$	$0.33\pm0.02^{a-i}$
MPC	4°C	0.44 ± 0.01^{a}	0.44 ± 0.06^{a}	$0.38\pm0.01^{a-f}$	$0.40\pm0.01^{a-c}$	$0.38\pm0.01^{a-f}$	$0.38\pm0.01^{a-f}$
	RT		$0.26\pm0.04^{b-k}$	$0.28\pm0.00^{a-k}$	$0.32\pm0.03^{a-i}$	$0.30\pm0.01^{a-j}$	$0.29\pm0.01^{a-k}$
2MPC /1SDL	4°C	$0.41\pm0.02^{a-c}$	0.44 ± 0.01^{a}	0.43 ± 0.02^{ab}	0.44 ± 0.01^{a}	$0.42\pm0.01^{a-c}$	$0.40\pm0.01^{a-c}$
	RT		$0.29\pm0.00^{a-k}$	$0.20\pm0.03^{g-k}$	$0.19\pm0.01^{h-k}$	$0.20\pm0.00^{g-k}$	$0.20\pm0.00^{g-k}$
1MPC /1SDL	4°C	$0.41\pm0.00^{a-c}$	$0.42\pm0.02^{a-c}$	$0.41\pm0.01^{a-c}$	$0.41\pm0.01^{a-c}$	$0.41\pm0.01^{a-c}$	$0.40\pm0^{a-d}$
	RT		$0.26\pm0.01^{b-k}$	$0.19\pm0.01^{h-k}$	$0.24\pm0.01^{c-k}$	$0.22\pm0.01^{e-k}$	$0.21\pm0.00^{f-k}$
1MPC /2SDL	4°C	$0.42\pm0.01^{a-c}$	$0.41\pm0.02^{a-c}$	$0.40\pm0.01^{a-c}$	$0.41\pm0.01^{a-c}$	$0.40\pm0.01^{a-c}$	$0.41\pm0.00^{a-c}$
	RT		$0.26\pm0.02^{b-k}$	$0.19\pm0.01^{h-k}$	0.14 ± 0.01^{k}	0.15 ± 0.00^{jk}	$0.16\pm0.00^{i-k}$

* Numbers are mean ± SD (n = 3). Means with different superscript letters indicate significant differences of all treatments ($P < 0.05$).

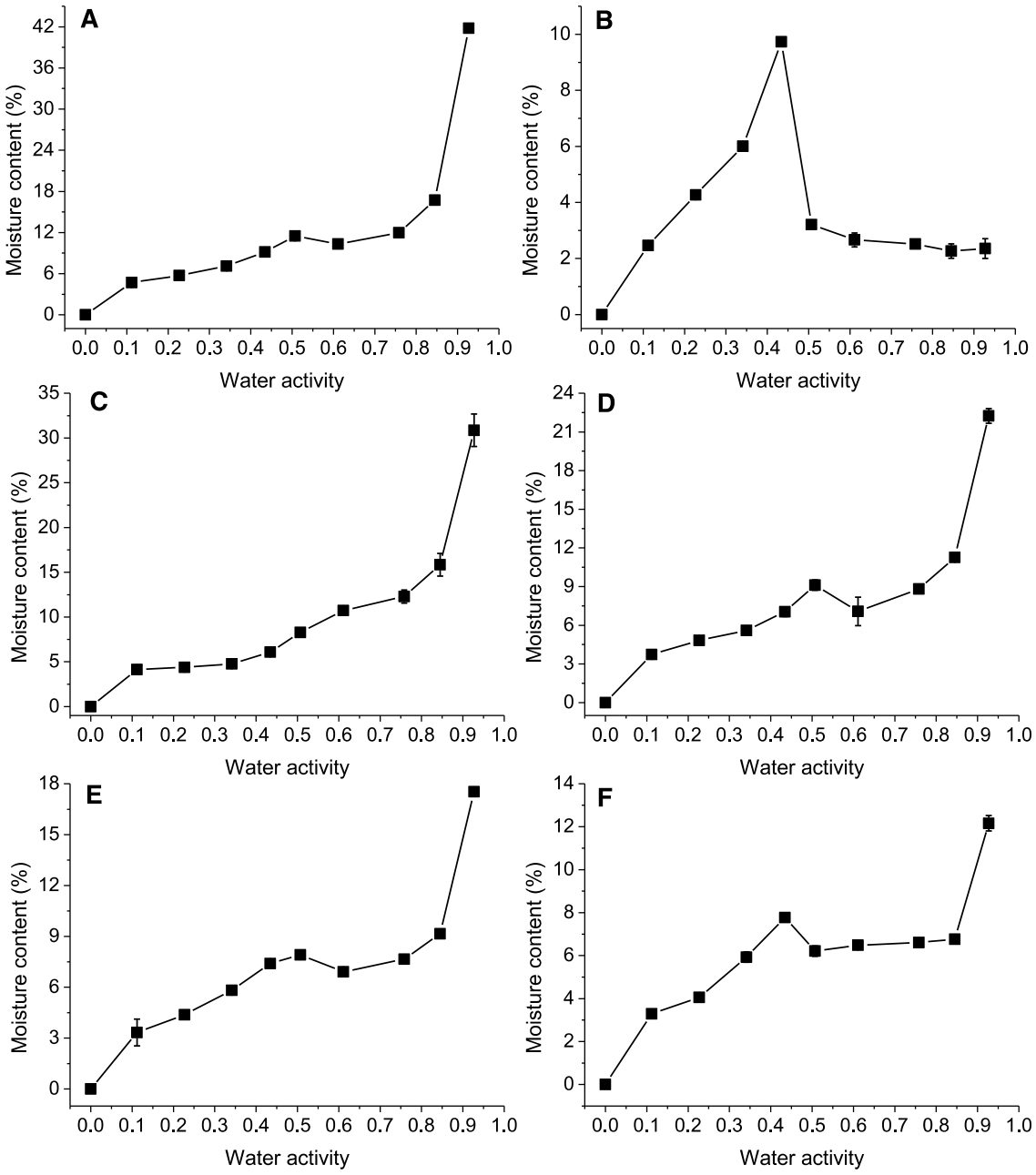

Figure 3-1 Water sorption isotherms showing equilibrium moisture content of skim milk powder (SMP, A), spray-dried lactose (SDL, B), milk protein concentrate (MPC, C), or MPC and SDL at a mass ratio of 2:1 (D), 1:1 (E), or 1:2 (F) incubated at different water activities at room temperature (~21 ºC). Error bars are SD (n = 2).

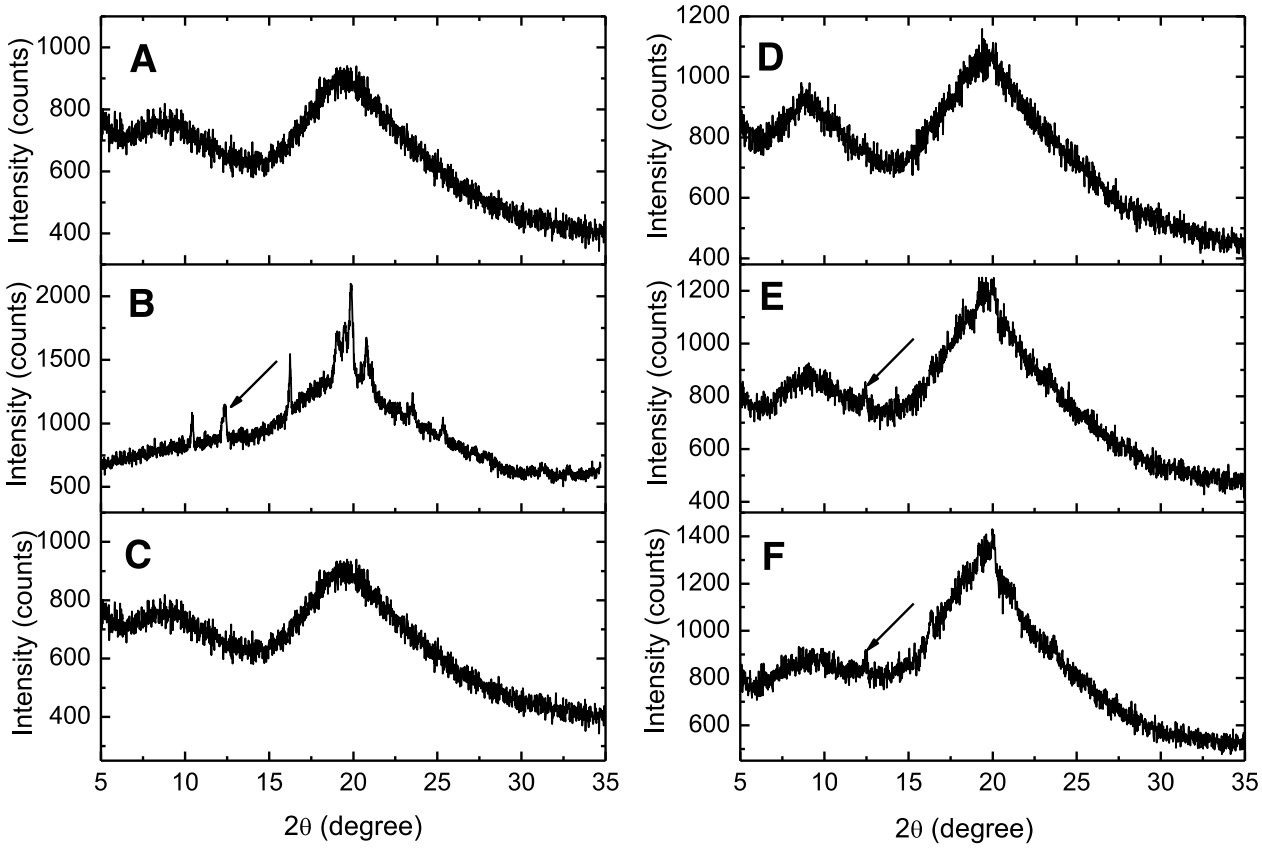

Figure 3-2 X-ray diffractograms of powdered *L. salivarius* prepared by mixing cell suspensions with skim milk powder (SMP, A), spray-dried lactose (SDL, B), milk protein concentrate (MPC, C), or MPC and SDL at a mass ratio of 2:1 (D), 1:1 (E), or 1:2 (F) at a volume (mL):mass (g) ratio of 1:25. Arrows in B, E, and F highlight the characteristic diffraction peak of α-lactose monohydrate crystals at 2θ of 12.4°.

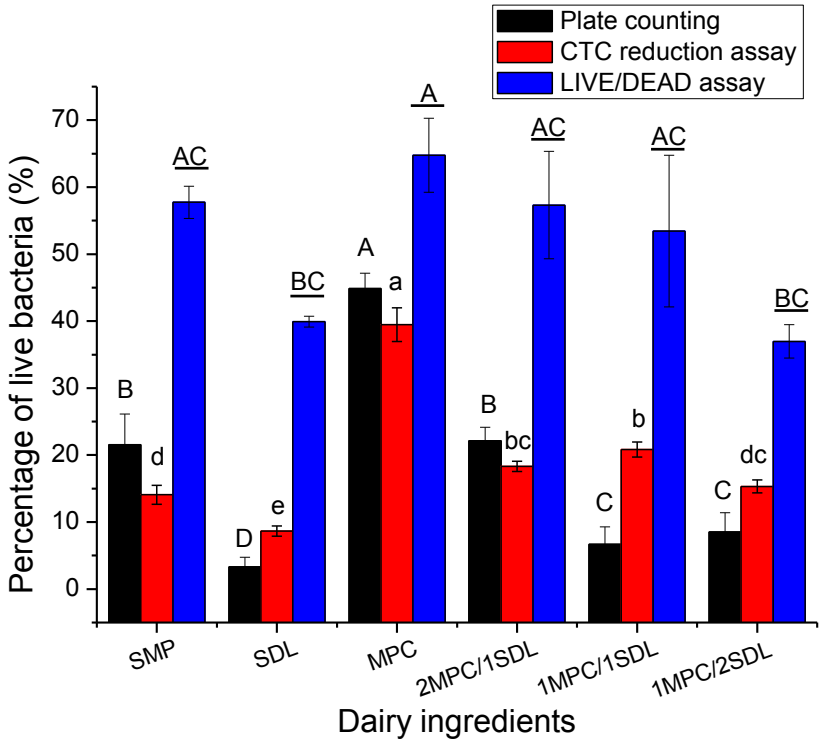

Figure 3-3 Percentages of viable *L. salivarius* in the probiotics powders prepared by mixing cell suspensions with different dairy powders (SMP: skim milk powder; SDL: spray-dried lactose; MPC: milk protein concentrate) at a volume (mL):mass (g) ratio of 1:25, as determined using plate counting, LIVE/DEAD® *Bac*Light™ assays, and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) reduction. The freshly prepared probiotics powders were placed in a desiccator for 12 h at room temperature (~21ºC) before the determinations. Error bars are SD (n = 3). Different letters above bars with the same color indicate significant differences ($P < 0.05$) among different treatments assayed with the same method.

Chapter 4 Synergistic effects of whey protein isolate and amorphous sucrose on improving the viability and stability of powdered *Lactobacillus salivarius* NRRL B-30514

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My primary contributions to this paper include sample preparation, data collection and analysis, results interpretation and writing.

4.1 Abstract

Amorphous sucrose, as an efficient protectant during dehydration of probiotics, can be prepared by spray drying aqueous solutions with both sucrose and whey protein isolate (WPI). The objective of this study was to characterize the synergistic effect of WPI and sucrose on protecting the survival of powdered *Lactobacillus salivarius* NRRL B-30514 prepared by directly mixing a cell suspension with spray-dried WPI/sucrose powders (WSP) with different WPI:sucrose mass ratios. In the prepared WSP-probiotics powders (WPP), differential scanning calorimetry, X-ray diffraction spectroscopy and scanning electron microscopy results indicated that WPI stabilized amorphous sucrose with the glass transition temperature above room temperature. WPP with the presence of amorphous sucrose showed higher probiotic viability and 30-day storage stability than the WPI only treatment. WPP with a higher amount of sucrose also resulted in better survival of *L. salivarius* with higher membrane integrity detected using the LIVE/DEAD® *Bac*Light™ assay after heating at 80ºC for 30 min. The present study showed WSP may protect probiotics better than individual components.

Keywords: probiotics, amorphous sucrose, whey protein isolate, dehydration, viability

4.2 Introduction

Probiotics are viable microorganisms which, when administrated in adequate amounts (10^8~10^9 CFU per dose), can confer beneficial effects on the host by improving the intestinal microbial balance, enhancing immunological functions, and alleviating intestinal barrier dysfunctions (Su et al., 2018). With the rising consumption and popularity of functional food products containing viable probiotics, producing probiotic ingredients with high viability and stability is essential for achieving optimal functionalities and convenient applications (Feng et al., 2018). Unlike probiotics in liquid preparations that are susceptible to environmental stresses, powdered probiotics with a water activity (a_w) low enough to suppress metabolic processes are more suitable to preserve viability during production, storage, transportation, and consumption (Ramos et al., 2018). The dehydration of probiotics is commonly done with spray drying and freeze drying in the microbiological industry due to their simplicity and scalability (Sarao & Arora, 2017). However, heating or freezing and water removal can lead to temperature-induced shocks and osmotic stress on cells, causing structural damage, loss of cellular functions, and consequently cellular mortality (Dianawati et al., 2016; Fiocco et al., 2019).

Incorporation of sucrose as an efficient protectant in the media has been reported as an effective approach to protect survival of probiotics during dehydration (Homayoni Rad et al., 2016; Stefanello et al., 2019). Sucrose can displace the water molecules lost during dehydration and interact with the phosphate head groups at the surface of cellular bilayers via hydrogen bonds to protect against membrane phase transitions (Vaessen et al., 2019). For example, addition of 10% (w/v) sucrose in skim milk as the spray drying medium increased the viability of *Lactobacillus plantarum* BM-1 by 75.70% (Zhu et al., 2016). However, amorphous sucrose in

dehydrated probiotics is metastable and hygroscopic. Absorption of water or increase of temperature above the glass transition temperature (T_g) can transform the physical state of amorphous sucrose to either rubbery or crystalline structures (Masavang et al., 2019). The transformation may provoke stickiness, collapse, caking, or recrystallization of sucrose (Li et al., 2019), which can be detrimental to the physical stability of powders and even the prolonged survival of probiotics. Therefore, drying aids with the ability to stabilize amorphous sucrose are usually required for spray or freeze drying of probiotics.

Whey protein isolate (WPI - with a protein content higher than 90%) has been studied as a drying aid to prepare spray-dried sucrose due to its surface active and film forming properties (Fang et al., 2013). When atomized into hot air, WPI preferentially migrates to the droplet surface and cover the powder particles to resist the cohesive stickiness of sucrose (Adhikari et al., 2009). Adhikari *et. al.* (2009) reported that the mass yield of solid amorphous sucrose was increased from 0% when spray dried alone to 80% when co-spray dried with 1% (dry basis) of WPI. In addition, WPI is a probiotic protectant by coating on the cell membrane as a film during drying to prevent cellular damage (Ramos et al., 2018). Khem *et al*. (2016) reported the survival rate of *Lactobacillus plantarum* after spray-drying in 10% (w/v) WPI solution was about 45% higher than spray-drying in 10% (w/v) lactose solution. Currently, sucrose and WPI are mainly incorporated in the media as the protectants during dehydration of probiotics. However, mixing a probiotic suspension directly with spray-dried sucrose powder stabilized with WPI to produce powdered probiotics, by utilizing water sorption properties of WPI and sucrose, has not been studied. Therefore we hypothesize that the spray-dried WPI/sucrose powders (WSP) can be used

to directly prepare powdered probiotics and synergistically protect probiotics during powder preparation and storage when compared to sucrose or WPI alone.

The first objective of this study was to investigate the effect of WPI on stabilizing amorphous sucrose by characterizing physical properties of sucrose in WSP before and after mixing with *Lactobacillus salivarius* NRRL B-30514 suspensions. The second objective was to study the efficiency of WSP protecting the storage and thermal survivability of powdered *L. salivarius* as affected by the WPI:sucrose mass ratio (WSR). The *L. salivarius* is a model probiotic bacterium (Messaoudi et al., 2013) previously adopted in our encapsulation and spray drying studies (Zhang et al., 2015; Zhang et al., 2016). The present study may be used to improve the viability of powdered probiotics during processing and storage.

4.3 Materials and methods

4.3.1 Materials

Unless noted otherwise, all chemicals were obtained from either Sigma-Aldrich Corp. (St. Louis, MO, USA) or Thermo Fisher Scientific Inc. (Pittsburgh, PA, USA).

4.3.2 Preparation of concentrated bacterial suspension

All glassware, pipet tips, and solutions were sterilized at 121 ºC for 15 min. Frozen stock culture of *L. salivarius* NRRL B-30514 (20 μL) was inoculated in 5 mL De Man, Rogosa, and Sharpe (MRS) broth (Oxoid Ltd, Altrincham, Cheshire, England) and was subsequently incubated at 37 °C for 18 h in an anaerobic jar with GasPak™ EZ anaerobe container system sachets (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The *L. salivarius* culture

was later inoculated in 100 mL MRS broth and grown to late-exponential growth phase under the same growth conditions. Cells were subsequently harvested by centrifugation at 4500 *g* for 30 min (Sorvall ST 16R, Thermo Scientific Company, Waltham, MA, USA) at 4 °C followed by washing twice with a phosphate-buffered saline (PBS, pH 7.4). The final cell suspension was at a concentration of about 10^{10} CFU/mL in PBS and stored at 4°C prior to use in the same day.

