MICROENCAPSULATION OF PROBIOTIC *LACTOBACILLUS RHAMNOSUS* GG WITH JELLYFISH GELATIN AND THE EFFECT OF ULTRA-HIGH-PRESSURE HOMOGENIZATION (UHPH) OF ENCAPSULATING MATERIALS ON THE SURVIVABILITY OF *LACTOBACILLUS PLANTARUM* NRRL B-1927 AFTER DRYING

by

NAN JIANG

(Under the Direction of Kevin Mis Solval)

ABSTRACT

The feasibility of microencapsulating *Lactobacillus rhamnosus* GG (LRGG) with jellyfish gelatin (JG) and maltodextrin (MD) *via* mixed-flow spray drying (MXSD) and/or freeze drying (FD) was first evaluated in this study. JG with low mineral content was successfully produced from salted and dried cannonball jellyfish. Higher cell viability of LRGG was observed after MXSD (26.69%) compared to FD (3.63%), which suggested the presence of heat-sensitive antimicrobial compounds in JG.

Then, the effect of ultra-high-pressure homogenization (UHPH) at 150 and/or 300 MPa of soymilk (SOY) and/or skim milk (SKIM) used to microencapsulate *Lactobacillus plantarum* NRRL B-1927 (LP) *via* concurrent (CCSD), MXSD and/or FD was studied. Higher cell survival (%) of LP was observed in powders microencapsulated with UHPH-treated than with non-UHPH-treated SOY and SKIM.

This demonstrated that LRGG can be successfully microencapsulated with JG and MD, and the potential of using UHPH to improve the microencapsulating ability of SOY and SKIM.

INDEX WORDS: Probiotics encapsulation, Jellyfish gelatin, Cannonball jellyfish, High pressure homogenization, Mixed-flow spray dry, Concurrent spray drying, Freeze drying, *Lactobacillus rhamnosus* GG, *Lactobacillus plantarum* NRRL B-1927, Probiotic survivability

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DEDICATION

To everyone who loved, helped, and supported me throughout my life.

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CHAPTER 1

INTRODUCTION

Probiotics are microorganisms that can provide health benefits to humans when ingested in adequate quantities. Therefore, they have been added in functional foods and nutritional supplements. Studies have shown that probiotics interact with host microbiota in the gastrointestinal (GI) tract, improves microbial balances, modulate immune function, produce antimicrobial compounds including organic acids, and better gut barrier integrity (Sanders, Merenstein, Reid, Gibson, & Rastall, 2019). Therefore, consumption of probiotic-containing foods and supplements is recommended by health professionals.

Conventionally, probiotics are delivered as lyophilized powders, which can be used in nutritional supplements and/or added in foods such as cheese, yogurt, ice cream, breakfast cereal, nutrition bars and infant formulas. Nevertheless, the survival of probiotic cells after the processing and storage of probiotic-containing foods and supplements and passage through the human GI tract is reportedly low. To observe health benefits, viable probiotic cells need to colonize the intestines which requires the delivery of significant numbers of viable cells. Microencapsulation of probiotics provides an effective protection against adverse environmental, processing and storage conditions.

There are two parts in this study. In the first part, we evaluated the feasibility of microencapsulating *Lactobacillus rhamnosus* GG (LRGG) with gelatin extracted from salted and dried cannonball jellyfish (SDJ) *via* mixed-flow spray drying (MXSD) and/or freeze drying (FD). Then, we studied the effect of ultra-high-pressure homogenization

(UHPH), as a pretreatment for encapsulating materials, on the survivability of *Lactobacillus plantarum* NRRL B-1927 (LP) after concurrent spray drying (CCSD), MXSD, and/or FD.

We found that SDJ is an excellent source of collagen, a fibrous protein and the raw material of gelatin which may be used as a foaming, emulsifying, and wetting agent due to its surface-active properties. Hence, we hypothesized that gelatin from SDJ could be used as a novel microencapsualting agent for LRGG.

Jellyfish gelatins (JG) were succesfully developed from SDJ. We found that SDJ has a high mineral content which greatly affected the quality of the resulting JG; hence, a method that successfully removed more than 85% of minerals in JG was developed. This allowed the production of high-quality JG. Then, JG with maltodextrin (MD) were used to microencapsulate LRGG *via* MXSD and/or FD. Higher cell survival (%) of LRGG were observed after MXSD than FD. This might suggest the presence of heat-sensitive antimicrobial compounds in JG which were inactivated during MXSD process. The cell counts of LRGG in microencapsulated powders were reduced by more than 99.9% after 15 days of storage at room temperature and 55 and/or 75% relative humidity conditions, and the LRGG powders absorbed significant amounts of moisture from the storage environment, which may be due to the high hygroscopicity nature of the products. These results indicated that LRGG can be succesfully microencapsulated with JG and MD *via* MXSD.

UHPH is a novel technology that forces liquids through a valve or other pressure release components with pressures higher than 100 MPa, exerting ultra-shear (Patrignani & Lanciotti, 2016). UHPH can inactivate microorganisms (Patrignani & Lanciotti, 2016),

modify proteins configurations (Paquin, 1999), promote intermolecular complexes formation (Li et al., 2019), and improve emulsion stability, while maintaining nutritional and sensory qualities of the food (Sidhu & Singh, 2016).

In the second part of this study, soymilk (SOY) and/or skim milk (SKIM) was treated by UHPH at 150 MPa and at 300 MPa before being used to microencapsulate LP *via* MXSD, CCSD and FD. Higher cell viability was observed in LP powders microencapsulated with UHPH-treated than with non-treated SOY and SKIM. While higher cell viability rates after microencapsulation were obtained when SOY was treated at 300 MPa, treating SKIM at 150-MPa was a better approach to increase the cell survival of LP in the microencapsulated powders.

The study demonstrated that UHPH has a promising potential to improve the cell viability of LP after microencapsulation. More importantly, the study showed that LP powders with high cell counts can be effectively produced *via* spray drying, a more cost-effective microencapsulating process than FD.

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CHAPTER 2

LITERATURE REVIEW

Probiotics

According to Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO), probiotics are live microorganisms which when consumed in adequate amounts confer a positive health effect to the host. Nowadays, probiotics are getting more popular in functional foods and nutritional supplements. It has been projected that by 2023, the global probiotics market will be valued $69.3 Billion (MarketsandMarkets™, 2019). Currently, most of probiotic containing products are manufactured by dedicated probiotic production, nutritional supplements and food companies. Probiotics supplements are conventionally sold as lyophilized powders and/or capsules. Furthermore, probiotic cultures are added to foods such as cheese, yogurt, ice cream, breakfast cereal, nutrition bars and infant formulas. A wide variety of probiotic foods were recently launched (Tripathi & Giri, 2014). Most common probiotic bacteria (mainly of the genus *Lactobacillus* spp. and *Bifidobacterium* spp.) are extracted from the human gastrointestinal (GI) tract or fermented foods, such as yoghurts, kefir, pickles, and grains. Some probiotic strains with clinically proven health benefits have been accepted as Generally Regarded as Safe (GRAS) in the U.S. (FDA, 2020) or have been regarded as Qualified Presumption of Safety status by the European Food Safety Authority (EFSA) (O'Toole, Marchesi, & Hill, 2017). Many health benefits have been attributed to the consumption of fermented food products containing probiotics. Therefore, consumption

of probiotic containing products are recommended by health care providers (Suez, Zmora, Segal, & Elinav, 2019). Several studies have shown that probiotics interact with host microbiota in the GI tract, modulate immune function, produce antimicrobial compounds and organic acids, enhance enzyme formation and better the gut barrier integrity (Sanders, Merenstein, Reid, Gibson, & Rastall, 2019). Moreover, probiotics can improve microbial balances in human intestines (Rokka & Rantamäki, 2010). Probiotic bacteria of the genus *Lactobacillus* are a gram-positive, microaerophilic, lactic acid-producing, non-spore forming, non-flagellated rods or coccobacilli which can be found in various environments, including fermented products, animal gastrointestinal mucosal surfaces, as well as animal feces. *Lactobacillus rhamnosus* GG (LRGG) was first isolated from the feces of healthy humans (Zhang et al., 2010), and has an extensive amount of clinically proven health benefits including an increased resistance to gastrointestinal and respiratory infections and a decreased occurrence of fever (Liptáková, Valík, & MedveĎová, 2008). It has also been shown that LRGG has positive effects on preventing and treating primary rotavirus infection, diarrhea, and atopic dermatitis in humans (Doron, Snydman, & Gorbach, 2005; Marteau, Vrese, Cellier, & Schrezenmeir, 2001). Collado, Meriluoto, and Salminen (2007) reported that LRGG is able to inhibit the adhesion of *Clostridium histolyticum, Cl. Difficile* and *Salmonella enterica*; and when LRGG is combined with other *Lactobacillus spp.*, the growth of *Staphylococcus aureus, E. coli, S. enterica, clostridia, Listeria monocytogenes*, yeasts and molds may be inhibited. It has been reported that LRGG can adhere to intestinal mucus and colonized the GI tract after three days of ingestion (Tuomola, Ouwehand, & Salminen, 2000). Unlike free-living cells, LRGG shows a biofilm mode of growth which can improve its

existing probiotic properties (*e.g.* antimicrobial and anti-inflammatory) (Aoudia et al., 2016; Jones & Versalovic, 2009; Rieu et al., 2014). In addition, the biofilm mode of growth can protect LRGG from lethal conditions, including antimicrobial treatments, host immune defenses, and sudden change in pH, salt, temperature, and nutrients (Flemming et al., 2016). Because of its extensive health benefits, probiotic and antimicrobial properties, LRGG is widely added in fermented and non-fermented foods (Liptáková et al., 2008).