4.3.3 Preparation of spray-dried WPI/sucrose powders and freeze-dried sucrose

The powder mixture of Hilmar^{TM} 9420 WPI (95.0% protein, dry basis, Hilmar Ingredients, Hilmar, CA, USA) and sucrose at WSRs of 1:0, 2:1, 1:1, or 1:2 was hydrated to a total solid content of 12% (w/v) in deionized (DI) water. After vigorously stirring at room temperature (RT, ~21°C) for 1 h, pH of the mixture was adjusted to 7.0 using 5.0 M NaOH. The mixtures were spray-dried using a Buchi-B290 Mini Spray dryer (BÜCHI Corporation, Flawil, St. Gallen, Switzerland) as described in Zhang *et al.* (2015) with minor modifications. With a pump rate of 30% and an aspiration setting of 100% (38 m^3/h), the inlet and outlet temperatures were controlled at 160ºC and 75ºC, respectively, to minimize WPI denaturation (Gaiani et al., 2010).

Sucrose solution alone cannot be converted into the powder form through spay drying due to the stickiness of sucrose (Adhikari et al., 2009). To improve the reproducibility, solid amorphous sucrose was prepared using freeze drying in the present study as a control to WSP, although spray-dried sucrose shall be the more appropriate control. Additionally, Jawad *et al.* (2018) reported similar thermal properties of freeze-dried and spray-dried sucrose. Sucrose was dissolved in DI water at 12 g/100 mL and freeze-dried (VirTis AdVantage Plus EL-85benchtop freeze dryer, SP Scientific Inc., Gardiner, NY, USA). The WSP and freeze-dried sucrose (FDS)

were collected for ambient storage in a vacuum desiccator containing phosphorus pentoxide before further use.

4.3.4 Preparation of WSP-probiotics powders

The *L. salivarius* suspension was dropped on the WSP at a volume (mL):mass (g) ratio of 1:25, which was determined as the optimum in maintaining bacterial viability in preliminary experiments, and then mixed using a food blender (Osterizer galaxie, Oster Inc., Fort Lauderdale, FL, USA) for 20 s, a mortar for 5 min, and a coffee grinder (Hamilton beach, Hamilton Inc., Glen Allen, VA, USA) for 20 s at ambient conditions. In preliminary experiments, the coefficient of variation of bacterial distribution was measured to be lower than 5%, indicating the uniform distribution of *L. salivarius* in powders prepared with the developed protocol. The WSP-probiotics powders (WPP) at day 0 were sampled within 30 min after mixing. The remainder powders sealed in zip-lock bags were placed in desiccators containing silica gels and stored at RT or 4ºC for up to 30 days. The same mixing protocol was also used to prepare FDS-probiotics powders. However, FDS immediately absorbed the water in cell suspensions and became extremely sticky, which was not feasible for developing powdered *L. salivarius* and conducting further characterizations.

4.3.5 Physical properties of WSP and WPP

Freshly prepared WPP were equilibrated in a desiccator for 12 h at RT before following characterizations.

4.3.5.1 Wettability

The wettability of WSP was determined at RT according to Gaiani *et al.* (2010) with some modifications. Each WSP sample (0.1 g) was poured on 100 mL DI water in a 250 mL beaker while the stop watch was started immediately. The time required for all the powder particles to enter bulk water was recorded as the wettability index (*WI*) (Schuck et al., 2012).

4.3.5.2 Water content

About 1 g of WSP, FDS, and WPP samples were weighed and put in a convection oven (model Precision 6958, Thermo Scientific, Inc., Waltham, MA, USA) at 100°C for 24 h (Shi & Zhong, 2015). The water content of samples on wet basis (wb) was calculated according to Eq. (1). Two independent replicates were measured twice each (n = 2).

$$\text{Water content (\%wb)} = \frac{\text{Mass before drying (g)} - \text{Mass after drying (g)}}{\text{Mass before drying (g)}} \times 100 \quad (1)$$

4.3.5.3 Differential scanning calorimetry (DSC)

The T_g of WSP, FDS, and WPP was characterized using DSC (model Q2000, TA Instruments Inc., New Castle, DE, USA) according to Shi *et al.* (2015). About 3 mg of a powder sample was sealed in a hermetic aluminum pan and heated from 10 to 100ºC at a rate of 10ºC/min. Nitrogen was used as the transfer gas at a flow rate of 50 mL/min, and an empty pan was used as a reference. The results were analyzed using TA Universal Analysis 2000 software (TA Instruments, Inc., New Castle, DE, USA). Three independent replicates were measured (n = 3).

4.3.5.4 X-ray diffraction (XRD)

XRD patterns of WSP, FDS, and WPP were characterized using an Empyrean 2 diffractometer (PANalytical Inc., Westborough, WA, USA) with Ni-filtered Cu-Kα radiation at a voltage of 45 kV and 40 mA. The measurement conditions included a 2θ scanning range of 5-35°, a step size of 0.013°, and a scanning speed of 0.05°/s. Three independent replicates were measured (n = 3).

4.3.5.5 Scanning electron microscopy (SEM)

The morphology of WSP and WPP was characterized using SEM. A small amount of powder was glued onto an adhesive tape mounted on a specimen stub. The sample was then coated with gold to avoid charging in the microscope. Imaging was performed with a LEO 1525 SEM microscope (SEM/FIB Zeiss Auriga, Oberkochen, Germany) at 5,000 times of magnification.

4.3.6 Enumeration of L. salivarius

The *L. salivarius* suspension was serially diluted in PBS, and then plated on MRS agar using the spread plate method. Plates were incubated at 37 ºC for 24 h in an anaerobic chamber before enumeration. For powdered *L. salivarius*, 0.1 g of WPP sample was suspended in 10 mL PBS by vigorously vortexing for 2 min followed by dilution, anaerobic incubation, and enumeration as the cell suspension. In order to study if sucrose and WPI would influence *L. salivarius* survivability during enumeration, 10 mL of *L. salivarius* suspension at a concentration of ~10^6 CFU/mL in PBS with or without 0.1 g WSP was enumerated as above. No significant difference

between the total viable counts of *L. salivarius* in PBS and WSP treatments was observed (data not shown), indicating the bacterial enumeration results of WPP samples were not affected by the presence of sucrose and WPI.

4.3.7 Viability of L. salivarius in WPP during storage

Viable cells in WPP samples after storage in desiccators at RT or 4ºC in a walk-in cooler for 10, 20, 30, and 365 days were enumerated with the method presented in section 2.5.

4.3.8 Viability of L. salivarius in WPP after heat treatment

Powdered probiotic ingredients may be incorporated in food products undergoing thermal treatments such as pasteurization at ~70 ºC (Rodriguez- Gonzalez et al., 2015), pelleting at ~80 ºC (Wang et al., 2019), and roasting at ~100 ºC (Hinneh et al., 2019). To evaluate the thermal survivability of *L. salivarius* in WPP, freeze-dried *L. salivarius* (FDL) prepared by suspending cell pellets obtained in Section 2.2 in 100 mL DI water at a level of ~10^8 CFU/mL was used as a control.

About 0.5 g of the WPP and FDL samples individually put in an AquaLab sample cup (Decagon Devices Inc., Pullman, WA, USA) as a thin layer were heated at 80°C for 5, 15, and 30 min under the relative humidity (RH) of 40% or 26%. The former RH simulating the ambient RH (40~60%) during food processing was achieved by setting an environmental chamber (Yamato IG420U, Yamato Scientific Co., Tokyo, Japan) at 40% RH and 80 ºC. The latter RH simulated a lower RH by coating probiotic ingredients before thermal treatments (Siracusa et al., 2008). However, the 26% RH was out of the humidity range (40-95% RH) allowed by the environmental chamber. Therefore, a chamber containing a saturated magnesium chloride

solution was equilibrated in the convection oven at 80 ºC for 24 h, in which the RH was estimated to be around 26.05% according to Greenspan (1977). Within the closed chamber maintaining 26% RH, the airflow, although much slower than in the 40% RH environmental chamber, simulates conditions of coating ingredients during processing (Molina Filho et al., 2016). After heating at the 40% and 26% RH and then cooling to RT within covered sample cups, the a_w of all samples was determined (Aqualab Series 3 meter, Decagon Devices Inc., Pullman, WA, USA) to be around 0.4 and 0.26, respectively, indicating validity of the approaches to maintain constant RH. Viable cell counts of samples before and after heating were enumerated using the method in Section 2.5.

4.3.9 LIVE/DEAD® BacLight™ assay for bacterial membrane integrity

The membrane integrity of *L. salivarius* in WPP before and after heating at 80ºC for 30 min under 40% or 26% RH was evaluated using a LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit L7012 (Life Technologies Corp., Eugene, OR, USA). The *Bac*Light™ assay was conducted according to the Fluorescence Microplate Readers protocol of Molecular Probes (Anonymous, 2004). The standard curve for analyzing relative viability of *L. salivarius* was established in a Synergy 2 multi-mode reader (BioTek Instruments Inc., Winooski, VT, USA). For powdered *L. salivarius*, 1.00 g of a powder sample was suspended in 10.0 mL of 0.85% NaCl solution (~pH 6.0) and centrifuged at 4500 *g* for 10 min at 4 °C. The obtained pellet was subsequently resuspended in 10.0 mL of 0.85% NaCl solution. The bacteria with a cell concentration of about 1×10^7 CFU/mL were then stained and evaluated following the protocol (Anonymous, 2004).

4.3.10 Statistical analysis

The mean and standard deviation (SD) were calculated from three independent replicates unless noted otherwise. The one-way analysis of variance (ANOVA) was conducted using the SAS version 9.4 (SAS Institute, Cary, NC, USA). The Fisher's least significant-difference (LSD) test was used to compare differences of mean values at a significance level of 0.05.

4.4 Results and discussion

4.4.1 Wettability of WSP

The wetting behavior of WSP was studied to indicate the surface composition of WPI and sucrose. The *WI* of WSP prepared with different WSRs is shown in Figure 4-1. As expected, WSP prepared with a greater amount of sucrose showed lower *WI* due to the better solubility of sucrose (2005 g/L) (Mathlouthi & Reiser, 2012) than WPI (~900 g/L) (Ishwarya & Anandharamakrishnan, 2017) in water at RT. In addition, a nearly linear reduction of *WI* was observed with the decrease of WSR from 1:0 to 1:2, verifying the surface content of sucrose in WSP was highly correlated with the content of sucrose in the solutions prior to spray drying.

4.4.2 Physical properties of sucrose in WSP and WPP

Physical properties of sucrose in WSP before and after mixing with cell suspensions were studied using DSC and XRD. The FDS showed a glass transition at 60.06ºC (Table 4-1) which is consistent with the T_g of amorphous sucrose reported previously (Jawad et al., 2018). The T_g of amorphous sucrose has been shown to be hardly changed by additives, including polymers with a much higher T_g such as proteins (Shamblin et al., 1996). In the present study, the significantly (*P*

< 0.05) lower T_g of sucrose in WSP prepared at the WSR of 2:1 and 1:1 than FDS can be attributed to the higher water content in WSP (> 3.22%) than FDS (2.06%) as shown in Table 4-1. However, the T_g of WSP prepared at the WSR of 1:2, with the highest water content (5.82%), was not significantly (P > 0.05) different from FDS, with the possible reasons to be studied. After mixing with cell suspensions, FDS was immediately plasticized by the absorbed water to become a rubbery state which cannot be transferred into DSC pans. On the contrary, all WPP samples were still in the powdered form and the amorphous nature of sucrose was characterized with the T_g above RT, indicating the stabilization of amorphous sucrose by WPI. The T_g of sucrose in WPP was lower at a smaller WSR, in which the lowest T_g in WPP prepared at the WSR of 1:2 can again be attributed to its significantly higher (P < 0.05) water content than WPP prepared with the other two WSRs. It was demonstrated that polymers, such as polysaccharides and proteins, can increase the system viscosity and reduce the molecular mobility of amorphous sucrose, which helps to delay the crystallization of solid amorphous sucrose (Potes et al., 2012). In addition, the polar groups of WPI can absorb water via hydrogen bonding (Ji et al., 2016), thus impeding the amorphous sucrose to absorb sufficient water for physical transition.

XRD diffractograms (Figure 4-2) of WPP samples corroborated the DSC results. No crystalline peak was observed in all freshly prepared WPP samples. In addition, sucrose in WPP did not crystallize after 30-day storage (data not shown) possibly because the physical transition of sucrose was suppressed by the low moisture content in desiccators. Overall, DSC and XRD results suggest that WPI can facilitate the stabilization of amorphous sucrose in WPP with the T_g above RT, which may be important to the viability and thermal stability of powdered *L. salivarius*.

4.4.3 Morphology of WSP and WPP

SEM images of WSP before and after mixing with cell suspensions are shown in Figure 4-3. Spray-dried WPI had a spherical shape, with some collapsed, with a diameter between 1 and 10 μm and displayed a smooth surface, similar to a previous study (Khem et al., 2016). Particles of WSP with an increasing amount of sucrose transitioned from wrinkled to mostly collapsed. Particle structures are affected by the air-water interfacial composition of the atomized droplets during spray-drying (Andersson et al., 2019; Millqvist-Fureby et al., 2001). Therefore, a lower amount of surface active WPI with a higher T_g than sucrose at the air-water interface is expected to result in an increased amount of collapsed particles at a smaller WSR.

After mixing with cell suspensions, all samples showed more fragments of hollow particles, likely caused by blending and grinding during sample preparation. No particles with sharp edges (crystalline structures) were observed in WPP samples, which agreed with the XRD results about the absence of sucrose crystallinity. Additionally, agglomerated particles were observed in WPP prepared with a WSR of 1:2, probably because the T_g (28.45ºC, Table 4-1, further plasticized by water from cell suspension) was close to RT to enable the sticking of adjacent particles to reform structures (Li et al., 2019).

4.4.4 Viability of L. salivarius in WPP after preparation and during storage

The viability of *L. salivarius* in WPP after preparation (day 0) is shown in Table 4-2. Treatments with sucrose consistently showed 0.7 log CFU/g or higher of viable cells than the WPI only treatment, suggesting the better effectiveness of sucrose than WPI protecting the bacteria during dehydration. After storage in desiccators for up to 365 days (Table 4-2), the *L.*

salivarius in WPP showed significantly ($P < 0.05$) higher stability at 4 ºC than that at RT due to the suppressed metabolic activities of bacteria at a low temperature, which agreed with several studies (Dianawati et al., 2016; Huang et al., 2017). The more effective protection of sucrose on bacterial survival than WPI was also supported by the storage stability of powdered *L. salivarius* (Table 4-2). WPP prepared with a WSR of 1:1 showed ~0.5 log CFU/g and ~1 log CFU/g higher than the WPI only treatment during short-term (30-day) storage at RT and 4 ºC, respectively. After long-term (365-day) storage at RT and 4 ºC, more than 3 and 6 log CFU/g of viable *L. salivarius* in the WPI only treatment was enumerated, respectively, and WPP with sucrose had even higher *L. salivarius* viability than the WPI only treatment. It has been proposed that the high viscosity of glassy sucrose can retard molecular mobility and therefore slow down the cellular metabolic rate during storage (Huang et al., 2017). Therefore, the amorphous sucrose stabilized by WPI in WSP can improve the viability of powdered *L. salivarius* during dehydration and long-term storage through a synergistic effect.

4.4.5 Viability of L. salivarius in WPP after thermal treatment

The population of FDL control and *L. salivarius* in WPP after heating at 80ºC and 40% RH for up to 30 min is shown in Figure 4-4A. The viability of FDL became very low after heating for 5 min, suggesting the poor survivability of *L. salivarius* after thermal treatment. Comparing with FDL, the WPI only treatment maintained more viable cells after 5-min heating but was also reduced to be undetectable at longer heating durations of 10 and 30 min, indicating the limited protective effect of WPI on the survival of *L. salivarius* during heating. The survivability of *L. salivarius* in WPP with sucrose increased by > 2 log CFU/g from the FDL and WPI only treatments after heating for 10 and 30 min, suggesting the more significant role of sucrose on

protecting cells against thermal damage. The viable cells in WPP prepared with a WSR of 1:2 was not enumerated because of the stickiness and structural collapse of the powder, which can be attributed to the physical state change of sucrose due to water molecules acting as a plasticizer and high temperature during thermal treatment (Fang et al., 2013). WPP at the other WSRs did not show collapse, which can be attributed to their significantly higher T_g (Table 4-1) and the stabilization by a higher amount of WPI (Shi et al., 2013).

The viability of FDL and *L. salivarius* in WPP after heating at 80ºC and 26% RH for up to 30 min is shown in Figure 4-4B. Overall, the results had the same trend as those heated at 40% RH (Figure 4-3A), but the survival of cells was generally improved. The less abundant water molecules at lower RH can greatly reduce molecular mobility of cells and help stabilize ribosomal units against irreversible thermal damage (Syamaladevi et al., 2016). In addition, *L. salivarius* in WPP prepared with a greater amount of sucrose showed a greater improvement in thermal survival, and the WPP with a WSR of 1:2 had the highest viable cell counts after heating for 30 min, with only 2.25 log CFU/g reduction. WPP with a WSR of 1:2 showing better physical stability at 26% RH than 40% RH is likely due to the reduced amount of water molecules plasticizing amorphous sucrose in the 26% RH treatment with lower airflow. Therefore, coating probiotic ingredients before thermal processing can be an effective way to protect probiotic viability and ingredient stability.

The possible causes of thermal inactivation of bacterial cells are the destroyed higher-ordered structures of proteins, nucleic acids, and enzymes in cells (Syamaladevi et al., 2016). Amorphous disaccharides can interact with phospholipids and proteins of cellular membrane via hydrogen bonding, thus maintaining membrane integrity and protein structures of cells when

subjected to thermal treatment (Ying et al., 2012). For example, *L. casei* L61 spray-dried with glucose and sucrose showed a higher viability than those dried without sugars (Zheng et al., 2019). Additionally, glassy sucrose helps to maintain the spatial distance of membranes against compressive stress due to elevated temperatures, which can also protect the integrity of cell membranes (Santivarangkna et al., 2008). This is further studied as below.

4.4.6 Membrane integrity of L. salivarius in WPP before and after thermal treatment

The cellular membrane integrity of *L. salivarius* in WPP before and after thermal treatment was estimated using the LIVE/DEAD® *Bac*Light™ assay kit (Figure 4-5). Cells with a damaged membrane can be stained by propidium iodide (PI, red) and therefore distinguished from intact cells stained only by SYTO 9 (green) (Anonymous, 2004). Before thermal treatment, the counts of viable *L. salivarius* in WPP detected by *Bac*Light™ generally demonstrated a similar trend as that by plate-counting enumeration, with all treatments showing more than 20% intact cells. The WPP prepared with a WSR of 1:2 showed the highest percentage of viable cells (29.93%), verifying the effectiveness of sucrose on maintaining cellular membrane integrity.

After heating at 80ºC for 30 min under 40% or 26% RH, viable cells in the WPI only treatment was reduced to a level below 0%. This phenomenon is consistent with the results in Figure 4-4 and indicates the limited protection of membrane integrity by WPI during extended thermal treatments. In contrast, the cellular membrane integrity of *L. salivariu*s was greatly preserved in WPP with the presence of sucrose (Figure 4-5). The percentages of live *L. salivarius* after heating for 30 min were higher in treatments with more sucrose, further verifying that the cellular membrane integrity maintained by sucrose is critical to the enhanced survival of bacteria during thermal treatments.

4.5 Conclusion

Spray-dried WSP after mixing with a cell suspension to prepare powdered *L. salivarius* improved the survivability of bacteria through a synergistic effect. WPI stabilized the amorphous sucrose structures in WPP to enable the T_g above RT. WPP treatments resulted in higher viability after dehydration and during subsequent storage, as well as the improved thermal stability of *L. salivarius* than the WPI only treatment. Lowering the RH from 40% to 26% further improved the survivability of powdered *L. salivarius* after heating at 80ºC for 30 min, and the thermal stability of the bacteria resulted from the cellular membrane integrity maintained by amorphous sucrose. This work demonstrates a simple and scalable method to prepare protectant ingredients by utilizing the combination of sucrose to maintain cellular membrane integrity and WPI to stabilize amorphous sucrose. The WSP with improved functionality and stability compared to individual components may be used to improve the viability of powdered probiotics during processing and storage. Future studies are needed to explore mechanisms of interactions between bacteria and sucrose or WPI before realistic food applications. To improve the scalability, a ribbon mixer or alike may be used to replace multiple steps used in the present study to prepare WPP. Coating probiotic powders is another direction to develop applications in food products undergoing thermal treatments.

4.6 Acknowledgments

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Appendix

Table 4-1 Water content and glass transition temperature (T_g) estimated in differential scanning calorimetry of freeze-dried sucrose (FDS) and spray-dried powders with different WPI:sucrose mass ratios, before and after mixing with cell suspensions at a volume (mL):mass (g) ratio of 1:25.[*#]

WPI:sucrose	Before mixing		After mixing	
(w:w)	Water content (%)	T_g (ºC)	Water content (%)	T_g (ºC)
0:1 (FDS)	2.06±0.12[d]	60.06±1.84[a]	N/A[‡]	N/A[‡]
2:1	4.00±0.06[b]	53.96±1.47[bc]	4.85±0.34[b]	46.43±1.41[a]
1:1	3.22±0.27[c]	53.05±1.71[c]	4.27±0.23[b]	48.56±0.71[a]
1:2	5.82±0.25[a]	58.35±2.40[ab]	6.51±0.28[a]	28.45±0.04[b]

* Numbers are mean ± SD (n = 3). Different superscript letters indicate significant differences in the average of all samples ($P < 0.05$) within the same column.

[#] The powdered *L. salivarius* was placed in a desiccator for 12 h at RT before measurements.

[‡] After mixing with a cell suspension, FDS became too sticky to be handled for measuring water content and T_g.

Table 4-2 Viable cell counts of powdered *L. salivarius* prepared by mixing cell suspensions with spray-dried powders prepared with various WPI:sucrose mass ratios at a volume (mL):mass (g) ratio of 1:25 during 365-day storage at 4ºC or room temperature (RT, ~21ºC) in desiccators.

WPI: sucrose (w:w)		Viable cell count (Log CFU/g) *				
		Day 0[#]	Day 10	Day 20	Day 30	Day 365
1:0	4 °C	$7.67\pm0.10^{a-d}$	$7.38\pm0.15^{a-f}$	$7.60\pm0.17^{a-e}$	$7.39\pm0.12^{a-f}$	$6.09\pm0.13^{g-i}$
	RT		$7.00\pm0.15^{c-h}$	$6.93\pm0.15^{d-i}$	$6.42\pm0.11^{f-i}$	3.01 ± 0.02^{j}
2:1	4 °C	8.21 ± 0.22^{ab}	8.19 ± 0.16^{ab}	8.13 ± 0.08^{abc}	$7.98\pm0.03^{a-d}$	$6.35\pm0.07^{f-i}$
	RT		$7.68\pm0.06^{a-d}$	$6.88\pm0.17^{d-i}$	$6.44\pm0.47^{f-i}$	5.84 ± 0.08^{i}
1:1	4 °C	8.45 ± 0.14^{a}	8.20 ± 0.10^{ab}	8.25 ± 0.10^{ab}	8.20 ± 0.08^{ab}	$6.44\pm0.01^{f-i}$
	RT		$7.76\pm0.20^{a-d}$	$7.17\pm0.13^{b-g}$	$7.03\pm0.99^{c-h}$	5.91 ± 0.02^{hi}
1:2	4ºC	8.21 ± 0.10^{ab}	$7.94\pm0.10^{a-d}$	$7.95\pm0.03^{a-d}$	$7.93\pm0.11^{a-d}$	$6.51\pm0.04^{e-i}$
	RT		$7.76\pm0.20^{a-d}$	$7.17\pm0.13^{b-g}$	$7.03\pm0.99^{c-h}$	5.97 ± 0.03^{hi}

[*] Numbers are mean ± SD (n = 3). Means with different superscript letters indicate significant differences of all treatments ($P < 0.05$).

[#] The powders at day 0 were sampled within 30 min after mixing.

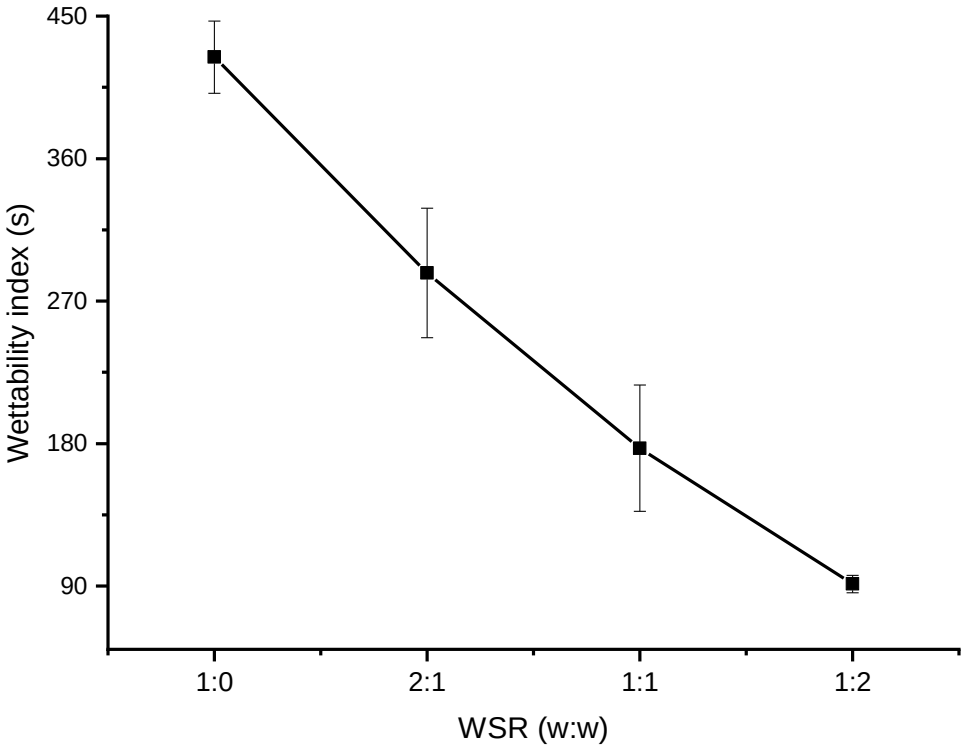

Figure 4-1 Wettability index of spray-dried powders prepared with various WPI:sucrose mass ratios (WSRs). Error bars are SD (n = 3).

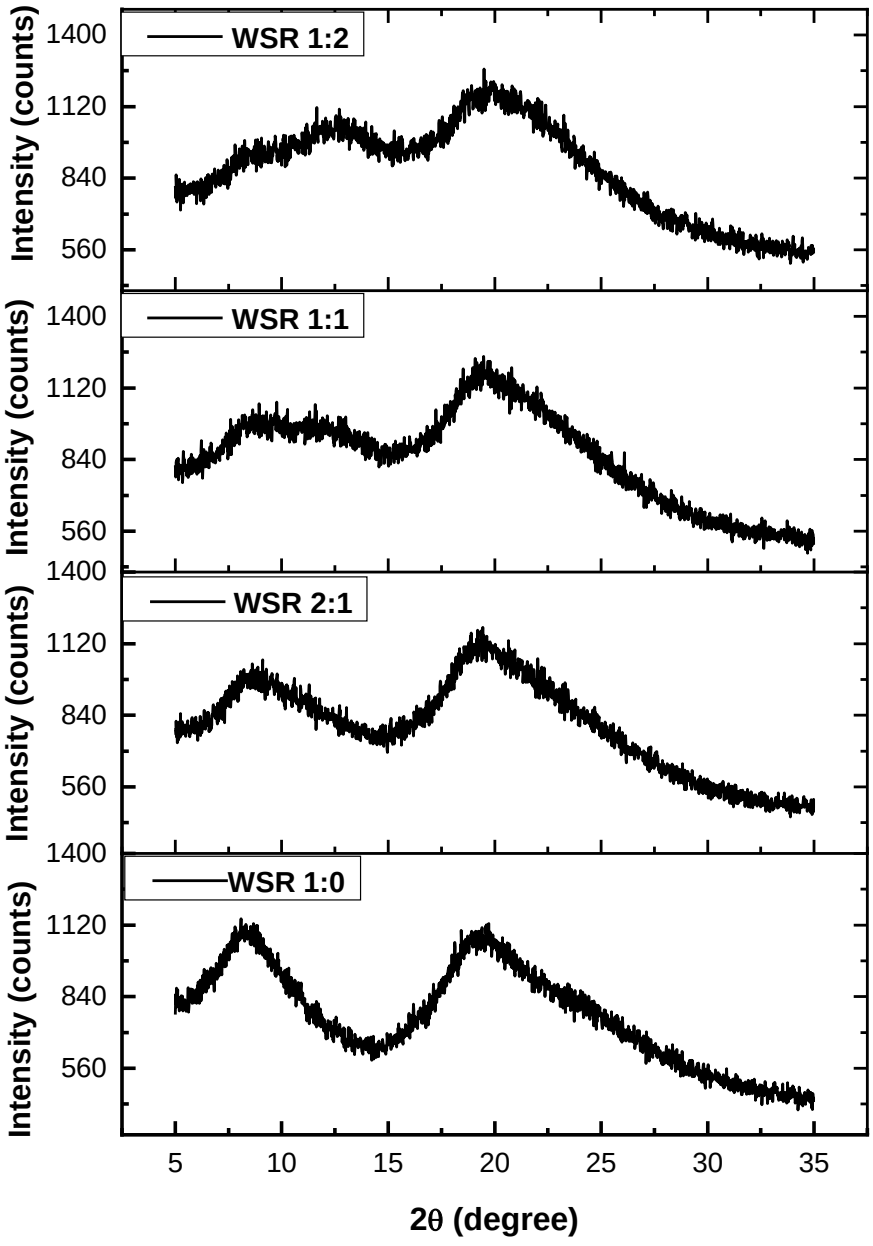

Figure 4-2 X-ray diffractograms of powdered *L. salivarius* prepared by mixing cell suspensions with spray-dried powders with various WPI:sucrose mass ratios (WSRs) at a volume (mL):mass (g) ratio of 1:25.

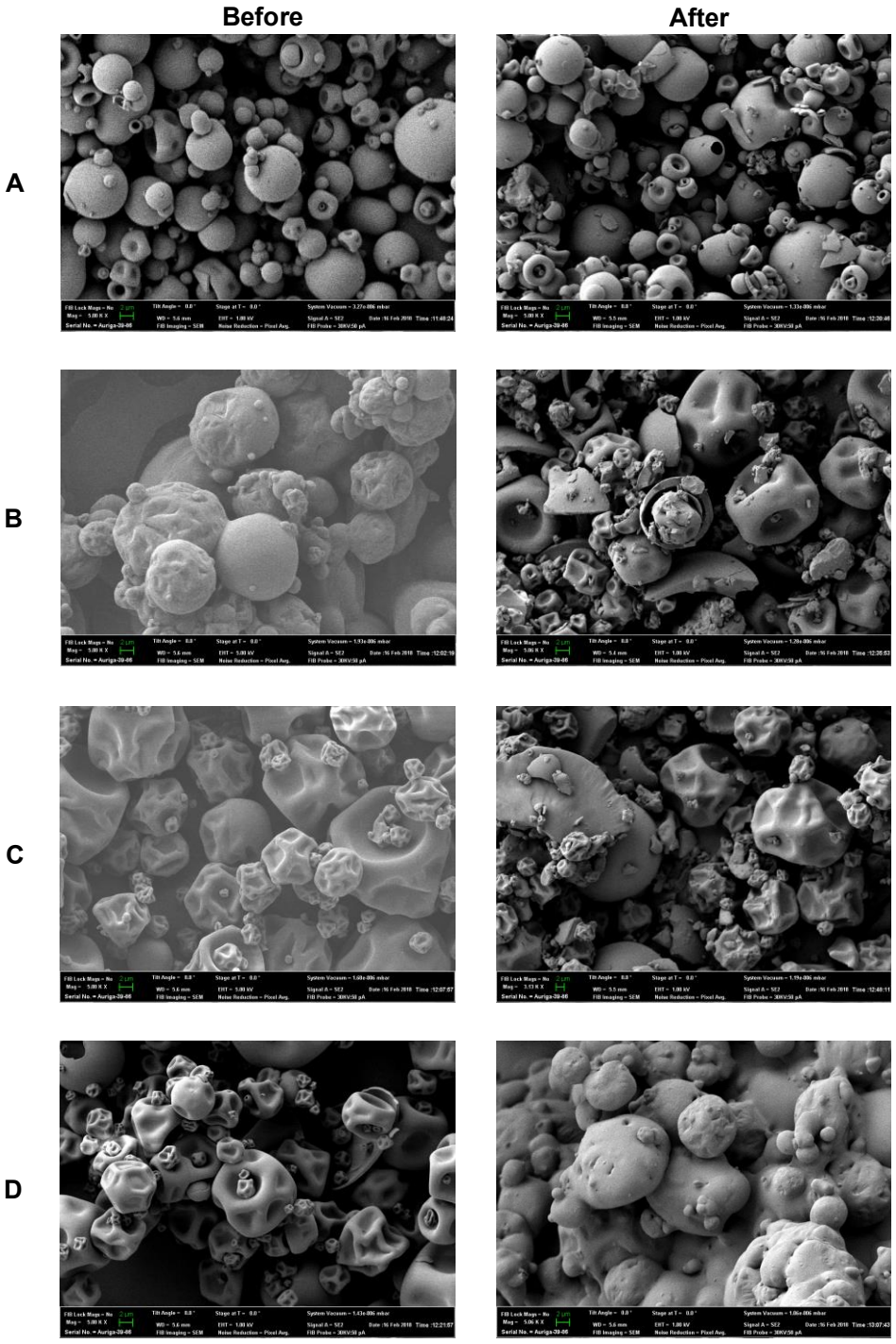

Figure 4-3 Scanning electron micrographs of spray-dried powders prepared at a WPI:sucrose mass ratio of 1:0 (row A), 2:1 (row B), 1:1 (row C), or 1:2 (row D) before mixing (left panel) and after mixing (right panel) with cell suspensions at a volume (mL):mass (g) ratio of 1:25.

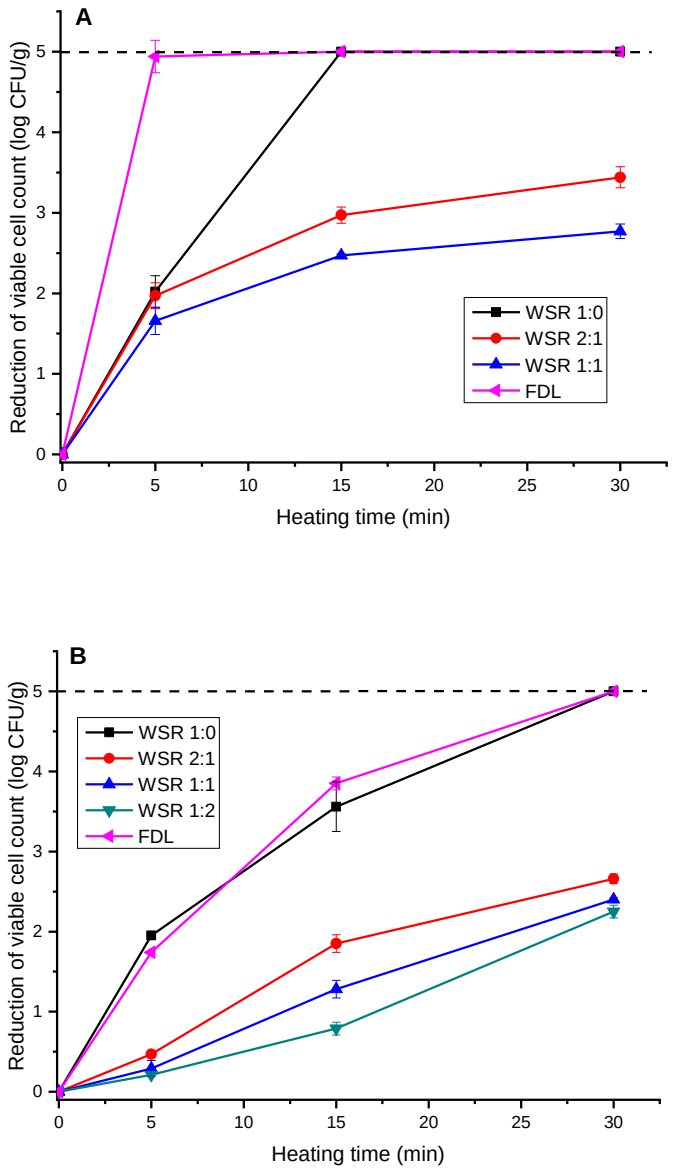

Figure 4-4 Reduction of freeze-dried *L. salivarius* (FDL) and powdered *L. salivarius* prepared by mixing cell suspensions with spray-dried powders with various WPI:sucrose mass ratios (WSR) at a volume (mL):mass (g) ratio of 1:25 after heating at 80ºC for up to 30 min under 40% (A) or 26% RH (B). The dashed line shows no viable cells were detected using the plating method with a detection limit of 3 log CFU/g. Error bars are SD (n = 3).