Lactobacillus plantarum NRRL B-1927 (LP) (accession numbers in other collections: ATCC 10241=NCDO 343=NCIB 7220=NCTC 7220) isolated from sauerkraut is another probiotic bacteria strain widely used in foods. LP have also shown health-benefits such as antioxidant, cholesterol-lowering, diarrhea prevention and irritable bowel syndrome (IBS) alleviation (Bested, Logan, & Selhub, 2013; Seddik et al., 2017). Some strains of *L. plantarum* can also produce bacteriocins that have a wide range of applications in the medical, food and veterinary areas. LP cells are straight rods with a relatively strong resistance to environmental stresses (Huang et al., 2017; Seddik et al., 2017). Therefore, LP can be found in different environments, suggesting its ability to adapting to various conditions, which can be explained by its large numbers of surface anchored proteins and genes encoding regulatory functions (Zuzana, Denisa, Sabína, & Ľubomír, 2016). LP has shown a high resistance against osmotic stress which is higher than those of *L. bulgaricus, Bradyrhizobium japonicum* and *Escherichia coli* (Huang et al., 2017; Mille, Beney, & Gervais, 2005). LP has the ability to survive and grow at low pH under the presence of bile salts, which is a characteristic environment found at both ends of the GI tract (saliva and feces) (Jiménez, 2009). Iaconelli et al. (2015) reported

that LP had higher survivability and better functionalities after spray, freeze and/or air drying than *Bifidobacterium bifidum* and *Lactobacillus zeae.*

Low survival rate and cell viability of probiotic cells after a) processing and storage of probiotic foods, and b) transit through high acidic conditions of the stomach and exposure to enzymes and bile salts in the small intestine is one of the main challenges of delivering probiotics into the intestine (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2012). Probiotic cells need to reach and colonize the intestines to show health benefits; however, after ingestion of probiotic foods, the number of viable cells significantly decrease because of the low pH of the gastric juice and bile salts. It has been reported that LRGG cells lose a high extent of viability in simulated gastric digestion, however, they are relatively more stable under the small intestine conditions (Burgain, Gaiani, Cailliez-Grimal, Jeandel, & Scher, 2013; Guerin et al., 2017).

Microencapsulation technology

To observe health benefits, viable probiotic cells need to colonize the intestines which requires the delivery of significant numbers of cells. The food industry and the U.S. Food and Drug Administration (FDA) recommends probiotic foods to have a minimum of 10^6 CFU ml^{-1} or g of viable probiotic cells. Several scientific papers have proposed that a total of 1 billion to 10 billion of probiotic cells should be consumed daily to observe health benefits (Karimi, Mortazavian, & Da Cruz, 2011). Microencapsulation of probiotics provides an effective protection against adverse environmental, processing and storage conditions. Freeze drying (FD) has been a common technique to microencapsulate probiotics. It has the advantage of having a high cell survival rate with the addition of cryoprotectants. However, FD is a costly and slow process (compared to

other microencapsulation technologies) and requires an additional milling step to pulverize the dried samples. In contrast, spray drying (SD) has been used as a cost-effective and more viable approach to microencapsulate probiotics. In SD, the liquid sample is atomized to fine mist before contacting with high velocity hot and dry air which quickly removes the moisture and produces fine powder that can be readily separated from the airstream *via* centrifugal separation and/or other techniques (Murugesan & Orsat, 2012). Based on contact configurations between the hot drying air and atomized droplets, three different spray drying designs are available: concurrent (CCSD), mixed flow (MXSD) and counter-current (COUSD). According to Barbosa and Teixeira (2017), CCSD is used to dry heat sensitive products while heat-stable products are dried in COUSD conditions. In CCSD, both the drying air and atomized droplets are introduced from the top of the drying chamber (DC) (Fig. 2.1); then, the dried powders and drying air exit at the outlet of DC. Meanwhile in MXSD, the liquid feed is atomized from the bottom of the DC, while the drying air is introduced from the top of the DC. In the case of COUSD, liquid foods are atomized from the top and collected at the bottom of the DC, while the drying air enters from the bottom and exits at the top of the DC. Although a rapid, continuous, and cost-effective microencapsulation method; low cell survival rate has been reported after the SD of probiotic cultures due to osmotic, heat, and oxidative stresses caused by the atomization of probiotic suspensions, high temperatures and conditions of the drying air (Anekella & Orsat, 2013; Paéz et al., 2012). Guerin et al. (2017) reported that the cell survival rates in the SD of probiotics is strain dependent. Currently, CCSD is the most popular SD design to microencapsulate LRGG with reported cell survival (%) between 10 to 60% at outlet temperatures below 72 ℃ (Avila-

Reyes, Garcia-Suarez, Jiménez, San Martín-Gonzalez, & Bello-Perez, 2014; Broeckx et al., 2017; Ying, Sun, Sanguansri, Weerakkody, & Augustin, 2012). However, SD processes that required outlet temperatures below 80°C are difficult to scale up because of the high moisture content of the resulting powders. Up to date, few studies have reported the production of microencapsulated LRGG powders under MXSD. Recently, we compared the effect of MXSD, CCSD and FD on the cell viability of LP using soybean protein and/or whey protein isolates as microencapsulating agents, higher cell survival rates were obtained in MXSD compared to CCSD and FD (Mis-Solval, Jiang, Yuan, Joo, & Cavender, 2019). And we also found that MXSD yielded higher cell survival rates than CCSD when LRGG was microencapsulated with fish gelatin and maltodextrin (Jiang, Dev Kumar, Chen, Mishra, & Mis Solval, 2020). Hence, our team hypothesized that MXSD may be better than CCSD to produce probiotic powders. In this research we studied the effect of MXSD and/or FD on the cell survivability of microencapsulated LRGG using cannonball jellyfish gelatin and/or mammalian gelatin combined with maltodextrin.

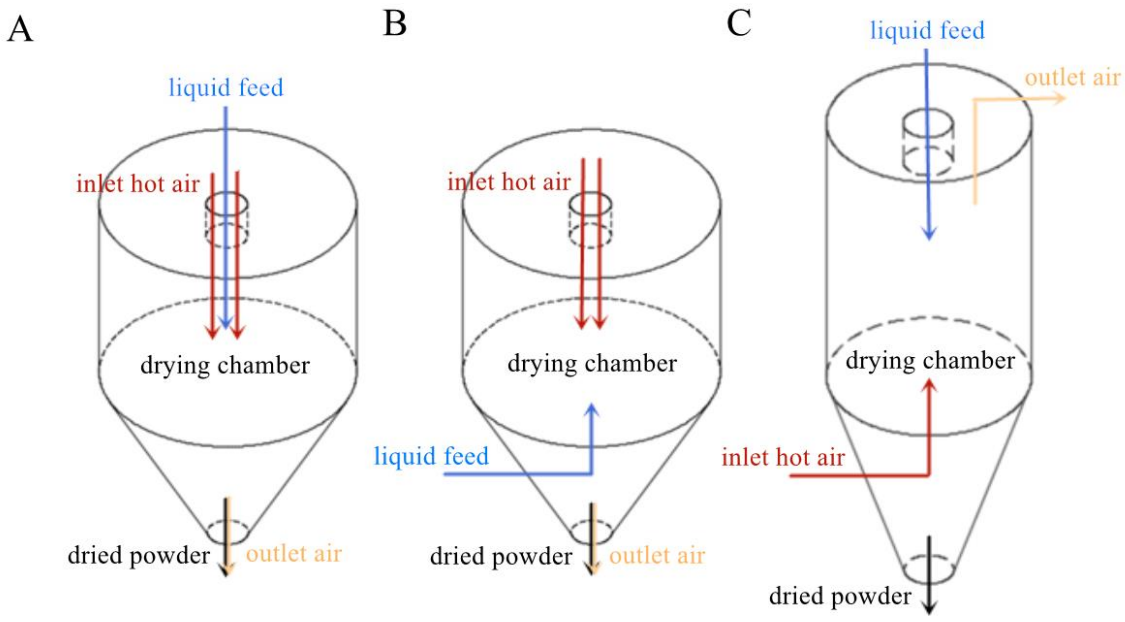

Fig 2.1. Spray drying designs: (A) concurrent spray drying (CCSD), (B) mixed-flow spray drying (MXSD) and (C) counter-current (COUSD)

Microencapsulation agents

Several biopolymers and food ingredients have been used as microencapsulating agents for probiotics, such as alginate, chitosan, gelatin, whey protein isolate, soy protein isolate, soymilk, skim milk and starch (Shori, 2017). In this project, we studied mammalian gelatins, gelatin from cannonball jellyfish, soymilk, and skim milk as microencapsulating agents.