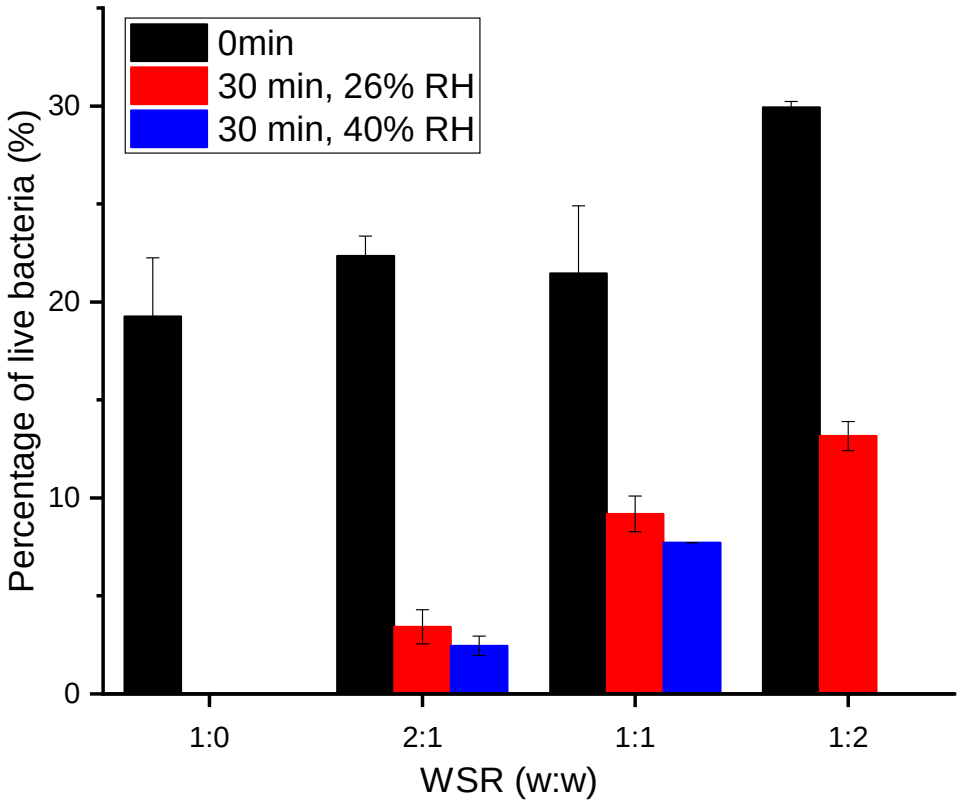

Figure 4-5 Percentage of viable *L. salivarius* with integral membranes in the powders, determined using the LIVE/DEAD® *Bac*Light™ assay, before (0 min) and after heating at 80ºC for 30 min under 26% or 40% RH. Probiotics powders were prepared by mixing cell suspensions with spray-dried powders with various WPI:sucrose mass ratios (WSR) at a volume (mL):mass (g) ratio of 1:25. The percentage of viable bacteria in the treatment at a WSR of 1:0 after heating at 80ºC for 30 min was reduced to a level below 0%. Error bars are SD (n = 3).

Chapter 5 Enteric rice protein-shellac composite coating to enhance the viability of probiotic *Lactobacillus salivarius* NRRL B-30514

5.1 Abstract

This study reports a novel modified rice protein (MRP)-ammonium shellac (NH_4SL) enteric composite coating on millimeter-sized pellets to protect the survival of probiotics during storage, thermal treatments, and simulated gastrointestinal (GI) digestions. An aqueous MRP solution at pH 7.0-13.0 was dropwise added into an aqueous ethanol NH_4SL solution at pH 8.2, and the mixture pH significantly affected the homogeneity of MRP-NH_4SL suspensions and formed films. The MRP_{pH13}-NH_4SL suspension with pH of 9.4 had smaller MRP particles and thus better stability than other suspensions with pH of ~8.4, predominantly due to the better solubility and stability of MRP at a higher pH. Atomic force microscopy, fluorescence spectroscopy, and dynamic light scattering results indicated the complexation between MRP and NH_4SL in all treatments, which increased the intermolecular repulsions to further facilitate the stability of MRP_{pH13}-NH_4SL suspension. The homogenous MRP_{pH13}-NH_4SL suspension resulted in smooth films with improved mechanical and enteric properties at a higher content of MRP having a pH-dependent solubility. Probiotics pellets coated with MRP-NH_4SL had significantly more viable *Lactobacillus salivarius* NRRL B-30514 than uncoated pellets after 30-day ambient storage, heating at 80 ºC for 20 min, and during simulated GI digestions. The composite coating also preserved the probiotics viability better than the NH_4SL-only coating after 2-h gastric digestion. Therefore, MRPs can be used to modify the enteric properties of shellac-based edible coatings to deliver powdered probiotics, which is significant to manufacturing solid probiotics-containing products.

Keywords: shellac; modified rice protein; enteric coating; probiotics; delivery

5.2 Introduction

Shellac (SL, MW of ca. 1000 Da) is a natural and biodegradable resin from lac insects (*Kerria lacca*) and is a mixture of polyesters consisting of mainly sesquiterpenoid acids (with the major one being shellolic acid) esterified with hydroxy fatty acids (with the major one being aleuritic acid) (Al-Gousous et al., 2015; Farag & Leopold, 2009). SL is highly soluble in ethanol and capable of forming films with high gloss and poor permeability to water vapor and gases (Pearnchob et al., 2003). SL has a high p*K*a value of 6.9-7.5, which results in the insolubility at highly acidic gastric pH but solubility at neutral intestinal pH (Limmatvapirat et al., 2007). The solubility characteristics of SL were primarily used for enteric delivery of nutraceuticals and pharmaceuticals that are degraded at gastric conditions (Penning, 1996). However, the use of SL as an enteric coating has significantly declined in recent decades, mainly caused by the continued polymerization and esterification among the hydroxyl and carboxyl groups of SL during film aging that cause a failure of SL coating to effectively dissolve at neutral pH (Limmatvapirat et al., 2004; Limmatvapirat et al., 2008).

Several strategies have been studied to modify the SL disintegration properties. Deprotonating carboxyl groups to prepare SL salts has been studied to impede polymerization process to improve film solubility at neutral pH. For example, films prepared from SL succinate were completely dissolved at pH 7.0 within 7 min, which was about 16 times faster than those prepared from SL in the acid form (Limmatvapirat et al., 2008). Furthermore, the dissolution properties of SL films are a function of the specific SL salt form (Al-Gousous et al., 2015), exemplified by highly water soluble potassium SL films that disintegrated even at acidic pH and thus lost the enteric feature (Al-Gousous et al., 2015). Incorporation of sorbic acid or

hydroxypropyl methylcellulose has also been reported to improve the disintegration of SL-coated soft gelatin capsules in simulated intestinal fluids while retaining gastric resistance (Pearnchob et al., 2004). Furthermore, fabrication of zein-SL complexes by antisolvent precipitation increased the release rate of encapsulated curcumin by about 30% when compared to that of curcumin in SL only treatment after simulated intestinal digestion (Sun et al., 2017). However, it is unknown if these composite particles have the enteric features after solvent evaporation to prepare coatings. Therefore, SL-based enteric composite coatings incorporated with generally-recognized-as-safe materials still need to be investigated for food applications.

Rice proteins (RPs) are known for potential hypoallergenicity and high nutritive values (Fabian & Ju, 2011) but have low water solubility due to high glutelin content (~80%) (Xia et al., 2012). Recently, Wang *et al.* (2015) modified the solubility of RPs in steps of suspension in an alkaline solution at pH 12.5, incubation at -20 ºC for 24 h, and milling to unfold protein and expose interior hydrophilic groups. The obtained modified RPs (MRPs) exhibit a pH-depend solubility with marginal solubility at acidic pH and a dramatically increased solubility from pH 6.0 to 7.0, which is desirable for designing enteric delivery systems (Wang, Liu, et al., 2015). MRPs were found to deposit on the surface of self-emulsified eugenol droplets through hydrophobic binding to control the release of encapsulated caffeic acid phenethyl ester (Wang et al., 2017). The MRP shell precipitated on soybean oil droplets was also reported to enable the limited release of encapsulated β-carotene during *in vitro* gastric digestion and the sustained release in subsequent intestinal digestion (Wang et al., 2016). Therefore, MRPs with the unique pH-dependent solubility may be used to prepare enteric composite films with SL to improve the disintegration properties, which, however, has not yet been studied.

One such coating application is for probiotics that may be deactivated at gastric conditions. Probiotics are live microbial species that have been fortified in functional foods to confer many beneficial effects in human, including maintaining intestinal microbial balance, enhancing immune system, and reducing gastrointestinal (GI) disorders (Ramos et al., 2018; Sarao & Arora, 2017). Compared to probiotics in liquid preparations that are susceptible to environmental stresses, such as pH, temperature, water activity (a_w), and oxygen (Liu et al., 2017; Papadimitriou et al., 2016), probiotics in the powdered form with low a_w are metabolically suppressed and thus can survive better in harsh conditions (Fu et al., 2018). Our recent study found that powdered *Lactobacillus salivarius* NRRL B-30514 prepared by mixing a concentrated cell suspension with spray-dried whey protein isolate (WPI)/sucrose powder maintained up to 6 log CFU/g viable cells after 12-month storage at 4 ºC or heating at 80 ºC for 30 min (Wang et al., 2020). However, the majority of cells adhered on the powder surface and may be inactivated after being exposed to harsh conditions in the GI tract. In a separate study, encapsulation of spray-dried *L. salivarius* in soybean oil emulsified with sugar beet pectin, solid/oil/water (S/O/W) emulsion, further cross-linked by divalent calcium ions, improved bacterial viability during *in vitro* GI digestions (Zhang et al., 2016b). These S/O/W emulsions might be suitable to formulate liquid probiotic products, which, however, usually have a short shelf-life and require refrigerated storage (Zhang et al., 2016a). Therefore, novel and scalable approaches to provide effective protection on powdered probiotics from processing to digestion need to be developed for convenient applications in solid probiotics-fortified food matrices.

The hypothesis of the present work is that enteric coating on millimeter-sized probiotic pellets prepared by direct compression of powdered probiotics ingredients can protect probiotics

during storage, thermal treatment, and simulated GI digestion. Direct compression with or without excipients to prepare probiotics pellets has been widely studied as a simple, inexpensive, and scalable method to supplement probiotics in the solid form for food and pharmaceutical applications (Chan & Zhang, 2002; Iniesta et al., 2012; Klayraung et al., 2009). The enteric coating can act as a moisture, oxygen, and mechanical barrier to protect probiotics in pellets against environmental stresses during storage, thermal processing, and gastric digestion to release probiotics in intestines. Furthermore, pellets with a millimeter dimension can be sprinkled on a solid food matrix such as snack bars. More importantly, considering the size threshold of ~1.4 mm during swallowing nut particles (Prinz & Lucas, 1995), small pellets may be directly swallowed to avoid mastication and therefore structural damage during oral processing (Jalabert-Malbos et al., 2007).

The first objective of this work was to study the possibility of preparing homogenous coating suspensions by stabilizing MRPs in alkaline aqueous ethanol solutions of ammonium SL (NH_4SL). Although SL can be liquidized by dissolving in aqueous alkaline solutions or melting at >77 ºC (Goswami, 1979), the major components (WPI and sucrose) of probiotics pellets can be dissolved in alkaline solutions and the high temperature can deactivate bacteria. Conversely, a brief immersion of pellets in alcoholic coating suspensions may not be detrimental to bacterial viability (Chambers et al., 2006). The second objective was to prepare and characterize physical, mechanical, and enteric delivery properties of films casted from MRP-NH_4SL coating suspensions formulated with various MRP concentrations. The third objective was to evaluate the potential of MRP-NH_4SL composite coating in improving the viability of powdered *L. salivarius* NRRL B-30514 in millimeter-sized pellets during storage, thermal treatment, and

simulated GI digestion. This study presents a novel SL-based enteric coating system for stabilizing and delivering powdered probiotics in small pellets, which may enable a convenient, scalable, and affordable way to supplement probiotics in solid food matrices with extended shelf-life.

5.3 Materials and methods

5.3.1. Materials

SSB® 55 Pharma SL flakes were kindly provided by Stroever GmbH & Co. (Bremen, Germany). MRP powder was kindly provided by Dr. Tao Wang in Jiangnan University (Wuxi, Jiangsu, China). de Man, Rogosa, and Sharpe (MRS) broth medium and agar (dehydrated) were from Oxoid Ltd (Altrincham, Cheshire, England). Unless noted, other chemicals were products of either Sigma-Aldrich Corp. (St. Louis, MO, USA) or Thermo Fisher Scientific, Inc. (Pittsburgh, PA, USA).

5.3.2. Preparation of MRP-NH_4SL suspensions

SL was dissolved at 20% (w/v) in ethanol at room temperature (RT, ~21 ºC) by stirring for overnight. A 2.0 M aqueous $(NH_4)_2CO_3$ solution was then added to adjust the suspension pH to ~ 8.2 with a final ethanol concentration of 90% (v/v) (Hagenmaier & Shaw, 1991). After centrifugation at 4000 *g* for 10 min at RT (Sorvall LYNX 6000, Thermo Scientific Company, Waltham, MA, USA), 10.0 mL of the supernatant was transferred into a vial. The 3.0% (w/v) MRP solution was prepared according to Wang *et al.* (2015) and adjusted to pH 7.0, 9.0, 11.0 or 13.0 using 0.10 M KOH, before dropwise addition of 1.0 mL MRP solution in the NH_4SL

aqueous ethanol solution with gentle stirring, and the corresponding mixture samples were termed as MRP_{pH7}-NH_4SL, MRP_{pH9}-NH_4SL, MRP_{pH11}-NH_4SL, and MRP_{pH13}-NH_4SL, respectively. The mixture pH was measured immediately after preparation. The MRP only treatments were prepared at the same pH as MRP-NH_4SL suspensions by adding 1.0 mL of 3.0% (w/v) MRP solution at pH 8.0 or 9.0 into 10.0 mL of 90% (v/v) aqueous ethanol at ~pH 8.2 followed by adjusting to the final pH using 0.10 M KOH. The NH_4SL only treatments were prepared similarly to MRP-NH_4SL suspensions by substituting the MRP solution with deionized water followed by pH adjustment using 0.10 M KOH. The physical stability of suspensions was observed after incubation at RT for up to 6 h.

5.3.3 Particle size and zeta (ζ)-potential measurement

Particle size distribution and ζ-potential of suspensions were measured using a Zetasizer Nano-ZS90 instrument (Malvern Instruments Ltd., Worcestershire, UK). The Z-average mean hydrodynamic dimeter (D_h) was calculated from the Stokes-Einstein equation. The ζ-potential of suspensions was calculated using the Henry equation through electrophoretic mobility measurements.

5.3.4 Morphological properties

The morphology of MRP-NH_4SL, MRP only, and NH_4SL only samples was characterized using atomic force microscopy (AFM, model Multimode 8, Bruker Corp., Santa Barbara, CA, USA). All samples were diluted 100 times to a NH_4SL concentration of 0.16% (w/v) or an MRP concentration of 0.0027% (w/v) using 81.8% (v/v) aqueous ethanol adjusted to the corresponding sample pH. After dropping 20 μL of each diluted sample onto a freshly cleaved mica sheet

mounted on a sample disk (Bruker Corp., Santa Barbara, CA, USA) and spinning using a P6700 spin coater (Specialty Coating Systems Inc., Indianapolis, IN, USA) for even spreading of the droplet, samples were dried at ambient conditions for at least 2 h. Then, samples were scanned at the tapping mode using a rectangular cantilever having a silicon tip on nitride lever (Bruker Corp., Camarillo, CA, USA) and a quoted force constant of 0.4 N/m. Images were generated with a preset scan area of 2.0 × 2.0 μm at a scanning speed of 1 Hz, and the height properties were analyzed using the NanoScope Analysis software (Bruker Corp., Santa Barbara, CA, USA).

The structure of undiluted suspensions was studied using confocal laser scanning microscopy (CLSM). MRP and NH_4SL solutions were stained with 10.0 mg/mL fluorescent isothiocyanate (FITC) and 1.0 mg/mL Nile red ethanol solutions, respectively, to a fluorophore concentration of 4.0 μg/mL before preparing suspensions as Section 2.2. The microscope (model Leica TCS SP8, Leica Microsystems, Heidelberg GmbH, Germany) was equipped with a white light supercontinuum laser at an excitation wavelength of 488 nm and 555 nm for FITC and Nile red, respectively (Martinez & Henary, 2016; Wang, Hu, et al., 2015). Images were analyzed using the LAS X software (Leica Microsystems, Heidelberg GmbH, Germany).

5.3.5 Fluorescence measurement

To study the intrinsic fluorescence intensity of MRPs as affected by solvent and system pH, 3.0% (w/v) aqueous MRP solutions and MRP only aqueous ethanol suspensions prepared as Section 2.2 were respectively diluted with KOH solution and 81.8% (v/v) aqueous ethanol both of which were adjusted to the same corresponding pH to fit within the instrument sensitivity range. To study the interactions between MRPs and NH_4SL, MRP-NH_4SL suspensions were prepared as Section 2.2 using solutions with 0, 5, 10, 15, and 20% (w/v) SL dissolved in ethanol.

These samples were subsequently diluted 100 times using 81.8% (v/v) aqueous ethanol adjusted to the corresponding pH to reach the instrument sensitivity range. The emission spectra of MRPs in all samples were recorded using a LS 55 fluorescence spectrometer (PerkinElmer Inc., Waltham, MA, USA) from 300 to 500 nm with an excitation wavelength of 280 nm. The slit width was set at 10 nm for both excitation and emission.

5.3.6 Preparation of MRP-NH_4SL films

The film-forming MRP-NH_4SL suspensions were prepared as in Section 2.2 by adding 0, 0.5, 1.0, and 3.0% (w/v) MRP solutions at the optimized pH into 18% (w/v) NH_4SL solution in 90% (v/v) aqueous ethanol at ~pH 8.2. Glycerol was added at 0.5% (v/v) into NH_4SL solutions as a plasticizer. Adapted from the method of Alkan *et al.* (2011), 4.0 mL of the prepared $MRP_{0\%}$-NH_4SL, $MRP_{0.5\%}$-NH_4SL, $MRP_{1\%}$-NH_4SL, and $MRP_{3\%}$-NH_4SL mixtures were immediately poured into a Fisherbrand^{TM} polystyrene antistatic weighting dish (8.9 cm in diameter). After drying in a desiccator containing a saturated lithium chloride solution at 11% relative humidity (RH) and RT for 24 h, the films were peeled off and aged in a desiccator containing a saturated magnesium nitrate solution at 50% RH and RT for at least 2 days before further study.

5.3.7 Characterization of MRP-NH_4SL films

5.3.7.1 Scanning electron microscopy (SEM) analysis

The morphology of film surface and cross-sections was imaged using SEM. Samples were mounted on a specimen stub using a double-sided adhesive tape and then coated with gold to

avoid charging in the microscope. Imaging was performed with a LEO 1525 SEM microscope (SEM/FIB Zeiss Auriga, Oberkochen, Germany) at 1.71 K times of magnification.

5.3.7.2 Color and opacity

Color and opacity of films were measured using a MiniScan XE Plus Hunter colorimeter (Hunter Associates Laboratory, Inc., Reston, VA). Color of films was measured for lightness (*L*) and chromaticity parameters *a* (red-green) and *b* (yellow-blue) in the Hunter Lab scale. Color measurements were performed over the standard white tile. Opacity was measured over the standard white tile and black glass. For each independent replicate, two film replicates were measured, and each tested in duplicates.