Gelatins

Gelatin is a food ingredient produced by the partial hydrolysis of collagen which is the main fibrous protein constituent in bones, cartilages and skins. Typically, collagen is composed of about 35% glycine, 11% alanine, and 21% proline and hydroxyproline. Because of the low contents of cysteine and the essential amino acid tryptophan, collagen is regarded as a protein of low nutritional value (Boran & Regenstein, 2010). Gelatin (water-soluble) is produced from collagen (water-insoluble) in a sequence of processing steps including a pretreatment to remove non-collagen impurities, the hydrolysis of collagen with an alkaline and/or acid solution to convert collagen into gelatin, and finally, a series of refinement and recovery processes to get a highly purified dried gelatin (Fig. 2.2). At last, gelatin is separated usually by filtration, evaporation, and deionization, followed by drying and grinding (Boran & Regenstein, 2010; Hinterwaldner, 1977). The quality and amino acid composition of raw collagen affects the gelatin quality. Also, the processing conditions such as hydrolyzation temperatures, concentration and types of acid or alkali solutions and the soaking time can dramatically affect the final quality of gelatin (Boran & Regenstein, 2010; Cho, Jahncke, Chin, & Eun, 2006; Hinterwaldner, 1977; Zhou & Regenstein, 2005).

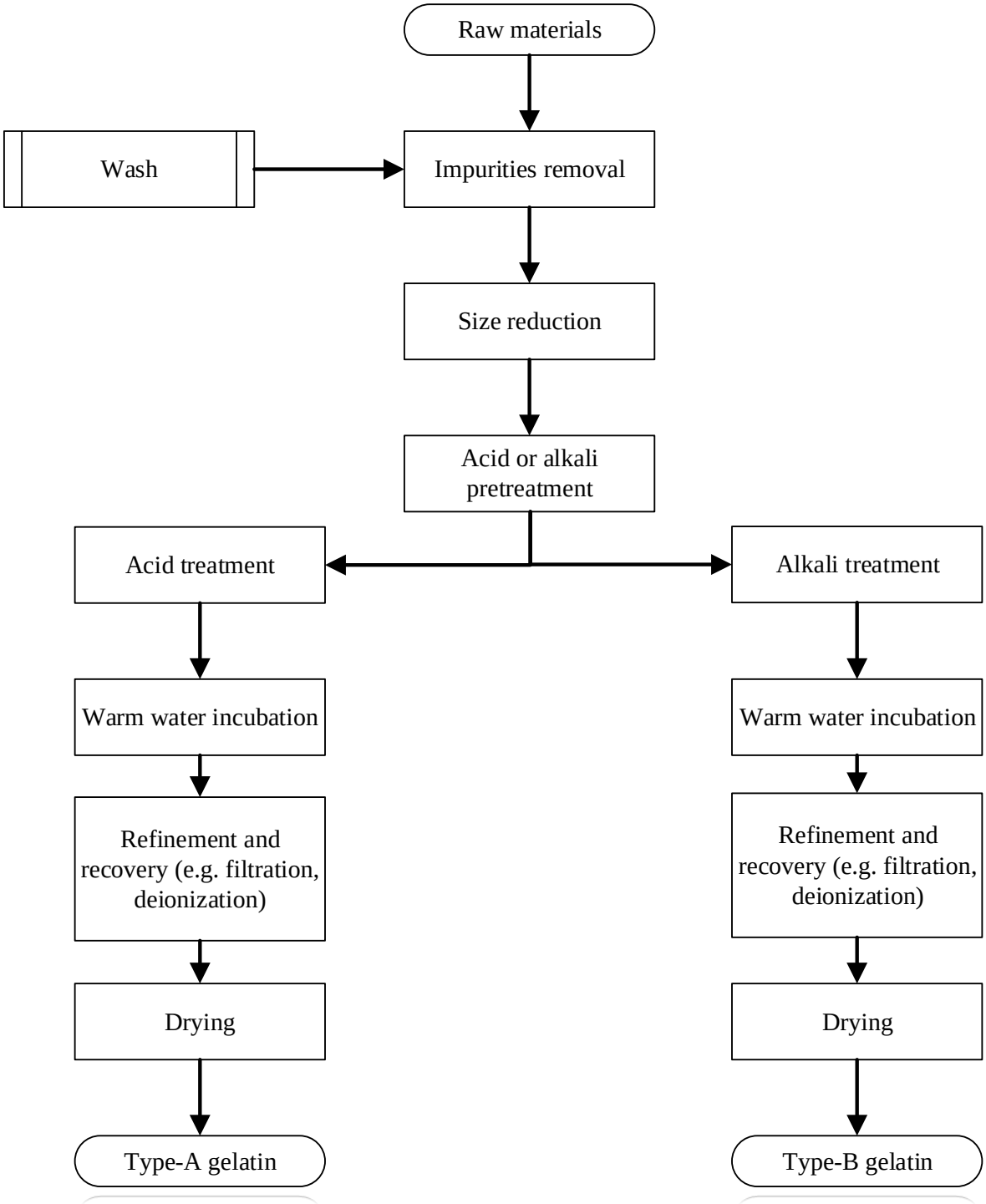

Fig. 2.2. General flowchart of gelatin production

For example, longer hydrolysis time and/or temperatures cause excessive damage to the collagen molecule and the resulting gelatins form weak gels with low viscosities. Similarly, excessive concentrations of acid and/or alkali can cause degradation of collagen structure giving a gelatin with lower functional values (Boran & Regenstein, 2010). Type-A gelatins are produced by hydrolyzing collagen with an acid solution, while type-B gelatins are produced by hydrolyzing collagen with an alkali solution (Boran & Regenstein, 2010; Hinterwaldner, 1977). The isoelectric point (pH at which solubility is minimal) of type-A is higher than that of type-B gelatin. The hydrolysis of collagen using weak organic acids is not capable to hydrolyze the amide nitrogen of glutamine and asparagine, which results in a high isoelectric point (as high as 9.4) for type-A gelatin. A stronger acidic hydrolysis of collagen may be able to hydrolyze some of the amide groups and therefore reducing the isoelectric point of the resulting gelatins (generally between 6 and 8). While, the isoelectric point of type-B gelatin might be as low as 4.8, as the alkali removes the amide groups in the collagen (Boran & Regenstein, 2010).

Most of commercial gelatins are produced from collagen obtained from bovine and porcine sources. The source, age of the animal, type of collagen and the processing methods are all factors influencing the properties of the gelatins. For mammalian gelatin production, skins are usually treated with an acid solution, while bones are usually treated with an alkali solution (Petersen & Yates, 1977). Mammalian gelatin is widely used by the food, cosmetic, pharmaceutical industries because of its great functional properties. Moreover, it is used in confections, low-fat spreads, dairy, baked goods, meats; as well as lowering caloric density in foods as a foaming, emulsifying, and wetting agent due to its

surface-active properties. Mammalian gelatin is a popular and effective microencapsulating agent for bioactives (Pech-Canul, Ortega, García-Triana, & González-Silva, 2020). Several studies have reported that mammalian gelatin can interact with various polysaccharides and it is an effective microencapsulating agent in several microencapsulation techniques, like SD, FD, extrusion, spray chilling, complex coacervation (Flores-Belmont, Palou, López-Malo, & Jiménez-Munguía, 2015; Nualkaekul, Cook, Khutoryanskiy, & Charalampopoulos, 2013; Paula et al., 2019; Pech-Canul et al., 2020; Silva et al., 2018; Silva et al., 2019; Yan & Zhang, 2014). Because of its linear structure, it is believed that mammalian gelatin can provide a better oxygen barrier than globular proteins when used as microencapsulating agent for probiotics (Borza et al., 2010; Kaushik & Roos, 2007; Li, Chen, Cha, Park, & Liu, 2009; Rajabi, Ghorbani, Jafari, Sadeghi Mahoonak, & Rajabzadeh, 2015). Borza et al. (2010) microencapsulated probiotics with genipin cross-linked gelatin-maltodextrin and found improved survival rates. Li et al. (2009) reported positive results when probiotics were microencapsulated with alginate–gelatin. Gelatin can form thermo reversible gels whose melting and gelling temperatures are below human body temperature (37°C); the gap between melting and gelling temperatures is narrower than carbohydrate-based gels; which make gelatins good microencapsulating agents to deliver probiotics.