5.3.7.3 Mechanical properties

Tensile strength (TS) and elongation at break (EB) of films were determined using a TA.XTplus Texture Analyzer (Texture Technologies Corp., Scarsdale, NY) in the tensile mode (Ma et al., 2016). Films were cut into 5 cm × 1 cm strips, and the initial gap and test speed were set as 4 cm and 1 mm/s, respectively. The TS and EB values were calculated using Eqs. (1) and (2), respectively. For each independent replicate, the measurements represent an average of four samples.

$$\text{TS (MPa)} = \frac{F}{S} \quad (1)$$

$$\text{EB (\%)} = \frac{\Delta l}{l_0} \times 100 \quad (2)$$

Where, F is the maximum force (N) and S is the cross-section area of each film (mm^2). Δl and l_o are the extension of the film at break (mm) and the original test length of the film (mm).

5.3.7.4 Water vapor permeability (WVP)

The WVP of films was determined by measuring mass changes of Fisher/Payne permeability cups (Fisher Scientific, Pittsburgh, PA) during incubation at RT, as reported previously (Ma et al., 2016; Zhai et al., 2018) with some modifications. Cups were filled with ~6.0 g dried silica gels (0% RH), sealed with films, and placed in a desiccator with 50% RH controlled by a saturated sodium bromide solution. The cup mass was measured daily for 7 days. The values of water vapor transmission rate (WVTR) and WVP were calculated using Eqs. (3) and (4), respectively. For each independent replicate, measurements were performed using two film replicates for each formulation.

$$\text{WVTR}\ (\text{g/m}^2 \cdot \text{h}) = \frac{\Delta m}{A \times t} \quad (3)$$

$$\text{WVP}\ (\text{g/m} \cdot \text{Pa} \cdot \text{h}) = \text{WVTR} \times \frac{x}{\Delta P} \quad (4)$$

Where, Δm is the weight gain of the cup (g), A is the exposed area (m^2), t is the time (h), x is the film thickness (m) measured using a digital microcaliper (Mitutoyo Corp., Kawasaki, Japan) with 0.001 mm precision, and ΔP is the partial water vapor pressure difference across the film (1583.7 Pa at 25 °C) (Zhai et al., 2018).

5.3.7.5 Disintegration test

The disintegration test of films cut into 1 cm × 1 cm was performed based on the United States Pharmacopoeia (2012) with some modifications. The test was composed of a 2-h stage where films were individually immersed into 2.0 mL of 0.1M HCl at 37 ºC followed by a 4-h stage where films were transferred into 2.0 mL of 0.1 M phosphate-buffered saline (PBS, pH 7.0) at 37 ºC. The images of films after each stage were recorded using an optical microscope

(BX51, Olympus, Tokyo, Japan) equipped with a digital camera (DP 70, Olympus, Tokyo, Japan).

The release profile of Rhodamine B (RB) from films at the above disintegration conditions was tested complementarily to study the disintegration properties of films. RB-loaded films were prepared by adding 1% (w/v) RB into MRP solutions at a volume ratio of 1:10 before preparing MRP-NH_4SL films as in Section 2.6 (Fujii et al., 1995). After each stage of the disintegration test, the mixtures were centrifuged at 300 *g* for 5 min (MiniSpin Plus centrifuge, Eppendorf Inc., Hauppauge, NY, USA) to precipitate film flakes and then the supernatant was mixed with ethanol at a volume ratio of 1:1 to completely dissolve the released RB. After centrifugation at 13,000 *g* for 5 min, the amount of released RB was determined by measuring the absorbance of the supernatant at 555 nm using an Evolution 201 UV-vis spectrophotometer (Thermo Scientific, Waltham, MA, USA) (Yuan et al., 2014).

5.3.8 Preparation of millimeter-sized probiotic pellets with and without MRP-NH_4SL coating

The WPI/sucrose-probiotics powders (WSPPs) prepared at the WPI:sucrose mass ratio of 1:1 as reported previously (Wang et al., 2020) were used in this study due to the high viability and thermal stability. Subsequently, to prepare probiotic pellets, the powdered probiotics were subjected to direct compression using a KBr pellet maker for FTIR analysis (Thermo Nicolet Corp., Madison, MI, USA). Around 0.1 g WSPPs at the a_w of ~1.3 were loaded into 7 mm die set and pressed using a press handle. The developed pellets were further cut into small cubic pellets with a side of ~1.75 mm using a multiple pill splitter (Cibolo Press LLC., Houston, TX, USA). The millimeter-sized pellets were randomly assigned to three treatments (n = 5): (1) uncoated pellets, (2) coated with $MRP_{0\%}$-NH_4SL suspensions, and (3) coated with $MRP_{3\%}$-NH_4SL

suspensions. To develop coating, pellets were dipped in a coating suspension for 10 s, put on a stainless steel net, and dried in a model Precision 6958 convection oven (Thermo Scientific Inc., Waltham, MA, USA) at 25 ºC for 60 min to ensure dryness (Fajardo et al., 2010). The pellets were turned over and dipped in the suspension for a second time, followed by drying in the same way to improve uniformity of coating. The mass, thickness, and *L. salivarius* viability of pellets before and after coating were measured.

5.3.9. Viability of L. salivarius in pellets during storage, thermal treatment, and simulated GI digestions

To evaluate storage stability under simulated retail conditions, viable cells in coated and uncoated pellets after storage in a desiccator containing a saturated potassium carbonate solution at 43% RH and RT for 10, 20, and 30 days were enumerated (Quodbach & Kleinebudde, 2015). To measure thermal stability, a single pellet was put in an AquaLab a_w measurement sample cup (METER Group, Inc., Pullman, WA) and heated at 80 °C and 60% RT for up to 20 min in an environmental chamber (Yamato IG420U, Yamato Scientific Co., Tokyo, Japan). To test the viability of probiotics during simulated GI digestion, coated and uncoated pellets were individually immersed in 2.0 mL of the simulated gastric fluid (SGF) composed with 1.0 mg/mL pepsin in 0.01 M HCl at pH 2.0. After incubation at 37 ºC for 2 h in a water bath (New Brunswick Scientific Co., Edison, NJ, USA) with mild shaking, the samples were mixed with 2.0 mL of 0.1 M PBS to adjust pH to 7.0 before enumeration. To simulate intestinal digestion, the samples after the SGF digestion were mixed with 2.0 mL of the simulated intestinal fluid (SIF) formulated with 4.0 mg/mL bile extract, 2.0 mg/mL pancreatin, and 1.0 mg/mL lipase in 0.1 M

PBS at pH 7.0. After incubation at 37 ºC in the water bath with mild shaking for 2 and 4 h, the digesta was placed on ice to stop the pancreatic reaction before enumeration.

L. salivarius in pellets before and after the above experiments were enumerated using the spread plating method. Each pellet after the storage and thermal stability tests was vigorously vortexed with 10.0 mL PBS to prepare a suspension, while the collected digesta was directly vortexed to dissolve the residual pellets. The obtained cell suspensions were serially diluted in PBS and plated on MRS agar for anaerobic incubation, enabled by GasPak™ EZ anaerobe container system sachets with indicator (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) in an anaerobic jar, at 37 °C for 24 h before enumeration.

5.3.10. Statistical analysis

Unless noted otherwise, the mean and standard deviation (SD) were calculated from results of three independent replicates. One-way analysis of variance (ANOVA) with the Fisher's least significant-difference (LSD) was conducted using the SAS version 9.4 software (SAS Institute, Cary, NC, USA) to determine differences between treatment mean values at a significance level of 0.05.

5.4 Results and discussion

5.4.1. Characteristics of MRP-NH_4SL suspensions and films prepared with MRP solutions at different pH

The pH and ζ-potential of MRP-NH_4SL suspensions prepared with MRP solutions at different pH are shown in Table 5-1. The aqueous ethanol solution of NH_4SL at pH 8.2 had a

negative ζ-potential of -18.1±0.9 mV due to the deprotonation of carboxyl groups of SL (Luangtana Anan et al., 2007). The addition of MRP solution at higher pH to NH_4SL solution significantly ($P < 0.05$) increased the mixture pH to a greater extent, and the increase of pH from 8.6 to 9.4 when the pH of MRP solution increased from 11.0 to 13.0 was caused by the rapid increase of pH near the equivalence point (~pH 9.6), as determined during titration of SL with KOH (Figure 5-1). As expected, the mixture suspensions were more negatively charged at higher pH, and the ζ-potential magnitude was all above 20. The particle size distribution (Figure 5-1A) of MRP_{pH7}-NH_4SL, MRP_{pH9}-NH_4SL, and MRP_{pH11}-NH_4SL suspensions showed multiple peaks with an increased proportion of large particles at a lower pH, which resulted from protein aggregation evidenced by the visual precipitation after incubation at RT for 6 h (Figure 5-1B). The MRP_{pH13}-NH_4SL treatment had the smallest particles with the narrowest distribution (Figure 5-1A), corresponding to the absence of precipitation (Figure 5-1B) and an increase of D_h from 390 ± 37 nm to 820 ± 70 nm after 6 h at RT. Nevertheless, precipitation of MRPs was observed in all treatments after overnight storage at RT, indicating the ζ-potential magnitude was insufficient to prevent aggregation of MRPs. These observations suggest that MRPs are not soluble at the studied solvent conditions but may be temporarily suspended for film preparation.

The kinetic dispersibility of MRPs in the mixture determined the properties of films (Figure 5-1B). The surface of films casted from the MRP_{pH13}-NH_4SL suspension was homogenous, continuous, and smooth because the small D_h enabled the suspension of MRPs during drying. Conversely, the quick aggregation of MRPs in other treatments resulted in heterogeneous and rough film surfaces, corresponding to a phase-separated matrix observed in SEM (Figure 5-1C). A lack of structural homogeneity can adversely influence the barrier and mechanical properties

of films to limit their performance in realistic applications (Galus & Kadzińska, 2016). Results in Figure 5-1 suggest that the pH of MRP-NH_4SL mixture suspensions, controlled by the pH of MRP solution in the present study, plays a significant role in suspending MRPs and forming films with varied functionalities.

5.4.2. Effects of pH on the solubility and structures of MRP in aqueous ethanol

The effects of pH on MRP solubility in aqueous ethanol was studied to understand possible complexation properties in MRP-NH_4SL mixtures. The pH of 8.4 and 9.4 was chosen to represent the poorest and best solubility of MRPs in the MRP-NH_4SL mixtures (Table 5-1, Figure 5-1), respectively. When the pH was increased from 8.4 to 9.4, the ζ-potential and D_h of MRPs significantly ($P < 0.05$) decreased from -15.3 ± 1.0 to -18.5 ± 0.5 mV and from 1223 ± 151 to 219 ± 6 nm, respectively, with smaller particles at pH 9.4 (Figure 5-3A). These results indicate better solubility of MRPs at a higher pH, which was further confirmed in AFM (Figure 5-3B). The MRP particles were mostly spherical and had a narrow height distribution from 0 to 2 nm at pH 9.4, while large irregular structures with a height of up to 6 nm were observed at pH 8.4. CLSM imaging of the undiluted MRP suspensions showed individual and spherical MRP particles at pH 9.4 but irregular and heterogenous aggregates at pH 8.4, consistent with the visual appearance of the two treatments after 1 h incubation at RT (Figure 5-3C). Therefore, MRPs had a better solubility and stability in aqueous ethanol at a higher pH. Addition of ethanol to water can lower the ionization of carboxyl and amino groups and therefore weaken the intermolecular electrostatic repulsion, leading to the reduced solubility of many water-soluble proteins in aqueous ethanol (Damodaran, 2017; Zhou & Pang, 2018). Differences in ionization properties of amino acids in aqueous ethanol can also change protein overall charge, as reported for the

increased isoelectric point of casein at an increased ethanol concentrations due to the greater effects on carboxyl groups than amino groups (Mezdour et al., 2006). The particle dimension and ζ-potential of MRPs as affected by pH confirmed the above speculation that the differences on MRP-NH_4SL mixtures in Figure 5-1 were mainly caused by MRPs.

The effects of pH on MRP structures were further investigated using fluorescence spectroscopy at the excitation wavelength of 280 nm (Figure 5-3D). In aqueous solutions, MRPs at pH 8.4 and 9.4 exhibited a similar emission peak at 353 nm due to the fluorescence emission of tryptophan (Trp) and tyrosine (Tyr) residues (Bortolotti et al., 2016). Whereas, in 81.8% (v/v) aqueous ethanol, the maximum emission wavelength of MRPs distinctly blue-shifted to 341 and 346 nm at pH 8.4 and 9.4, respectively, and the fluorescence intensity increased appreciably. These phenomena result from the increased exposure of Trp and Tyr residues due to conformational changes of MRPs in a less polar solvent (Chattoraj et al., 2014; Faizullin et al., 2017). Furthermore, the maximum fluorescence intensity of MRPs increased and red-shifted as the aqueous ethanol pH increased from 8.4 to 9.4, reflecting the increased polarity around Trp and Tyr (Faizullin et al., 2017). This may be related to a dimer-monomer transition that favored the MRP stability in aqueous ethanol at an increased pH (Renard et al., 1998). Taken together, MRPs had a better solubility and stability in 81.8% (v/v) aqueous ethanol at a higher pH, which can play a significant role in suspending MRP in the MRP-NH_4SL mixtures.

5.4.3. MRP-NH_4SL complex structures studied with AFM and fluorescence spectroscopy

The structures of MRP-NH_4SL suspensions imaged using AFM are shown in Figure 5-4A. All treatments showed more than one type of structures based on morphology and height information. Large spherical or irregular aggregates with a height of 30~40 nm were observed in

MRP_{pH7}-NH_4SL, MRP_{pH9}-NH_4SL, and MRP_{pH11}-NH_4SL treatments, whereas smaller and more regular particles with a smaller height of less than 10 nm were discretely distributed for the MRP_{pH13}-NH_4SL treatment. The monolayer coverage on mica surface observed in all samples was likely due to the self-assembled SL (Figure 5-5) driven by lateral intermolecular hydrogen bonding (Benítez et al., 2008). The AFM results agree with the D_h data in Figure 5-1A and CLSM images of the undiluted MRP-NH_4SL suspensions in Figure 5-6 where the MRP_{pH13}-NH_4SL treatment had small and spherical particles, but coarse aggregates were evident in other treatments. The larger structures of MRP-NH_4SL treatments than the MRP only treatments observed in AFM (Figure 5-3B) suggests the formation of MRP-SL complexes.

The complexation between MRPs and SL in aqueous ethanol was confirmed using fluorescence spectroscopy. When the excitation wavelength was 280 nm, a greater reduction in the fluorescence intensity of MRPs with an increase in SL concentration (Figure 5-7) indicated the binding between MRPs and SL that reduced the exposure of Trp and Tyr residues to the polar medium (Li et al., 2019; Wei et al., 2018). The fluorescence quenching spectra were analyzed using the Stern-Volmer equation in Eq. (5) (Zhang et al., 2013).

$$F_o/F = 1 + k_q \times \tau_o \times [Q] = 1 + K_{sv} \times [Q] \quad (5)$$

Where, F_o and F are fluorescence intensities in the absence and presence of a quencher at a concentration [Q]; k_q is the fluorescence quenching rate constant; τ_o is the lifetime fluorescence of fluorophore without the quencher and equals 10^{-8} s; and K_{sv} is the Stern-Volmer quenching constant.

The corresponding results in Table 5-2 show that the k_q of all treatments at an excitation wavelength of 280 nm was higher than the maximum dynamic quenching constant (2×10^{10} M^{-1}

s^{-1}) for quenchers interacting with biopolymers (Lange et al., 1998). The results suggest the fluorescence quenching is static and therefore complexes are formed when MRP solutions at pH 7.0, 9.0, 11.0, and 13.0 are added into NH_4SL aqueous ethanol solutions. Furthermore, when the initial SL concentration in aqueous ethanol was 20% (w/v), a distinct redshift in the wavelength of maximum emission was observed for the MRP_{pH13}-NH_4SL treatment that had a higher fluorescence intensity than other treatments (Figure 5-4B). This is consistent with the fluorescence spectra of the MRP only treatments in Figure 5-3D, indicating that the better MRP solubility at a higher pH may predominantly result in the better stability of the MRP_{pH13}-HH_4SL treatment than other treatments at lower pH.

The formation of MRP-SL complexes was found to improve the stability of MRPs in aqueous ethanol when compared to the MRP only treatment at the same pH. As shown in Figure 5-4C, the D_h of the MRP_{pH13}-NH_4SL treatment (390 ± 37 nm) was initially larger than that 210 ± 9 nm of the MRP only treatment at pH 9.4 due to the MRP-SL complexation. However, after 2 h at RT, the D_h of the former increased to 602 ± 39 nm without visual changes while the latter showed MRP aggregates with the drastic increase of D_h to 1767 ± 74 nm. Additionally, MRP-NH_4SL samples were more negatively charged than MRP only samples at the same pH, as discussed previously. These results indicate that the formation of MRP-SL complexes with some SL molecules on the particle surface may have strengthened intermolecular electrostatic repulsion to enhance the stability against aggregation, most evident for the MRP_{pH13}-NH_4SL suspension.

5.4.4. Characteristics of MRP_{pH13}-NH_4SL films prepared at different MRP concentrations

5.4.4.1. Appearance and mechanical properties

The MRP_{pH13}-NH_4SL suspension showing the best stability was used to prepare films with different MRP contents. Shortly after mixing MRP solutions with NH_4SL solution, all suspensions showed a narrow and monodispersed distribution with the average D_h of around 200-400 nm (Figure 5-8A). The small and monodispersed particles facilitated system stability during casting and drying processes to result in films with smooth, uniform, and homogenous appearance (Figure 5-8B). The cross-sections of films (Figure 5-8B) appeared to be dense and compact without phase separation, indicating uniform suspension of MRPs in the continuous SL phase to enable film homogeneity. The self-assembly of SL into a network possibly prevented the aggregation of MRPs during drying (Figure 5-4A). Due to the evaporation of volatile NH_3 and CO_2 formed from excessive $(NH_4)_2CO_3$ during drying (Penning, 1996), all films had neutral pH (Table 3) and yellow appearance without significant differences ($P > 0.05$) in lightness (~89 in L values), which is important for realistic food coating applications. As expected, a higher amount of MRPs with yellow color increased not only b values (yellowness) but also opacity of the prepared films due to the increased D_h (Table 3). MRP-NH_4SL films prepared in the present study were visually transparent to translucent and were more opaque than the transparent chitosan-based films (Ma et al., 2016).

As summarized in Table 3, the thickness of films was around 0.1 mm for all treatments. A higher amount of MRPs increased the tensile strength (TS) and elongation at break (EB) of films, indicating improved coupling strength and ductility and reduced stiffness of films (Skurtys et al., 2014). SL films without a plasticizer have been reported to be brittle and stiff due to excessive

intermolecular interactions (Farag & Leopold, 2009). The improved film strength and reduced brittleness of wax films were also reported after incorporation of sodium caseinate (Fabra et al., 2008). Furthermore, an improved TS of a film is typically accompanied by a sacrifice of EB (Skurtys et al., 2014). However, the EB value of MRP-NH_4SL films was also higher at a higher amount of MRPs. The TS-EB correlation exception was also reported for gelatin/gellan gum-based films loaded with different amounts of red radish anthocyanins (Zhai et al., 2018). In the present study, the continuous SL network in films is strengthened by uniformly distributed MRP-SL complexes (Figure 4) with some SL on the surface (as discussed above based on ζ-potential), which may be responsible for both the improved TS and EB of MRP-SL composite films. Particularly, the $MRP_{3\%}$-NH_4SL treatment having the highest TS (7.64 MPa) and EB (7.7%) can be used to protect probiotics pellets with enhanced mechanical handling properties.