Although most of commercial gelatins are produced from porcine and bovine sources, they are under constraints and skepticism because of social, cultural and health-related concerns (Karim & Bhat, 2009). Thus, gelatins produced from marine collagen (including fish and jellyfish) has been investigated recently. However, the quality of marine gelatins is highly variable while their prices are higher than mammalian gelatins

(Lin, Regenstein, Lv, Lu, & Jiang, 2017). According to Karim and Bhat (2009), marine gelatins have interesting properties such as low melting temperatures which allows a faster dissolution in the mouth with no residual 'chewy' mouthfeel, this effect may be desirable for a fast release of flavor and other bioatives in some food systems; therefore, providing technological and sensory advantages over mammalian gelatins (Choi & Regenstein, 2000). Also, some marine gelatins have shown acceptable gel strength and viscosities.

Most marine gelatins are extracted from fishery and aquaculture by-products including fish skin, bones and scales. Fish skins and bones may represent around 30% of the total weight of live fish (Gómez-Guillén et al., 2002). Depending on the fish species, size and processing conditions, the quality and properties of fish gelatin varies highly. Gelatin production from several fish species have been comprehensively studied. Fish skins have a great potential to yield gelatins that have similar gel strengths, viscosities and wider range of gelling and melting temperatures than mammalian gelatins (Boran & Regenstein, 2010).

Shyni et al. (2014) reported the extraction of type-A gelatin from the skins of dog shark (*Scoliodon sorrakowah*), skipjack tuna (*Katsuwonus pelamis*) and rohu (*Labeo rohita*) and found that the yield, molecular weight, viscosity, melting point, and hydroxyproline content of dog shark gelatin were higher than those of tuna and rohu skin gelatins. Furthermore, the foaming properties, water holding capacity, odor, color and clarity of dog shark gelatin were in general better than those of the tuna and rohu skin gelatins. Gelatin from tilapia scales extracted at different pH conditions (from 3 to 9) was reported by Weng, Zheng, and Su (2014). The study reported that the ash and calcium

contents of gelatins extracted at pH 3 was higher compared to gelatins extracted at higher pH conditions; while, the amino acid composition of gelatin extracted at pH 3 was similar to that extracted at pH 9, but was different to those extracted from pH 5 or pH 7.

According to Jeya Shakila, Jeevithan, Varatharajakumar, Jeyasekaran, and Sukumar (2012), the extraction of type-A fish bone gelatin from red snapper and grouper had melting and gelling temperatures of 26 °C and 16 °C, respectively. The high viscosity, bloom strength, foaming abilities/stabilities and fat binding capacity (FBC) and water holding capacity of the fish bone gelatin indicates that it can be used as a good alternative to mammalian gelatin. Wangtueai and Noomhorm (2009) reported the production of high-quality type-B gelatin from lizardfish (*Saurida* spp.) scales that could be a good alternative to mammalian gelatin.

Gelatin can act as an emulsifier by absorbing the immiscible liquids; therefore, reducing the interfacial tension between them and facilitate the production of small micelles. It can improve the long-term stability of emulsions by generating repulsive forces between droplets on droplet surface and by forming interfacial membranes resistant to rupture around the droplets (Surh, Decker, & McClements, 2006). Moreover, gelatin has been used as emulsifier in oil-in-water (O/W) emulsions to protect lipids from iron-catalyzed oxidation. When gelatin is dispersed with pH values below its isoelectric point (pI), it forms positively charged interfacial membranes around the micelles that electrostatically repel Fe^{2+} and Fe^{3+} ions in the continuous phase, this prevents iron from catalyzing oxidation of the lipids in the micelles (Hu, McClements, & Decker, 2003). Type-A gelatin have a relatively high isoelectric point and most fish gelatins are type-A gelatin, most fish gelatins are more effective at positively charging, over a wider range of

pH values, the surface of droplets than other protein emulsifiers, *e.g.* casein, whey proteins, land mammalian bone gelatin and soybean protein.

Because of its emulsifying properties, gelatin has been used as microencapsulating agent of bioactives. According to Dickinson and Lopez (2001), the emulsifying properties of fish gelatin are useful in products like microencapsulated powders. However, fish gelatin often makes relatively large micelles when used alone (Dickinson & Lopez, 2001; Lobo, 2002; Taherian, Britten, Sabik, & Fustier, 2011). Therefore, researchers have modified fish gelatin with polar or nonpolar groups (Huang et al., 2018; Toledano & Magdassi, 1998), or used fish gelatin in conjunction with anionic surfactants or other encapsulants to improve its rheological behavior and emulsifying effectiveness (Aewsiri et al., 2009; Olijve, Mori, & Toda, 2001; Surh, Gu, Decker, & McClements, 2005; Taherian et al., 2011). Duan, Zhang, Liu, Cui, and Regenstein (2018) compared the performance of gelatin from channel catfish (*Ictalurus punctatus*) skin and calf bone gelatin in ice-cream and beer. The study found that catfish gelatin had more high molecular weight components (β and γ chains) than calf bone gelatin, and the viscosity of catfish gelatin was three times more than that of calf bone gelatin. Compared to calf bone gelatin, catfish gelatin showed higher emulsion capacity and stability, as well as higher foaming stability. The resulting catfish gelatin containing ice cream and beer had better mouth feel and clarification effects, respectively.

The gelation and stabilization properties of marine gelatins are important for the microencapsulation of bioactives (Karim & Bhat, 2009). For example, Soper (1997) reported the microencapsulation flavor particles using fish gelatin. Moreover, fish gelatin is used in both hard and soft capsules production. The gelatin prevents the drug

degradation during transportation and storage. Apfel, Ghebre-Sellassie, and Nesbitt (1991) reported the use of Teleostean (fish) gelatin (soluble in water at 5°-10° C) to encapsulate water insoluble drugs. Lately, our team used commercial fish gelatin and maltodextrin to microencapsulate LRGG through different SD designs and observed satisfactory cell viability and biofilm formation properties (Jiang et al., 2020).

Recently, jellyfish populations have developed quickly around the world as a result of overfishing, climate change, eutrophication, and habitat modifications (Purcell, Uye, & Lo, 2007; Richardson, Bakun, Hays, & Gibbons, 2009). This phenomenon known as 'jellyfish blooming' can cause serious problems including stings to humans (sometimes deadly), decline in coastal tourism, clogging of cooling equipment and disabling of power plants, burst fishing nets, and contaminate fish catches (Barzideh, Latiff, Gan, Benjakul, & Karim, 2014; Dong, Liu, & Keesing, 2010). The consumption of some species of jellyfish (*Acromitus hardenbergi*, *Rhopilema hispidum* and *Rhopilema esculentum*) have been associated with many health benefits, including anti-hypertensive, anti-hyperlipidemic, UV-protective and immunostimulant properties (Khong et al., 2016; Omori & Nakano, 2001). Edible jellyfish is a good source of collagen, vitamins, and minerals. Moreover, conversion of jellyfish collagen into gelatin using acidic and alkaline methods has been reported (Chancharern, Laohakunjit, Kerdchoechuen, & Thumthanaruk, 2016; Cho, Ahn, Koo, & Kim, 2014; Karim & Bhat, 2009; Khong et al., 2016; Rodsuwan, Thumthanaruk, Kerdchoechuen, & Laohakunjit, 2016).

For example, Cho et al. (2014) reported that although rheological properties of gelatin from jellyfish (*Rhopilema Hispidum*) are not as good as mammalian gelatins, they can potentially replace mammalian gelatins in products that do not require strong gelling

properties. Both type-A and type-B gelatins have been extracted from desalted jellyfish (*Lobonema smithii*). Type-A has shown better gelling properties than type-B gelatin and can be a good alternative of mammalian gelatin in food or cosmetic products (Chancharern et al., 2016; Rodsuwan et al., 2016).