5.4.4.2. Barrier and enteric properties

Incorporating a higher amount of hydrophilic MRPs had an insignificant impact ($P > 0.05$) on the WVP of MRP-NH_4SL composite films (Table 3). The WVP of a film is correlated with its chemical structure, morphology, and hydrophilicity (Zhai et al., 2018). The results in Table 5-3 indicate that the WVP of composite films is dominated by hydrophobic SL present as the continuous phase and the complexation between MRPs and SL may further weaken the impact of MRPs on water diffusion in the film. In addition, the WVP value of MRP-NH_4SL films (~3×10^{-8} g/m·Pa·h) was ~10 times lower than that of the unplasticized films prepared from aqueous NH_4SL solutions (Luangtana Anan et al., 2007), likely due to the lower water permeability of SL films cast from alcoholic solutions (Hagenmaier & Shaw, 1991). The attempt to measure oxygen permeability of films using an oxygen permeability test instrument was not

successful in this study because high vacuum could strip out the plasticizer (glycerol) to break films. Thus, the oxygen permeability was indirectly evaluated below by monitoring survival of anerobic *L. salivarius* during ambient storage.

The enteric properties of MRP-NH_4SL films were evaluated by monitoring film integrity in acidic (0.1 M HCl) and neural (PBS) pH (Figure 5-9A). The film prepared without MRP was disintegrated in 0.1 M HCl due to the high water-solubility of NH_4SL. Before the acid had the time to completely protonate the carboxylate groups of NH_4SL, a high degree of film dissolution, swelling, and structure loosening occurred with the $MRP_{0\%}$-NH_4SL film (Al-Gousous et al., 2015). Incorporation of a higher amount of MRPs that are insoluble at acidic pH greatly improved the film resistance to dissolution in the acid, and the $MRP_{3\%}$-NH_4SL treatment maintained an intact film after 2-h incubation in 0.1 M HCl (Figure 5-9A). All films were then disintegrated during the subsequent PBS stage because of the increased solubility of MRPs and NH_4SL at neutral pH. Therefore, incorporation of MRPs can help to maintain the integrity of NH_4SL-based films in the acid without influencing film disintegration in neutral pH, giving rise to the $MRP_{3\%}$-NH_4SL film with pH-dependent solubility which is desirable for enteric delivery of probiotics. The enteric properties of MRP-NH_4SL films were further confirmed by measuring the accumulative release of Rhodamine B (RB) from films (Figure 5-9B). During incubation in 0.1 M HCl, the gradually decreased release rate of RB verified the less dissolution of films loaded with more MRPs. When samples were transferred into PBS, the release rate of RB from all films was quickly increased by ~40% within 1 h and remained stable afterwards, indicating the efficient disintegration of films to release a large amount of RB. The accumulative release of RB after 4-h incubation in PBS was lower in films prepared with more MRPs, likely because

MRP-NH_4SL films were disintegrated into larger flakes than NH_4SL films, thus releasing less RB. Overall, the $MRP_{3\%}$-NH_4SL film with a compact structure and satisfactory machinal, barrier, and enteric properties is desirable for developing enteric coating to protect probiotics against environmental stresses.

5.4.5. Application of MRP-NH_4SL coating to protect L. salivarius in millimeter-sized pellets during storage, thermal treatment, and simulated GI digestions

The WSPPs were used to prepare probiotics pellets due to their high *L. salivarius* viability (Wang et al., 2020) and the dry binding property of amorphous sucrose used as a direct compression excipient (Sugimoto et al., 2006). Pellets after $MRP_{0\%}$-NH_4SL and $MRP_{3\%}$-NH_4SL coating showed weight gains of 18.6 ± 2.38 and 20.6 ± 5.33%, thickness gains of 3.12 ± 1.56 and 3.64 ± 1.04%, and *L. salivarius* viability loss of 0.74 ± 0.05 and 0.31 ± 0.06 log CFU/g, respectively. Combining with the microscopic images of pellet cross-sections before and after coating (Figure 5-10A), the present coating protocol was proved to be efficient to prepare an evenly developed coating on the pellet surface without severely deactivating bacteria.

As shown in Figure 5-10B, after storage at RT and 43% RH for 30 days, the viability of *L. salivarius* in uncoated pellets showed 1.06 log CFU/g reduction due to the synergistic protection from amorphous sucrose and WPI in WSPPs (Wang et al., 2020). Compared to the uncoated pellets, *L. salivarius* in pellets coated with $MRP_{0\%}$-NH_4SL and $MRP_{3\%}$-NH_4SL had the significantly improved ($P < 0.05$) stability, showing only 0.60 and 0.45 log CFU/g reduction, respectively, and there was no significant difference ($P > 0.05$) between these two formulations. The significant protective effect of MRP-NH_4SL coatings on bacterial storage stability further verify their satisfactory water vapor and oxygen barrier properties. After heating at 80 ºC and

60% RH for up to 20 min, the reduction of probiotic viability was 2.65 log CFU/g for the uncoated pellets which was about three times higher than that with the MRP-NH_4SL coating (Figure 5-10C). The results are consistent with our previous study (Wang et al., 2020) that lowering RH from 40% to 26% with slower air flow to simulate coating conditions improved the survivability of powdered *L. salivarius* after heating at 80ºC for 30 min. Therefore, the MRP-NH_4SL coating provides an effective physical and water vapor barrier to inhibit plasticization of amorphous sucrose (Fang et al., 2013) and suppress cellular metabolic activities (Maltini et al., 2003) to preserve bacterial viability during heating.

Viable cell counts of *L. salivarius* in uncoated and coated pellets during simulated GI digestions are shown in Figure 5-10D. During the first 2-h incubation in the SIF, uncoated pellets were quickly dissolved with a significant reduction of 4.08 log CFU/g mainly caused by the gastric acid stress on damaging cellular substances (Papadimitriou et al., 2016). As expected, $MRP_{0\%}$-NH_4SL coated pellets were partially dissolved due to the high solubility of NH_4SL at acidic pH and the viability reduction was 1.37 log CFU/g. The pellets coated with $MRP_{3\%}$-NH_4SL also showed slight dissolution and the viability reduction (0.16 log CFU/g) was the lowest, likely attributed to the insolubility of coatings at acidic pH (Figure 5) and the limited hydrolysis of MRPs by pepsin (Wang et al., 2016). During the subsequent incubation in the SIF, all coated pellets were completely dissolved and the viability was appreciable reduced, with the former caused by the coating disintegration at neutral pH (Figure 5) and the latter due to the antimicrobial effects of bile salts (Urdaneta & Casadesús, 2017) and the bile susceptibility of *L. salibarius* NRRL B-30514. *L. salivarius* in the uncoated pellets became undetectable after 2-h SIF digestion. In contrast, the $MRP_{3\%}$-NH_4SL coating had ~1 log CFU/g more viable cells than the $MRP_{0\%}$-NH_4SL coating

treatment after 2-h SIF digestion, and the two coating treatments maintained comparable ~4 log CFU/g viable cells after 4-h SIF digestion. These results concur with another study where NH_4SL microcapsules doped with pH-sensitive polyelectrolytes protected the entrapped yeast cells in acidic pH followed by triggered release at higher pH (Hamad et al., 2012). Therefore, the MRP-NH_4SL enteric coating can appreciably increase the survivability of pelleted probiotics after simulated GI digestions, which will require future *in vivo* verifications.

5.5 Conclusion

MRP-NH_4SL complexes formed after dropwise adding MRP solutions into NH_4SL aqueous ethanol solutions at pH 8.2, and the higher pH manipulated by the pH of MRP solutions lowered the complex dimension and improved suspension stability and therefore film homogeneity. Smooth, continuous, and homogenous films formed by casting MRP_{pH13}-NH_4SL suspensions exhibited the satisfactory moisture barrier property, and the mechanical and enteric properties were improved when a higher amount of MRPs was loaded. These characteristics of the MRP-NH_4SL enteric coating on millimeter-sized probiotics pellets resulted in excellent protection of *L. salivarius* viability during storage, thermal treatment, and simulated GI digestions. This study presents a simple and scalable method to utilize MRPs with pH-dependent solubility to effectively improve the disintegration property of SL-based coatings for enteric delivery of powdered probiotics. These findings may be significant to preparing solid probiotics-fortified products and delivering other sensitive bioactives to facilitate the development of functional foods.

5.6 Acknowledgments

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Appendix

Table 5-1 pH and zeta (ζ)-potential of fresh suspensions prepared by adding 3.0% (w/v) modified rice protein (MRP) solutions at different pH into 18% (w/v) ammonium shellac in 90% (v/v) aqueous ethanol solutions at pH 8.2.*

MRP solution pH	Mixture suspension pH	ζ-potential (mV)
7.0	8.3 ± 0.0^{c}	-22.1 ± 1.1^{c}
9.0	8.4 ± 0.1^{c}	-25.7 ± 0.7^{ab}
11.0	8.6 ± 0.0^{b}	-23.7 ± 1.0^{bc}
13.0	9.4 ± 0.1^{a}	-28.9 ± 1.7^{a}

* Numbers are mean ± SD (n = 3). Means with different superscript letters indicate significant differences of treatments within the same column ($P < 0.05$).

Table 5-2 Stern-Volmer quenching rate constants (k_q) of fresh suspensions prepared by adding 3.0% (w/v) modified rice protein (MRP) solutions at different pH into ammonium shellac in 90% (v/v) aqueous ethanol solutions at pH 8.2.

MRP solution pH	k_q ($\times 10^{10}$/M·S)	Correlation coefficient (R)
7.0	5.5267	0.9701
9.0	6.9053	0.9914
11.0	4.0551	0.9834
13.0	5.4822	0.9702

Table 5-3 pH, thickness, color, opacity, tensile strength (TS), elongation at break (EB), and water vapor permeability (WVP) of films cast from suspensions prepared by adding 0, 0.5, 1.0, and 3.0 (w/v) of modified rice protein (MRP) solutions at pH 13.0 into 18% (w/v) ammonium shellac in 90% (v/v) aqueous ethanol solutions at pH 8.2.*

MRP concentration (% w/v)	pH	Thickness (mm)	*L*	*a*	*b*	Opacity (%)	TS (MPa)	EB (%)	WVP ($\times 10^{-8}$ g/m·Pa·h)
0	7.38±0.01^{a}	0.107±0.01^{a}	89.52±0.18^{a}	-1.89±0.21^{b}	610.87±0.86^{b}	2.39±0.35^{b}	5.62±0.33^{b}	2.80±0.64^{b}	2.29±0.43^{a}
0.5	7.38±0.06^{a}	0.098±0.01^{a}	89.22±0.26^{a}	-1.97±0.07^{ab}	11.61±0.80^{ab}	2.78±0.46^{ab}	6.26±0.47^{b}	4.47±0.80^{b}	3.27±0.60^{a}
1.0	7.35±0.03^{a}	0.113±0.01^{a}	89.22±0.08^{a}	-1.95±0.04^{ab}	11.14±0.76^{ab}	2.56±0.47^{b}	6.02±0.77^{b}	7.34±1.26^{a}	3.25±0.18^{a}
3.0	7.32±0.01^{a}	0.107±0.00^{a}	89.31±0.38^{a}	-2.07±0.05^{a}	12.45±1.35^{a}	3.27±0.21^{a}	7.64±0.29^{a}	7.70±1.38^{a}	3.55±0.11^{a}

* Numbers are mean ± SD (n = 3). Means with different superscript letters indicate significant differences of treatments within the same column ($P < 0.05$).

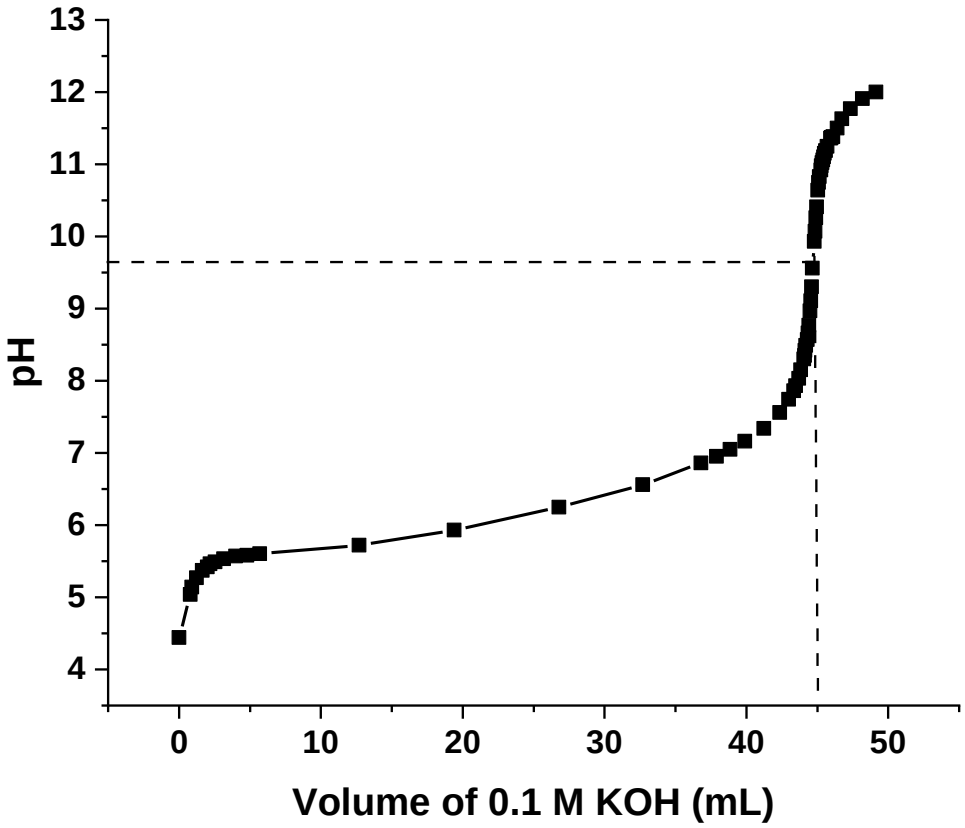

Figure 5-1 Acid-base titration curve of shellac ethanol solution. The dashed line indicates the equivalence point.

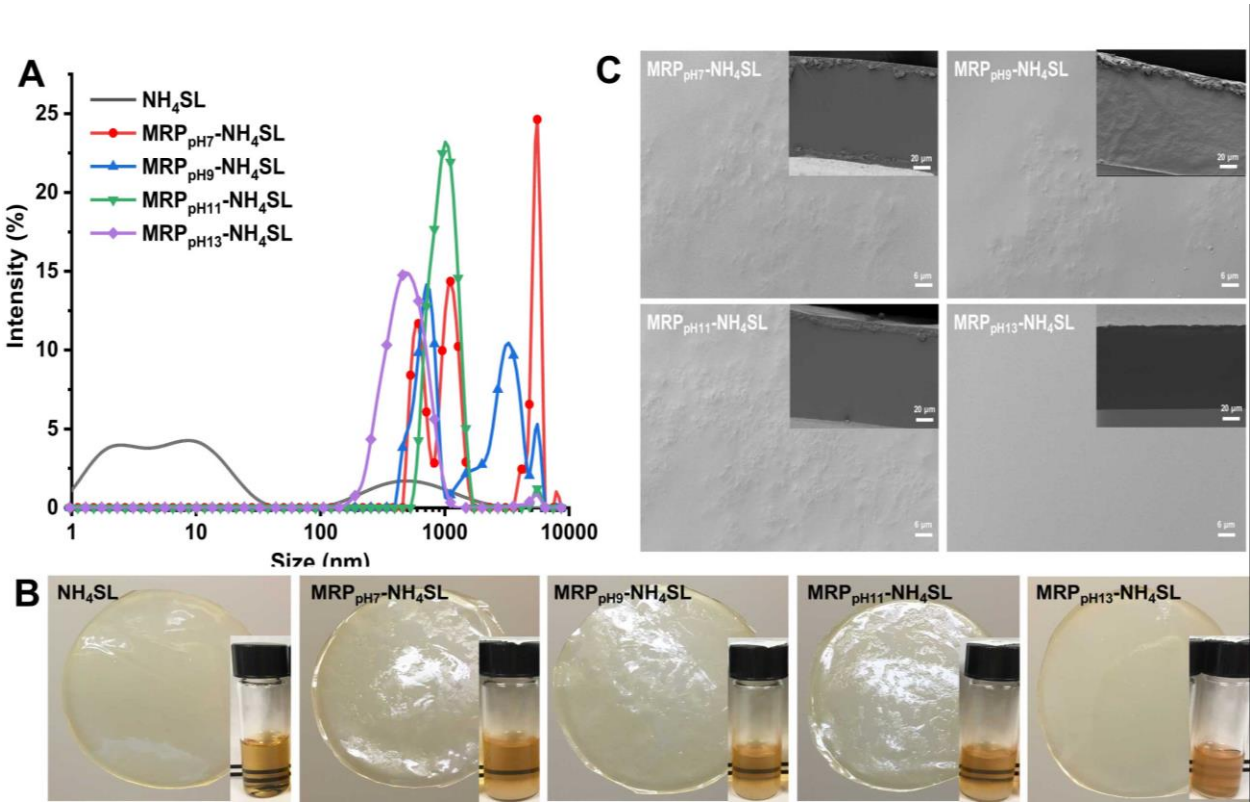

Figure 5-2 (A) Particle size distribution of suspensions prepared by adding 3.0% (w/v) modified rice protein (MRP) solutions at different pH into 18% (w/v) ammonium shellac (NH_4SL) in 90% (v/v) aqueous ethanol solutions at pH 8.2. (B) Appearance of films cast from the above suspensions, with the inset photo showing the suspension appearance after ambient storage for 6 h. (C) SEM images of surface and cross-section (inset) of films cast from the above suspensions.

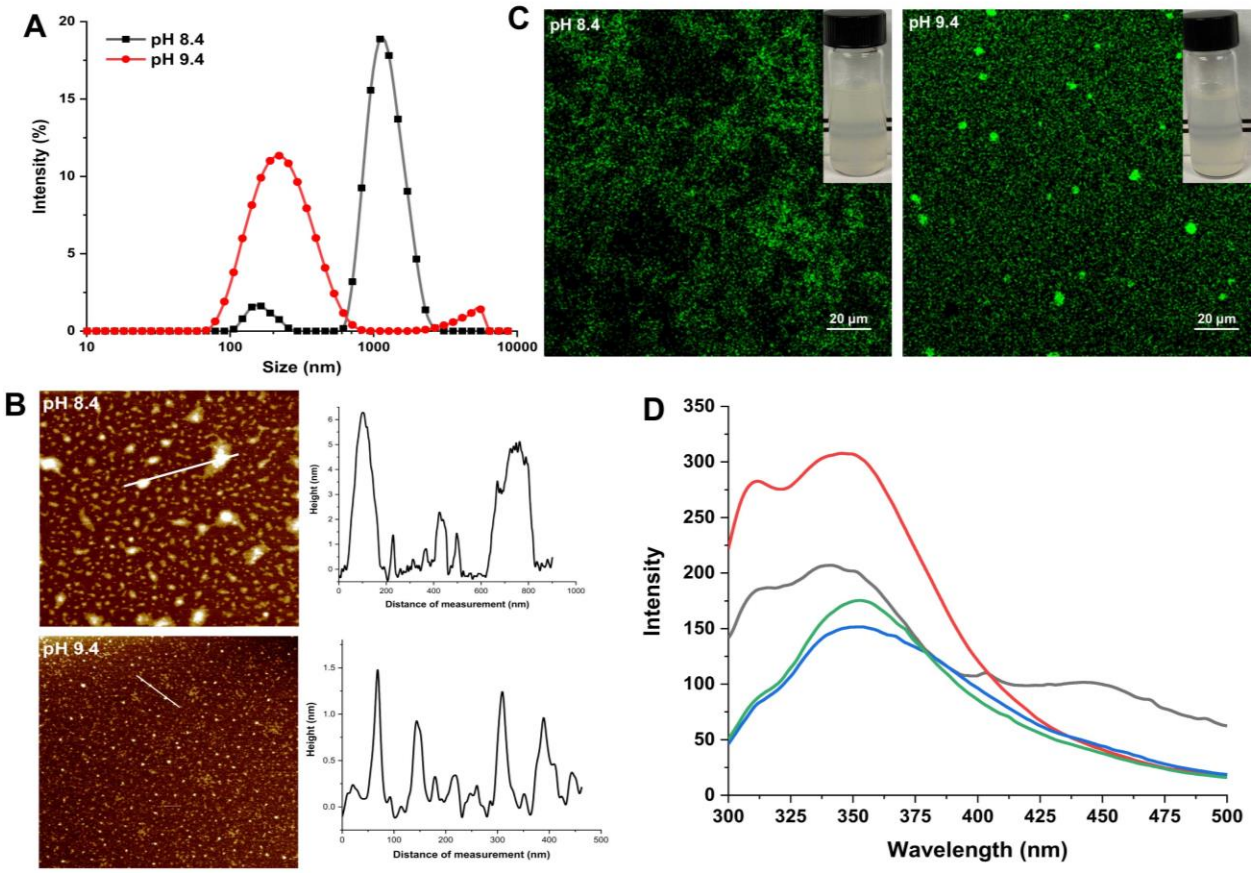

Figure 5-3 (A) Particle size distribution, (B) AFM images (2×2 µm; plots present the height distribution of particles along the white line), and (C) CLSM images (insets show the suspension appearance after ambient storage for 1 h) of 0.27% (w/v) modified rice protein (MRP) in 81.8% (v/v) aqueous ethanol at pH 8.4 and 9.4. Figure (D) compares fluorescence emission spectra of 0.27% (w/v) MRP in 81.8% (v/v) aqueous ethanol at pH 8.4 (black) and 9.4 (red) and in aqueous solutions at pH 8.4 (blue) and 9.4 (green).

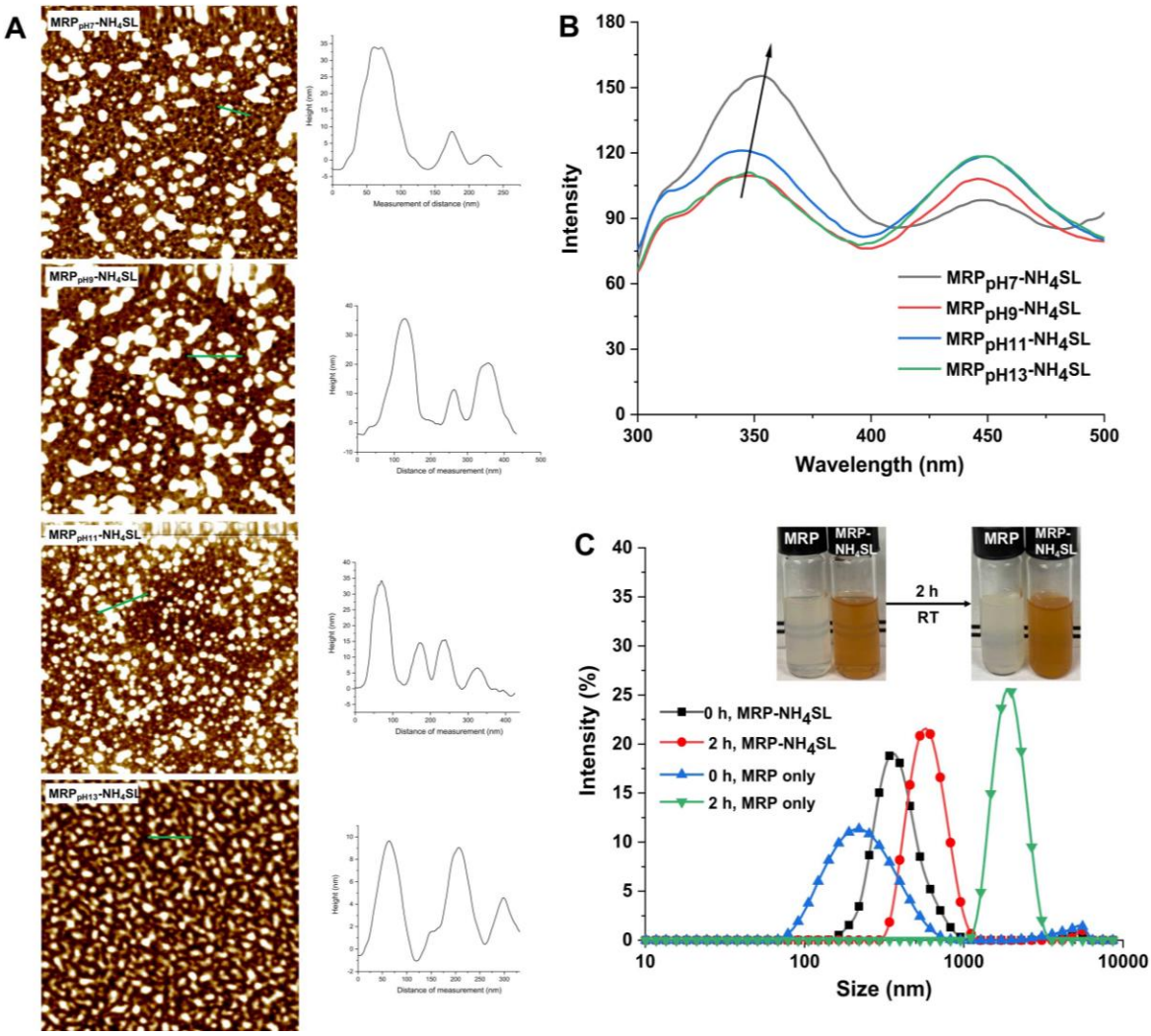

Figure 5-4 (A) AFM images (2×2 μm; plots present the height distribution of particles along the green line) and (B) Fluorescence emission spectra of suspensions prepared by adding 3.0% (w/v) modified rice protein (MRP) solutions at different pH into 18% (w/v) ammonium shellac (NH_4SL) in 90% (v/v) aqueous ethanol solutions at pH 8.2. (C) Particle size distribution of MRP$_{pH13}$-NH_4SL suspension and MRP only suspension at pH 9.4 before and after 2 h incubation at ~21 ºC (insets show the appearance).

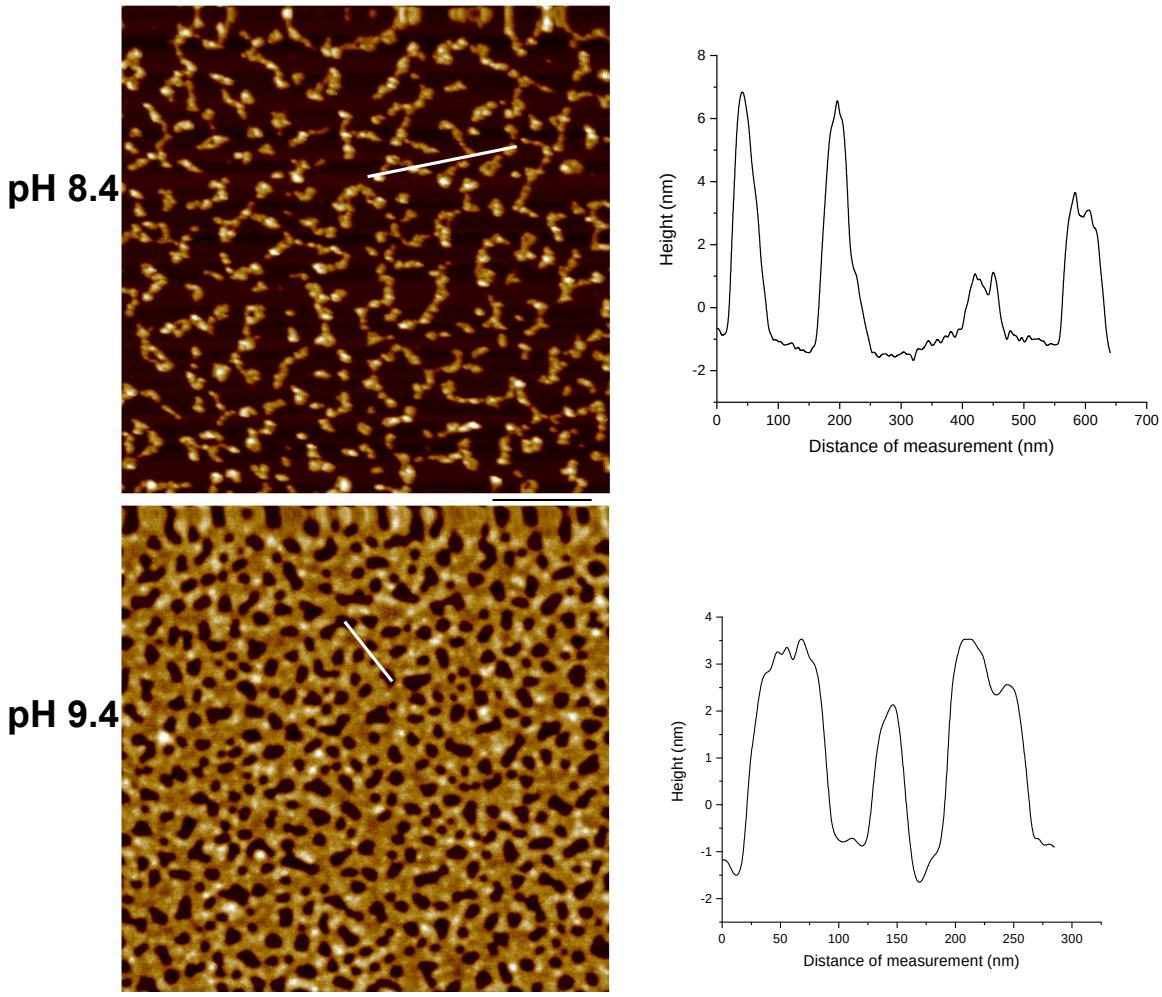

Figure 5-5 AFM images (2×2 μm; plots present the height distribution of particles along the white line) of 16.4% (w/v) ammonium shellac (NH_4SL) in 81.8% (v/v) aqueous ethanol solutions at pH 8.4 and 9.4.

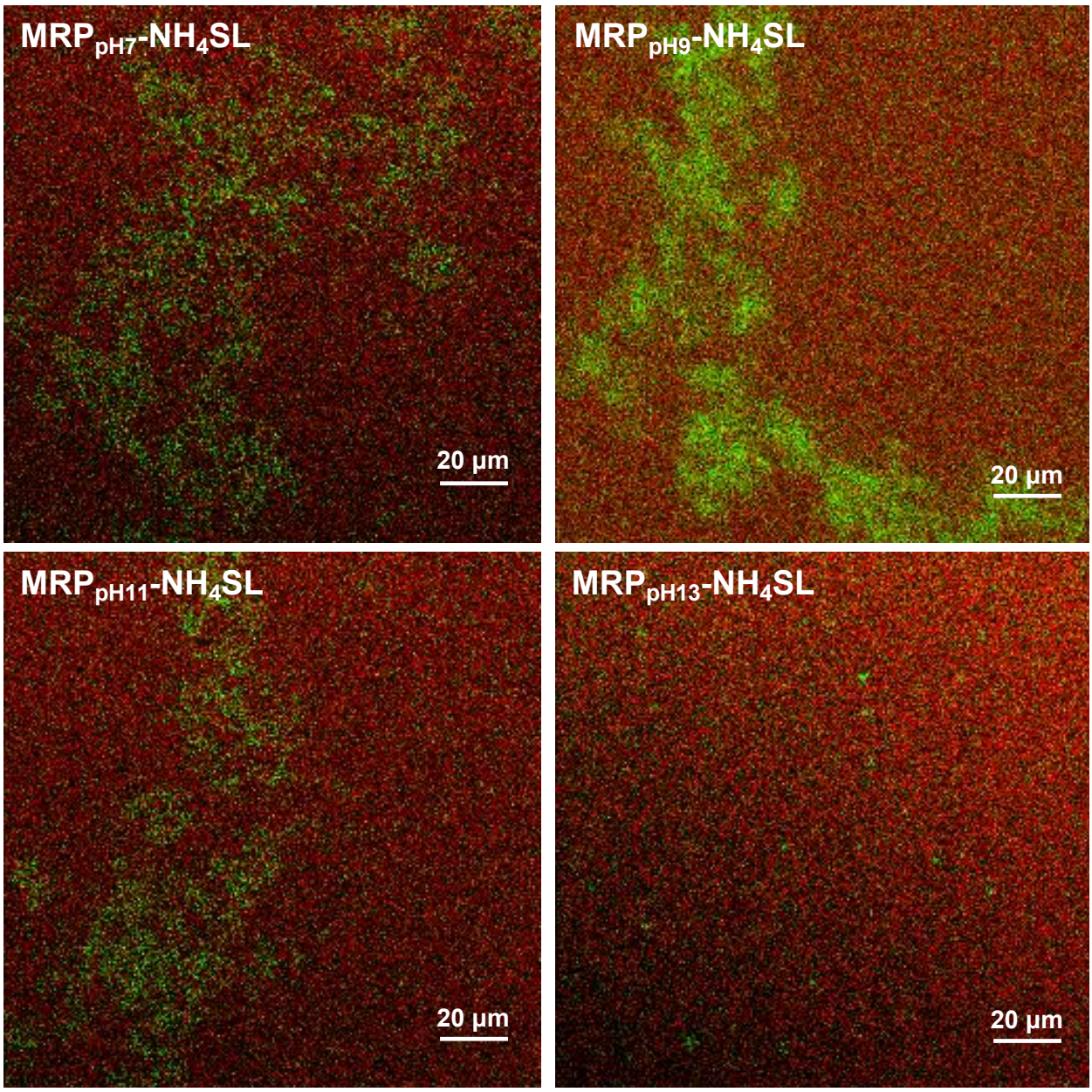

Figure 5-6 Confocal laser scanning microscopy images of suspensions prepared by adding 3.0% (w/v) modified rice protein (MRP) solutions at different pH into 18% (w/v) ammonium shellac (NH_4SL) in 90% (v/v) aqueous ethanol solution at pH 8.2.

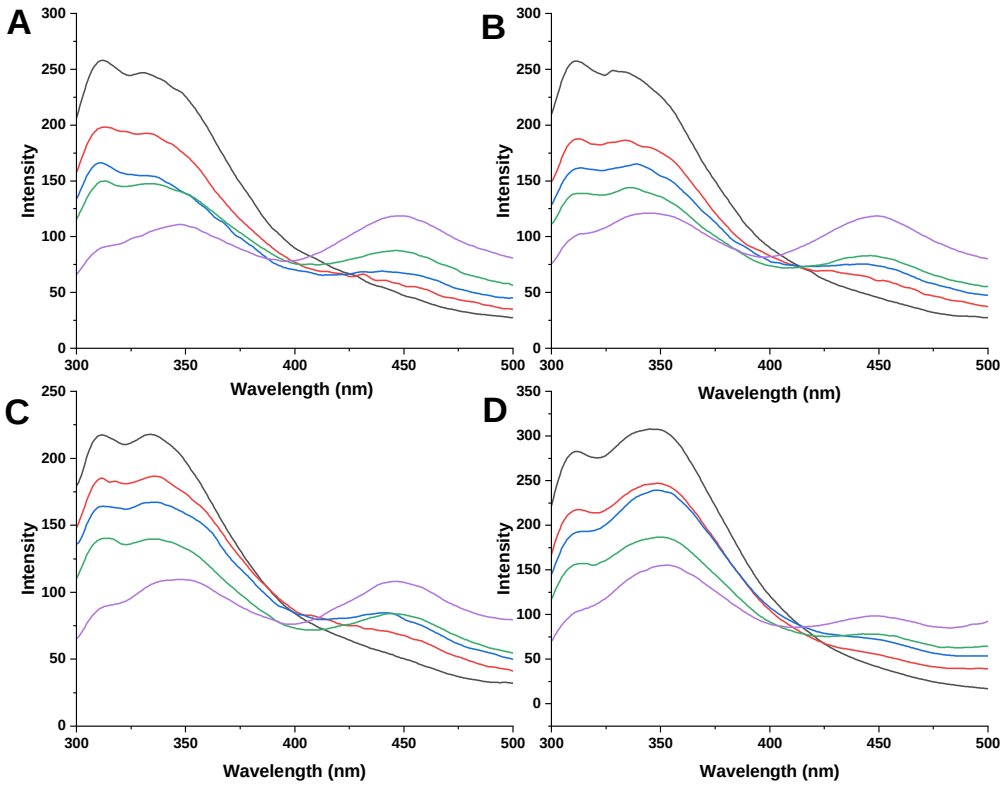

Figure 5-7 Fluorescence emission spectra of suspensions prepared by adding 3.0% (w/v) modified rice protein (MRP) solutions at pH 7.0 (A), 9.0 (B), 11.0 (C), and 13.0 (D) into 90% (v/v) aqueous ethanol solutions at pH 8.2 containing, from top to bottom, 0, 5, 10, 15, and 20% (w/v) of ammonium shellac (NH_4SL).