Cannonball jellyfish (CJ) (*Stomolophus Meleagris*), one of the largest commercial fisheries in Georgia by landings (kg), has been consumed in several Asian countries for centuries where it is considered a delicacy (Fluech, 2018). Collagen is the main protein in CJ. It is estimated that about 80-90% of all the protein in jellyfish is collagen (Hsieh & Rudloe, 1994). To date, limited scientific literature is available on the conversion of CJ collagen into gelatin. Therefore, our team has hypothesized that collagen from CJ can be converted into gelatin. The resulting gelatin from CJ can be used as an effective microencapsulating agent for probiotic bacteria.

Soymilk

Soybean is as an excellent source of protein (~40% on dry basis), enriched dietary fiber, nutritive minerals and vitamins. Soy proteins are highly digestible after heat treatment and have a balanced amino acid profile that meets the nutritional requirements of humans. Soy-based foods became more popular after the soy protein health claim were approved by the Food and Drug Administration (FDA) in 1999 (FDA, 1999). Consumption of soy-based foods has increased in recent years because their consumption has been associated with lower incidence of heart diseases (Giri & Mangaraj, 2012). Soymilk is the liquid extract of whole soybean and it has a similar appearance, physical properties and composition to cow's milk. It is an oil-in-water (O/W) emulsion with a high protein content. Soymilk is produced with a sequence of processing steps including

the selection, soaking, and wet grinding of soybeans, followed by filtration, heating, fortification, formulation, and packaging (Giri & Mangaraj, 2012). The physical properties, composition and nutritional values of soymilk are influenced by many factors such as soybean quality, formulation, processing methods and storage conditions. In US, no federal standards have been established for soymilk products. Table 2.1 is the nutritional values of soymilk (per 100 g) from the USDA's Food and Nutrient Database for Dietary Studies (FNDDS) (U.S. Department of Agriculture & Agricultural Research Service, 2018), which can provide an approximate idea of the composition and nutritional values of soymilk. From the table we can learn that soymilk is a good source of protein, minerals (like Fe, Zn) and vitamins (like vitamin B and vitamin K). Additionally, it contains lecithin, free amino acids, and polypeptides.

Table 2.1 Nutritional values of soymilk (per 100 g) from USDA's Food and Nutrient Database for Dietary Studies (FNDDS) (U.S. Department of Agriculture & Agricultural Research Service, 2018)

Component	Amount	Component	Amount
Water	90.36 g	Niacin	0.425 mg
Energy	43 kcal	Vitamin B-6	0.031 mg
Protein	2.6 g	Folate, total	9 µg
Total lipid	1. 47 g	Folate, food	9 µg
Carbohydrate, by difference	4.92 g	Folate, DFE	9 µg
Fiber, total dietary	0.2 g	Choline, total	23.6 mg
Sugars, total	3.65 g	Vitamin B-12	0.85 µg
Calcium, Ca	123 mg	Vitamin B-12, added	0.85 µg
Iron, Fe	0.42 mg	Vitamin A, RAE	55 µg
Magnesium, Mg	15 mg	Retinol	55 µg
Phosphorus, P	43 mg	Carotene, beta	2 µg
Potassium, K	122 mg	Vitamin E (α-tocopherol)	0.11 mg
Sodium, Na	47 mg	Vitamin D (D2 + D3)	1.1 µg
Zinc, Zn	0.26 mg	Vitamin K (phylloquinone)	3 µg
Copper, Cu	0.165 mg	Fatty acids (FA), total saturated	0.205 g
Selenium, Se	2.3 µg	FA, total monounsaturated	0.382 g
Thiamin	0.029 mg	FA, total polyunsaturated	0.858 g
Riboflavin	0.184 mg		

Soy isoflavones (with antioxidant capacity and estrogenic activity) are associated with a lower incidence of menopausal symptoms, hormone-dependent breast cancer, prostate cancer, cardiovascular disease, colon cancer, and osteoporosis (Mazumder & Hongsprabhas, 2016). With enriched nutrients and pH being 6.7, soymilk is a very suitable medium for microencapsulation of probiotics. According to Hati, Patel, and Mandal (2018), *Lactobacillus acidophilus*, *Lactobacillus pentosus*, *Lactobacillus delbrueckii* ssp. *Leuconostoc mesenteroides* and *Streptococcus thermophilus* can grow well in soymilk. Soymilk and fermented soymilk products were regarded as good nutritional supplements and suitable economical plant-based alternatives to dairy products for people who avoid animal food and who are allergic to dairy product (Abou-Dobara, Ismail, & Refaat, 2016).

Because of their functional properties (*e.g.* gelation and emulsification), soy protein isolate and soy extract are good microencapsulating agents for probiotics. Shi, Zheng, Zhang, Liu, and Tang (2015) encapsulated *Enterococcus faecalis* HZNU P2 with soy protein–alginate microspheres successfully, and found that soy protein–alginate microsphere showed a good potential as an encapsulation carrier for the protection of *E. faecalis* HZNU P2 during the storage and simulated transit in GI tract. Besides, Dianawati, Mishra, and Shah (2013) also reported that the survival of *Bifidobacterium longum* 1941 microencapsulated with soy protein isolate and maltodextrin reached almost 90% after freeze drying, more than 85% after bile tolerance test and more than 60% after acid tolerance test. Praepanitchai, Noomhorm, and Anal (2019) have reported the use of soy protein isolate with alginate for the microencapsulation of *L. plantarum* by extrusion which had probiotic cell survival rates above 90%. Furthermore, *L. acidophilus* has been

successfully microencapsulated with soy extract and maltodextrin using spray drying (Acordi Menezes et al., 2018). Additionally, microencapsulation of LP with ultra-high pressure homogenization-treated soy protein isolate alone as microencapsulating agent was achieved by SD and FD, with cell survival rates around 32% after SD and 33% after FD (Mis-Solval et al., 2019).

Skim milk

Cow's milk contains lactose (about 5%, wet basis) and proteins (about 3.3%, wet basis) mainly casein and whey proteins. With a water activity (a_w) value close to 1.0 and pH between 6.6–6.7, cow's milk is a great medium for probiotics. Commercially, there are four types of cow's milk (based on fat content): skim milk (0.1% fat, wet basis), low fat milk (1% fat, wet basis), reduced-fat milk (2% fat, wet basis) and whole milk (>3.3% fat, wet basis). Table 2.2 shows the nutritional composition of skim milk.

Table 2.2 Nutritional values of skim milk (per 100 g) from USDA National Nutrient Database for Standard Reference, Legacy (U.S. Department of Agriculture, Agricultural Research Service, & Nutrient Data Laboratory, 2018).

Component	Amount	Component	Amount
Water	90.84 g	Betaine	1.9 mg
Energy	35 kcal	Vitamin B-12	0.5 µg
Energy	144 kJ	Vitamin A, RAE	2 µg
Protein	3.37 g	Retinol	1 µg
Total lipid	0.18 g	Carotene, beta	7 µg
Ash	0.75 g	Vitamin A, IU	15 IU
Carbohydrate, by difference	4.86 g	Vitamin E (alpha-tocopherol)	0.01 mg
Sugars, total including NLEA	5.09 g	Fatty acids, total saturated	0.117 g
Lactose	5.09 g	Fatty acids, total monounsaturated	0.047 g
Calcium, Ca	122 mg	Fatty acids, total polyunsaturated	0.007 g
Iron, Fe	0.03 mg	Cholesterol	2 mg
Magnesium, Mg	11 mg	Tryptophan	0.043 g
Phosphorus, P	101 mg	Threonine	0.144 g
Potassium, K	156 mg	Isoleucine	0.174 g
Sodium, Na	42 mg	Leucine	0.319 g
Zinc, Zn	0.42 mg	Lysine	0.282 g
Copper, Cu	0.013 mg	Methionine	0.088 g
Manganese, Mn	0.003 mg	Cystine	0.021 g
Selenium, Se	3.1 µg	Phenylalanine	0.175 g
Fluoride, F	3.1 µg	Tyrosine	0.17 g
Vitamin C, total ascorbic acid	0 mg	Valine	0.221 g
Thiamin	0.045 mg	Arginine	0.096 g
Riboflavin	0.182 mg	Histidine	0.102 g
Niacin	0.094 mg	Alanine	0.114 g
Pantothenic acid	0.357 mg	Aspartic acid	0.288 g
Vitamin B-6	0.037 mg	Glutamic acid	0.757 g
Folate, total	5 µg	Glycine	0.067 g
Folate, food	5 µg	Proline	0.332 g
Folate, DFE	5 µg	Serine	0.203 g
Choline, total	15.6 mg		

According to Fu and Chen (2011), skim milk can effectively protect the probiotic cells from dehydration when used as microencapsulating agent. Several studies have reported the use of skim milk or reconstituted skim milk as microencapsulating agent for probiotics and have found good survival rates of probiotic cells after the microencapsulation process (Ananta, Volkert, & Knorr, 2005; Fu & Chen, 2011; Gardiner et al., 2000; Gardiner et al., 2002; Lian, Hsiao, & Chou, 2002; Riveros, Ferrer, & Borquez, 2009). Ananta et al. (2005) reported that when reconstituted skim milk was used as microencapsulating agent for *Lactobacillus rhamnosus* GG, a microbial survival rate of 60% was achieved with concurrent spray drying at an outlet temperature of 80 ℃. *Lactobacillus salivarius* CTC 2197 was microencapsulated with reconstituted skim milk *via* concurrent spray drying at outlet temperature of 70 ℃ and showed survival rate almost 100% during drying (Silva, Carvalho, Teixeira, & Gibbs, 2002). Reconstituted skim milk containing *Lactobacillus paracasei* NFBC 338 was dried by pilot scale spray dryer with air inlet and outlet temperatures of 175 ℃ and 68 ℃, respectively, which yielded a probiotic survival of 84.5% (Gardiner et al., 2002). The survival of *Lactobacillus casei* Shirota microencapsulated with 30% reconstituted skim milk was 94% after spray drying at outlet temperature ranging between 64-68 ℃ (Gul, 2017). Golowczyc, Silva, Abraham, De Antoni, and Teixeira (2010) studied the viability of *Lactobacillus plantarum* CIDCA 83114, *Lactobacillus kefir* CIDCA8348 and *Saccharomyces lipolytica* CID-CA 812, all from kefir, separately, microencapsulated with 11% (w/v) reconstituted skim milk during spray drying at outlet temperature of 70 ℃, and the survival rates were about 10%, 2% and 0.52% respectively. The protection ability of skim milk for probiotics during drying varied with different probiotic strains.

As the composition of skim milk is complex (Table 2.2), the exact protecting mechanism of skim milk remains unknown.

Ultra-high pressure homogenization (UHPH)

UHPH is a novel technology that forces liquids through a valve or other pressure release components with pressures more than 100 MPa, exerting ultra-shear (Patrignani & Lanciotti, 2016). UHPH can inactivate microorganisms (Patrignani & Lanciotti, 2016), modify proteins configurations (Paquin, 1999), promote intermolecular complexes formation (Li et al., 2019), and improve emulsion stability (Cruz et al., 2007; Hebishy, Zamora, Buffa, Blasco-Moreno, & Trujillo, 2017; Sidhu & Singh, 2016), while maintaining nutritional and sensory qualities of the food (Sidhu & Singh, 2016). It has been reported that the microbial, sensorial, physicochemical and nutritional properties of UHPH-treated cow's milk is similar or better than heat-treated milk (Amador-Espejo, Gallardo-Chacon, Nykänen, Juan, & Trujillo, 2015). UHPH dissociates casein micelles and partial denatures whey protein. UHPH-treated skim milk at 186 MPa, modifications in the casein micelle surface are observed (without the complete disruption of the casein micelle), as well as induced calcium phosphate solubilization because of casein micelle modification (Sandra & Dalgleish, 2005). Moreover, UHPH improves the binding efficiency of various compounds, like α-tocopherol acetate, triclosan, curcumin, vitamins, or other nutritional hydrophobic compounds (Benzaria, Maresca, Taieb, & Dumay, 2013; Chevalier-Lucia, Blayo, Gràcia-Julià, Picart-Palmade, & Dumay, 2011; Roach, Dunlap, & Harte, 2009). However, UHPH at pressures >200 MPa can cause casein micelle aggregation, which may be due to the increased solubilization of micellar calcium phosphate (Hayes & Kelly, 2003). And the pressure level of UHPH also affects the

denaturation/aggregation of whey proteins (globular proteins) (Trujillo, Roig-Sagués, Zamora, & Ferragut, 2016). In addition, UHPH promotes interactions between milk fat globule membrane components and whey proteins and/or caseins that were in the aqueous phase originally (Trujillo et al., 2016; Zamora, Ferragut, Guamis, & Trujillo, 2012). Cruz et al. (2007) reported the effect of UHPH on the quality of soymilk, the authors found that treating soymilk at pressures of 200MPa partially denatured soymilk proteins and the UHPH-treated soymilk was more stable than non-treated and ultra-high temperature treated soymilks. Interestingly, UHPH didn't affect the nutritional composition and sensory characteristics of soymilk; nevertheless, higher protein denaturation was observed with increased pressures (Ferragut et al., 2015; Sidhu & Singh, 2016). Li et al. (2019) reported that UHPH promoted more exposure of hydrophobic residues on the surface of soybean protein isolate and the interaction between soybean protein isolate and phosphatidylcholine in emulsions. Additionally, UHPH treating raw almond milk at 350MPa altered the physicochemical characteristics like particle size, and accessibility of antigenic epitopes and free -SH groups (Briviba, Gräf, Walz, Guamis, & Butz, 2016).

UHPH-treated milk can potentially improve the grow of probiotics in fermented dairy product (Burns et al., 2008; Patrignani et al., 2009; Patrignani et al., 2007). Patrignani et al. (2009) demonstrated that the counts of viable *Strep. Thermophilus* and *Lb. delbrueckii subsp. Bulgaricus* were higher in high pressure homogenization (HPH)-treated milk at 60MPa than non-HPH treated samples after fermentation and a period of refrigerated storage (35 days). UHPH-treated milk at 100MPa before inoculating can also

improve the viability of probiotic bacteria in cheese during the refrigerated storage (Burns et al., 2008).

We have found an increase in cell survival of probiotics microencapsulated with 150MPa-treated whey protein isolate and/or soy protein isolate after SD and/or FD (Mis-Solval et al., 2019). Based on the available scientific information about the effect of UHPH on the chemical, nutritional and physical properties of milk, we have hypothesized that UHPH-treated soy milk and/or skim milk can be good microencapsulating materials for probiotics. In this research we used 150MPa and 300MPa-treated soy milk and/or skim milk to microencapsulate *Lactobacillus plantarum* NRRL B-1927 *via* MXSD, CCSD and FD.

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CHAPTER 3

MICROENCAPSULATION OF PROBIOTIC *LACTOBACILLUS RHAMNOSUS GG* WITH JELLYFISH GELATIN

3.1 Materials and Methods

3.1.1 Materials

Salted and dried (SD) cannonball jellyfish (*Stomolophus Meleagris*) was obtained from Golden Island International (Brunswick, GA, USA), maltodextrin (MD) (dextrose equivalent = 9-13) from Now Foods (Bloomingdale, IL, USA), *L. rhamnosus* GG (ATCC 53103) (LRGG) from Culturelle (Cromwell, CT, USA), unflavored mammalian gelatin (MG) from Knox (Kraft Heinz Foods Company, Chicago, IL, USA), Butterfield's phosphate buffer from Hardy Diagnostics (Santa Maria, CA, USA), de Man, Rogosa and Sharpe (MRS) broth and MRS agar from Thermo Fisher Scientific (Waltham, MA, USA) and citric acid from Milliard (Lakewood, NJ, USA). Other chemicals were analytical grade and were obtained from Sigma Aldrich (St. Louis, MO USA).

3.1.2 Proximal composition of SD jellyfish

SD jellyfish was divided into umbrellas and oral arms. Its moisture content, crude protein, and ash content were determined by following the AOAC Official Method 934.01 (oven drying), 976.05 (automated Kjeldahl method), and 938.08 (furnace combustion), respectively (AOAC, 2019). Crude protein was quantified by measuring total nitrogen following a dry combustion method with a nitrogen analyzer (Rapid N Exceed, Elementar, Germany). Conversion factor of 5.8 was used to determine crude

protein (Khong et al., 2016). Water activity (a_w) values were obtained with a water activity meter (AquaLabSeries 3 TE, Decagon Devices, Inc., WA, USA). Color values (L*, a*, b*, chroma, and hue angle) were determined using a CIE colorimeter (Hunter associates lab. Inc., VA, USA).