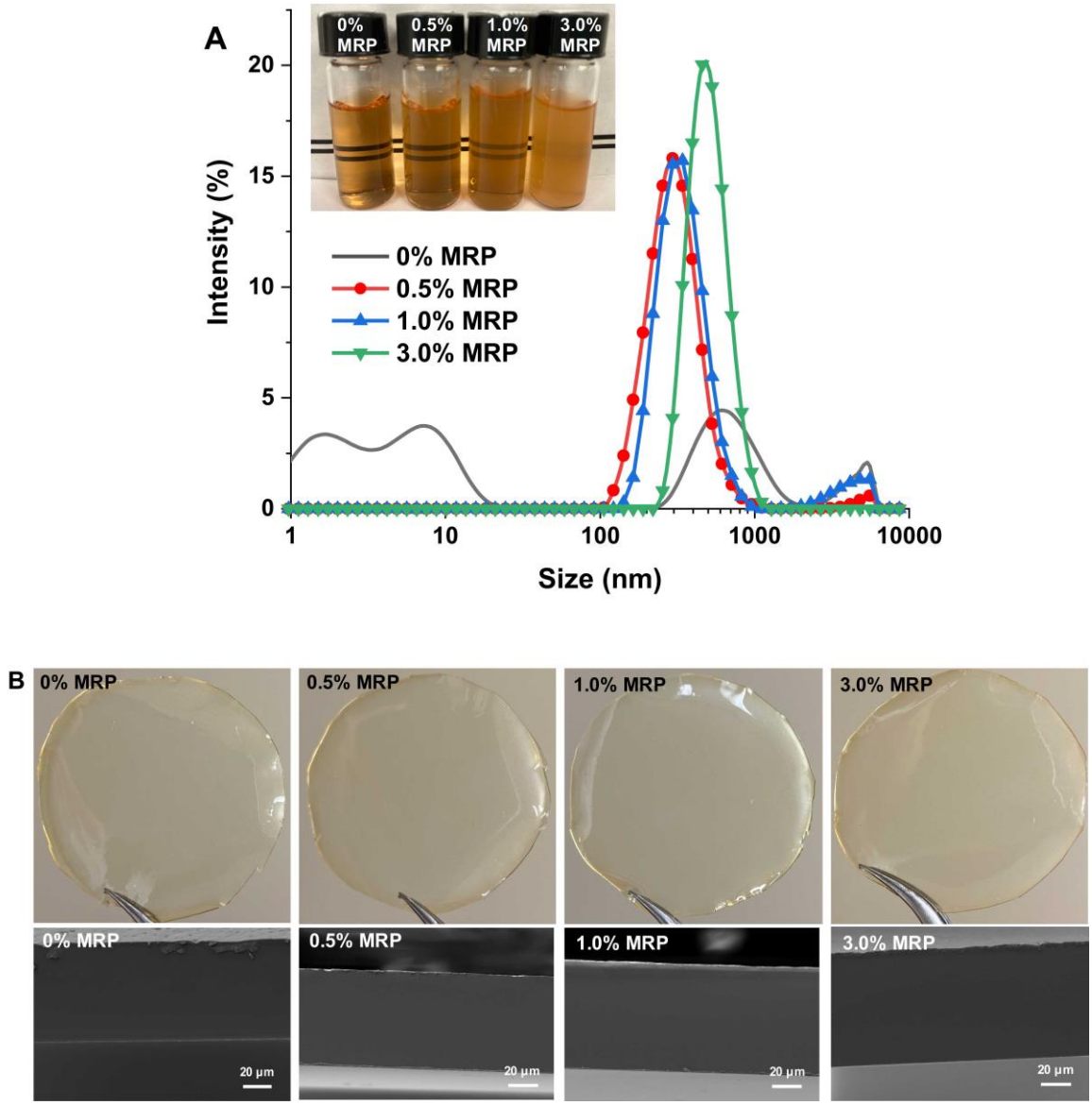

Figure 5-8 (A) Particle size distribution of suspensions (appearance shown in the inset) prepared by adding different concentrations (w/v) of modified rice protein (MRP) solutions at pH 13.0 into 18% (w/v) ammonium shellac (NH_4SL) in 90% (v/v) aqueous ethanol solutions at pH 8.2. (B) Appearance and cross-section SEM images of films cast from the above suspensions.

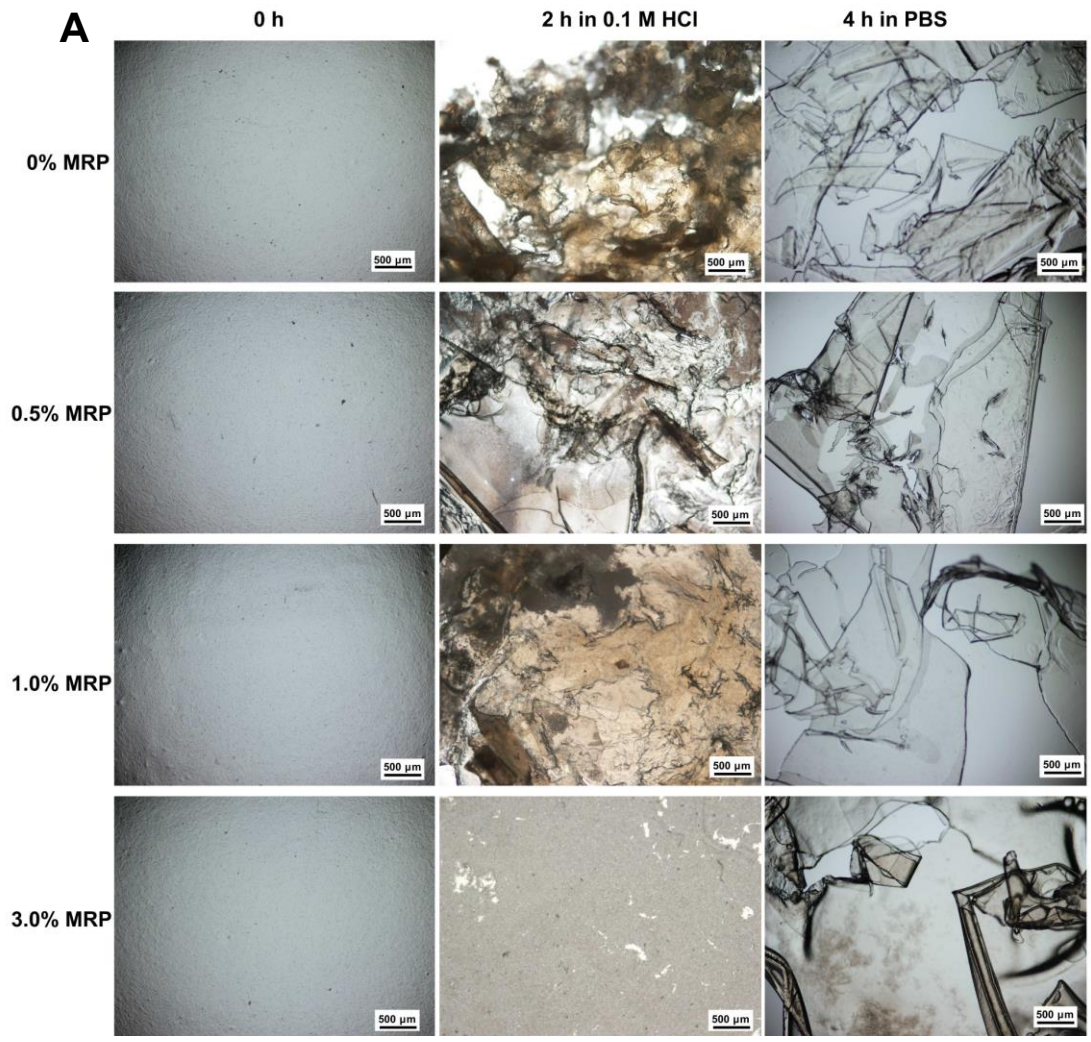

Figure 5-9 (A) Microscopic images of films and (B) release of Rhodamine-B (RB) from films after incubation in 0.1 M HCl for 2 h and then 0.1 M PBS at pH 7.0 for up to 4 h. The films were cast from suspensions prepared by adding solution with 0, 0.5, 1.0, and 3.0 (w/v) of modified rice protein (MRP) at pH 13.0 into 18% (w/v) ammonium shellac (NH_4SL) in 90% (v/v) aqueous ethanol solutions at pH 8.2.

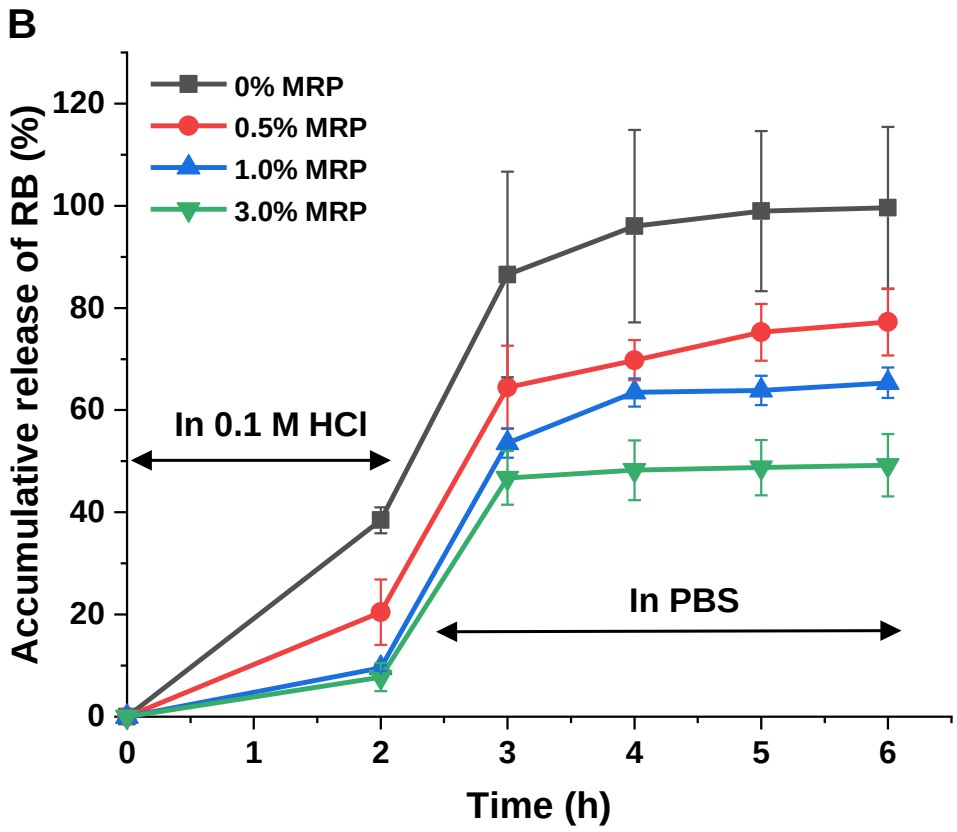

Figure 5-9 continued

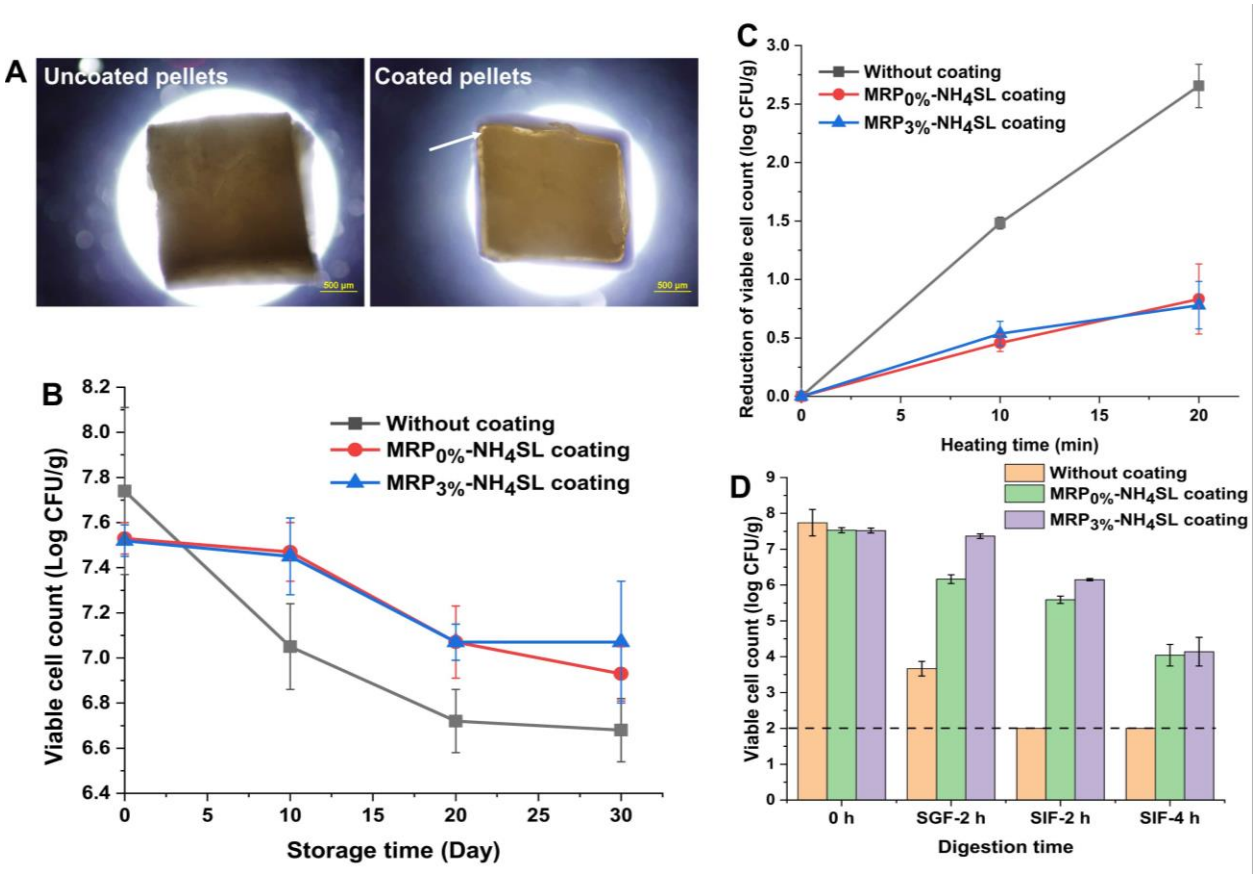

Figure 5-10 (A) Cross-section of probiotics pellets with and without coating (the arrow in the right figure highlights the surface coating); Viability of L. salivarius in uncoated and coated pellets during (B) 30-day storage at room temperature (~21ºC) and 43% relative humidity (RH), (C) heating at 80 ºC and 60% RH for up to 20 min, and (D) sequential *in vitro* digestions in simulated gastric fluid (SGF) for 2 h and simulated intestinal fluids (SIF) for 2 and 4 h. Coated pellets were prepared by immersing pellets into suspensions formulated by adding 0 or 3.0 (w/v) of modified rice protein (MRP) solution at pH 13.0 into 18% (w/v) ammonium shellac (NH_4SL) in 90% (v/v) aqueous ethanol solution at pH 8.2.

Chapter 6 Concluding remarks and future work

6.1 Conclusions

This dissertation demonstrated that the protective effects and water sorption properties of dairy-based ingredient powders can be utilized to prepare powdered probiotics by directly mixing a concentrated cell suspension with dehydrated powders to maintain a high level of viability during storage and thermal treatments. The enteric modified rice protein (MRP)-ammonium shellac (NH_4SL) composite coatings can further protect the enclosed probiotics pellets prepared by direct compression of powdered probiotics during preparation, storage, and simulated gastrointestinal (GI) digestions.

Mixing amorphous spray-dried lactose (SDL) and a concentrated cell suspension can be used to form chemically bound water to prepare powdered *L. salivarius* with up to 6.89 log CFU/g viable cells after 6-month storage at 4 ºC. A higher amount of cell suspension facilitated lactose crystallization and the lactose dissolved in excessive water resulted in a higher hypertonic stress and therefore lower viable bacterial counts initially and during subsequent storage. The hypertonic stress and reduced water activity (a_w) during the mixing procedure appeared to have induced the viable but non-culturable (VBNC) state of powdered *L. salivarius*.

The relative significance of milk proteins and lactose having different water sorption properties on survival of *L. salivarius* was then studied by preparing powdered *L. salivarius* using dehydrated milk protein concentrate (MPC) and SDL at different mass ratios. Treatments with more MPC showed up to 1 log CFU/g higher than the SDL only treatment after preparation and during 180-day storage at 4 ºC. A higher amount of milk proteins in dairy powders delayed the hydration of SDL by predominately absorbing the water in cell suspensions, resulting in a

lower hypertonic stress on adhered *L. salivarius* and thus more effective protection on probiotic survival. The more significant protective effects of MPC than SDL were further found in preserving the membrane integrity and metabolic activity of powdered *L. salivarius*.

The synergistic effect of whey protein isolate (WPI) and sucrose on protecting the survival of powdered *L. salivarius* during storage and thermal treatments was further studied. WPI stabilized the amorphous sucrose structures in powdered *L. salivarius* to enable the glass transition temperature (T_g) above room temperature (RT, ~21 ºC). Compared to the WPI only treatment, *L. salivarius* viability in treatments with the presence of amorphous sucrose increased by ~3 log CFU/g after 365-day storage at RT. Treatments with a higher amount of sucrose also resulted in better thermal stability of *L. salivarius* with higher membrane integrity.

Stable and homogenous MRP-NH_4SL enteric coating suspensions at pH 9.4 were prepared by dropwise adding MRP solutions at pH 13.0 into NH_4SL aqueous ethanol solutions at pH 8.2. MRP exhibited better solubility and stability in aqueous ethanol at a higher pH and the formation of MRP-SL complexes further stabilized the suspension of MRP likely due to strengthened intermolecular electrostatic repulsions. The homogenous MRP_{pH13}-NH_4SL suspension resulted in smooth films with improved mechanical and enteric properties at a higher content of MRP. The MRP-NH_4SL enteric coating on millimeter-sized probiotics pellets significantly improved the *L. salivarius* viability during storage, thermal treatments, and simulated GI digestions.

Overall, the developed mixing protocol consisting of simple procedures and equipment may be significant to preparing probiotic ingredients to facilitate the development of functional foods. Further development of the novel, simple, and scalable method utilizing MRP to modify the

enteric properties of SL-based edible coatings to deliver probiotics is significant to preparing solid probiotics-fortified products and delivering other sensitive bioactives.

6.2 Future work

The results presented in this dissertation show that powdered probiotic ingredients can be prepared by directly mixing a concentrated cell suspension with dehydrated dairy-based ingredient powders. However, future studies are needed to explore mechanisms of interactions between probiotic cells and different food ingredients. Specifically, the physicochemical properties of cellular surface of *L. salivarius* and the adhesive interactions between cells and different dairy ingredients analyzed using atomic force microscopy can be studied. In addition, future *in vitro* and *in vivo* studies are needed to study the mechanisms causing the VBNC state of *L. salivarius* and their possible physiological functions. *In vitro* and *in vivo* studies can also be conducted to explore the viability of probiotics after reconstitution, including the possibility of recovering from the VBNC state.

VITA

Anyi Wang was born in a small town in Shandong province, China on March 9th, 1992. After graduating from high school in 2010, she pursued a Bachelor of Science degree in Food Safety at China Agricultural University. She became fascinated with scientific research and analytical techniques during an undergraduate research program, when she was working with her partner to determine urinary equol using thin-layer chromatography and high performance liquid chromatography. She was then admitted to the MS program in Food Science and Engineering at China Agricultural University without examination in 2014 and worked on developing a solid-state fluorescent sensor for rapid detection of toxins in food. After obtaining her Master of Science degree, she was fortunate to join Dr. Qixin Zhong's group to pursue a Doctor of Philosophy degree in Food Science at the University of Tennessee, Knoxville, TN in 2016. After receiving her doctoral degree, she will stay optimistic, keep her primal belief, and continue to work hard to build on her existing research experience. She wants to be an independent research scientist and dedicate her life to making a better world!