3.1.3 Production of gelatin powders from SD jellyfish

Approximately, 1 kg (about 8 units) of SD jellyfish was rinsed and soaked in 8 L of tap water overnight for rehydration and to remove excess salt. Rehydrated SD jellyfish was rinsed with clean tap water, chopped, soaked in 3 L of 1.5% citric acid solution for 10 min., homogenized with a blender (Model BL610, NINJA, SharkNinja Operating LLC, Needham, MA, USA) in medium power for about 8 min and in high power for about 4 min, and processed with an ultra-shearing homogenizer (Homogenizer 850, Fisherbrand, Fisher Scientific UK Ltd, Loughborough, UK) at 8000 rmp for 6 min, and 10000 rmp for 8min, until the jellyfish sample was totally liquified. Afterwards, the liquified jellyfish sample was placed in water bath (Model 2872, Precision, Thermo Electron Corporation, Waltham, MA, USA) at 60°C for 270 min to hydrolyze the jellyfish collagen. These conditions were selected based on preliminary work conducted by our research group (data not shown). The resulting liquid hydrolyzed jellyfish collagen (LHC) was dialyzed to remove excess minerals using a regenerated cellulose dialysis tube (MWCO: 6-8 kD) (Spectra/Por® 1 Dialysis Membrane, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) at room temperature, following manufacturer instructions with deionized (DI) water as the dialysis buffer. 400 mL of LHC were placed in a dialysis tube which was submerged in 3.6 L of DI water. The dialysis buffer was replaced every 3 hours. In total, DI water was replaced three times

(Total dilution factor = 10^3, total time of dialysis = 9 h). Then, the resulting dialyzed LHC (DLHC) was frozen at -18°C for 12 h and freeze dried at -55°C for 96 h. Freeze-dried samples were subsequently powdered using a blender to obtain jellyfish gelatin powders (JG) which were immediately stored in a desiccator under room temperature.

3.1.4 Characterization of LHC and DLHC

3.1.4.1 Moisture content and ash content

Moisture and ash content of LHC and DLHC were determined with AOAC Official Method (AOAC, 2019). Moisture contents were measured with AOAC Official Method 934.01 (oven drying) with modification. Samples were dried to a constant weight (~16 h) at 100℃ in a mechanical oven (MO1440SC, Lindberg/Blue M, Asheville, NC, USA). The solid residues from moisture content measurement were used to determine ash contents according to AOAC Official Method 938.08 (furnace combustion). The solid residues were ashed to constant weight (about 8 h) at 550 ℃ in muffle furnace (Model NO. F-A1730, Thermolyne™, Sybron Corporation, Dubuque, Iowa, USA).

3.1.4.2 Mineral composition

Mineral composition of the samples was analyzed at the University of Georgia Soil, Plant and Water Laboratory under the Agricultural and Environmental Services Laboratories. Phosphorus, K, Ca, Mg, Mn, Fe, Al, B, Cu, Zn, Na, Pb, Cd, Ni, Cr and Mo were quantified using a microwave-acid (HNO3) digestion, ICP method using CEM Mars5 microwave digestion system model 61E ICP (Thermo Jarrell-Ash, Franklin, MA, USA).

3.1.5 Microencapsulation of *L. rhamnosus* GG (LRGG)

3.1.5.1 Preparation of LRGG cultures

LRGG cultures were prepared according to a modified method reported by Klu, Williams, Phillips, and Chen (2012). 1 gram of freeze-dried LRGG cultures (~10 Log CFU/g) was dissolved in 9 mL sterile Butterfield's phosphate buffer and 100 µL of this mixture were pour plated on MRS agar-containing petri dishes. The inoculated plates were wrapped in Ziploc bags (S.C. Johnson & Son, USA) and incubated at 37 °C for 24 h. To harvest LRGG cells, 2 mL of sterile Butterfield's phosphate buffer were added to an incubated plate, the surface of the plate was scraped and the liquid buffer containing LRGG cells was collected and centrifuged using a microcentrifuge (MiniSpin plus, Eppendorf, Hamburg, Germany) at 13,000 × *g* for 1 min. The collected LRGG pellet was washed with sterile Butterfield's phosphate buffer twice.

3.1.5.2 Preparation of LRGG suspensions

LRGG suspensions were prepared using a modified method previously reported by our lab (Mis-Solval et al., 2019). Two suspensions were prepared: a) 50 g of MD and 50 g of JG were homogenized in 1 L of DI water, and b) 50 g of MD were homogenized with 50 g of MG in 1 L of DI water. The suspensions were autoclaved (Model SM510, Yamato Scientific, Co. LTD, Japan) at 121°C for 15 min. Afterwards, LRGG pellets were suspended in cooled sterile suspensions to produce LRGG suspensions (~9 Log CFU/g solids). In this study, LRGG cells were also microencapsulated with MG to compare the performance of JG as a microencapsulating agent.

3.1.5.3 Drying of LRGG suspensions

LRGG suspensions were dried under mixed-flow spray drying (MXSD) conditions using a pilot-scale spray dryer (Anhydro, PSD 52, Denmark) using similar conditions previously reported by our group (Mis-Solval et al., 2019). Inlet and outlet air temperatures were set at 140°C and 80±1°C, respectively. Outlet air temperature was kept constant by adjusting the feed flow rate between 1.5 – 2.0 L/h. Alternatively, LRGG suspensions were frozen at -18°C for 12 h and FD at −55 °C for 96 h using a pilot-scale lyophilizer (Virtis, the Virtis Company, Gardiner, NY, USA) to produce FD samples which were subsequently powdered using a high-performance blender (Vitaminix 7500, Olmsted Township, OH, USA) to produce FD LRGG powders. MXSD and FD LRGG powders were kept in a desiccator and characterized within five days after the drying procedure.

3.1.5.4 Characterization of LRGG powders

3.1.5.4.1 Enumeration of LRGG cultures

Plate counts of viable cells were determined in LRGG suspensions and LRGG powders right before and immediately after the drying process. 100 µL of LRGG suspensions or 1 g of dried powders were serially diluted in sterile Butterfield's phosphate buffer and 100 µL of each dilution was pour plated on 100 mm diameter MRS agar plates in triplicate. Before counting cell colonies, plates were put in Ziploc bags (S.C. Johnson & Son, USA) and incubated for 40 h at 37°C. For ease of comparison, results were expressed as Log of colony forming units (CFU) per gram of dried solids (Log CFU/g). Cell survival rate of the probiotic cells was calculated using Eq. (1)

$$Cell\ survival\ (\%) = \frac{POWD}{SUSP} * 100 \quad (1)$$

where, POWD = cell counts (CFU/g solids) in LRGG powders; SUSP= cell counts (CFU/g solids) in LRGG suspensions before drying.

3.1.5.4.2 Moisture content and water activity (a_w)

Moisture contents of the LRGG suspensions and microencapsulated LRGG powders were measured using a moisture analyzer (HR73 Halogen Moisture Analyzer, Mettler-Toledo GmbH, Greifensee, Switzerland). Water activity (a_w) values of the microencapsulated LRGG powders were determined by a water activity meter (AquaLabSeries 3 TE, Decagon Devices, Inc., Pullman, WA, USA).

3.1.5.4.3 Color

The color of the microencapsulated LRGG powders was analyzed using a Lab Scan XE Colorimeter (Hunter Associates Laboratory, Inc., Resbon, VA, USA). The results were reported in CIE Lab color scales (L*, a*, and b* values). Chroma and hue Angle (°) values were calculated with method previously reported by Mis Solval, Sundararajan, Alfaro, and Sathivel (2012).

3.1.5.4.4 Particle size distribution

Particle size distribution of the microencapsulated LRGG powders was recorded with a particle size analyzer (Model PSA 1190, Anton Paar GmbH, Graz, Austria). Powders were illuminated with three lasers from different angles, the whole light scatter pattern was collected and used to calculate the particle size distribution by the system with Modified Michelson Interferometer method. The results were reported for D_{10}, D_{50}, and D_{90} which are the volume diameter of the particles at 10%, 50%, and 90%

cumulative volume respectively and the Span value (spread of particles) was calculated by following the method referred to Mis Solval, Bankston, Bechtel, and Sathivel (2016).

3.1.5.4.5 Shelf life

In order to determine stability, LRGG powders were placed in disposable polystyrene petri dishes and stored at a relative humidity (RH) of 50-55% and 75% which were maintained constant by saturated magnesium nitrate solution and saturated sodium chloride solution respectively in sealed glass containers. RH was measured with Temp/RH data logger (DIGI-SENSE 20250-43, Cole-Parmer Instrument Company LLC, IL, USA). The samples were kept under room temperature for 15 days. After the designated storage periods, samples were taken, and cell counts (CFU/g solids) of LRGG in the powders, moisture contents and water activities were determined using the methods described above. LRGG cells were enumerated as previously described.

3.1.7 Statistical analysis

Means and standard deviations (SD) of test results were reported. Analysis of Variance (ANOVA) and post-hoc Tukey's studentized range tests ($\alpha = 0.05$) were employed to determine the statistical significance of observed differences among means using RStudio statistical software version 1.2.5033 (RStudio, Inc. Boston, MA, USA).

3.2 Results and discussion

3.2.1 Characterization of SD Jellyfish

The oral arms of SD jellyfish had a slightly higher moisture content (71.28 g/100g) than the umbrellas (70.28 g/100 g) (Table 3.1). To produce SD jellyfish, freshly caught jellyfish is cured and dehydrated with a mix of table salt (NaCl) and alum ($KAl(SO_4)_2$) at room temperature for ~35-37 days. This process reduces the moisture

content of fresh jellyfish from 95-98 to about 68-70 (%, wet basis) and allows the conversion of a gel-like into a characteristic rubber-like texture with a crunchy feel (Hsieh & Rudloe, 1994). Similar results are reported by Khong et al. (2016) for other species of edible jellyfish. Higher crude protein content (%, dry basis) and ash content (%, dry basis) were found in oral arms than in the umbrellas (Table 3.1). It has been reported that fresh jellyfish contains ~ 2-3 % (wet basis) of salt (which is close to the osmotic equilibrium with sea water) and ~1 % (wet basis) of protein (25-35 g protein/100 g, dry basis); whereas SD jellyfish contains ~25 % (wet basis) of salt, and 5.5 % (wet basis) of crude protein (Hsieh & Rudloe, 1994).

Table 3.1 Preliminary characterization of SD jellyfish

		Umbrellas	Oral arms
Moisture (g/100g, wet basis)		70.28±0.59[b]	71.28±0.07[a]
Crude protein (g/100g, dry basis)		6.43±0.67[b]	7.96±0.55[a]
Ash (g/100g, dry basis)		88.40±0.04[b]	88.67±0.01[a]
Water activity (aw)		0.76±0.01[a]	0.76±0.02[a]
Color	**L***	23.88±1.41[b]	34.48±0.29[a]
	a*	5.56±0.83[a]	1.06±0.40[b]
	b*	19.21±2.96[a]	13.18±0.80[b]
	hue	73.87±0.71[b]	85.39±2.05[a]
	Chroma	20.00±3.07[a]	13.22±0.77[b]

Values are means ± SD of triplicate determinations.
[ab]Means with different letters in the same row are significantly different (P<0.05).

Furthermore, the a_w values of both oral arms and umbrellas were ~ 0.76 which indicates that the products are microbiologically stable at room temperature. Lower saturation (chroma values) was observed in umbrellas than in oral arms; while umbrellas were more yellowish and oral arms were more greenish (hue angle values). The change in color observed in SD jellyfish samples may be due to the release of polyphenols that may be entrapped in different tissues of fresh jellyfish. Understanding the composition of SD jellyfish is critical for developing customized food ingredients with tailored functional properties.

3.2.2 Ash content and mineral profile of LHC and DLHC

Preliminary results revealed that the amount of salts and minerals in LHC greatly affected the quality of the resulting jellyfish gelatin. It has been reported that dialysis can be used to remove minerals from macromolecule solution and purify active ingredients (Andrew, Titus, & Zumstein, 2001; Evans, Romero, & Westoby, 2009; Lupano, Dumay, & Cheftel, 1992; Phillips & Signs, 2004; Tan, Li, Xu, & Xing, 2012; Wang et al., 2016). Dialysis separates molecules based on size by allowing the diffusion of only small molecules (smaller than the pore size of the semipermeable membranes) through the membranes in solution (Evans et al., 2009). Phillips and Signs (2004) reported protocols of removing excessive salts from protein solutions with dialysis tubing. Lupano et al. (1992) removed calcium from whey protein isolate (WPI) and found that the water-holding capacity and elasticity of thermally induced WPI gels increased with decreasing calcium concentration. Tan et al. (2012) used dialysis membrane to remove salts during the extraction and purification of aloe polysaccharides from aloe leaves. Therefore, we decided to remove excess minerals observed in LHC using dialysis membrane to improve

the quality of resulting jellyfish gelatin gels. The dialysis procedure successfully reduced the solid content (g/100 g, wet basis) and ash content (g/100g, dry basis) of LHC from 4.42 to 1.83, and from 39.60 to 5.77, respectively. This indicated that the dialysis procedure was able to remove ~85% of minerals from LHC.

Table 3.2 Solid and ash content of liquid hydrolyzed jellyfish collagen before and after dialysis

Hydrolyzed jellyfish collagen	Ash content (g/100 g, dry basis)	Solid content (g/100 g, wet basis)
LHC (Before dialysis)	39.60 ± 0.35	4.42 ± 0.13
DLHC (After dialysis)	5.77 ± 0.32	1.83 ± 0.15

Values are means ± SD. LHC = liquid hydrolyzed jellyfish collagen, DLHC= dialyzed LHC.

The mineral profile of hydrolyzed jellyfish collagen before and after the dialysis process is shown in Table 3.3. Eighteen elements were identified: Al, B, Cd, Ca, Cr, Cu, Fe, Pb, Mg, Mn, Mo, Ni, P, K, Si, Na, S, and Zn. The mineral profile of marine animals, including cannonball jellyfish, are affected by the nutritional status, migratory habitats and marine environmental conditions (*e.g.* salinity, temperature and the level of pollution of the oceans) (Özden & Erkan, 2011). The levels of regulated toxic heavy metals (Cd and Pb) were found to be low. Fresh cannonball jellyfish is cured with a combination of salt and alum, therefore, the most abundant mineral found in LHC was Na, followed by Mg, K, Ca, S and P.

Table 3.3 Mineral profile of liquid hydrolyzed jellyfish collagen before and after dialysis

Element	**LHC - Before dialysis (ppm, dry basis)***	**DLHC - After dialysis (ppm, dry basis)***
Aluminum (Al)	28.22 ± 0.36	13.76 ± 0.29
Boron (B)	38.85 ± 1.62	1.20 ± 0.05
Cadmium (Cd)	<1.24	<0.18
Calcium (Ca)	3424.41 ± 195.73	59.81 ± 1.35
Chromium (Cr)	14.18 ± 0.84	3.79 ± 0.27
Copper (Cu)	5.43 ± 0.17	1.45 ± 0.04
Iron (Fe)	164.93 ± 10.36	194.16 ± 2.61
Lead (Pb)	<0.48	<0.07
Magnesium (Mg)	10385.50 ± 381.38	163.35 ± 2.37
Manganese (Mn)	5.88 ± 0.31	1.26 ± 0.02
Molybdenum (Mo)	3.05 ± 0.16	0.95 ± 0.00
Nickel (Ni)	14.61 ± 0.73	4.70 ± 0.13
Phosphorus (P)	1370.75 ± 63.56	199.99 ± 1.80
Potassium (K)	7122.65 ± 51.24	25.76 ± 3.30
Silicon (Si)	62.17 ± 3.36	18.61 ± 0.45
Sodium (Na)	51734.43 ± 1600.84	119.32 ± 4.49
Sulfur (S)	2753.59 ± 148.13	19.13 ± 0.61
Zinc (Zn)	25.01 ± 1.65	2.55 ± 0.07

Values are means ± SD, n=2. See Table 3.2 for description of LHC and DLHC.

Interestingly, the amount of Na decreased from 51734.43 to 119.32 ppm (dry basis) after dialysis. Furthermore, K and S contents were reduced more than 100 times in LHC after dialysis, from 7122.65 to 25.76 ppm (dry basis) and from 2753.59 to 19.13 ppm (dry basis), respectively. Significant amounts of Mg and Ca were also removed from LHC through dialysis. Except for Fe, the quantities of all the detected elements were reduced after dialysis. The quantities of Fe, an element that can attach to proteins and peptides, increased from 164.93to 194.16 ppm (dry basis) after dialysis. The increase in Fe after dialysis may be due to its ability to attach to some peptides while other free elements were easily removed *via* dialysis. After dialysis, element contents dropped below 200 ppm (dry basis); while P was the most abundant element followed by Fe, Mg, Na, Ca and K. The dialysis procedure allowed an effective removal of minerals (especially Na, Mg and K) and improved the observed palpable fishy flavor associated with SD jellyfish.

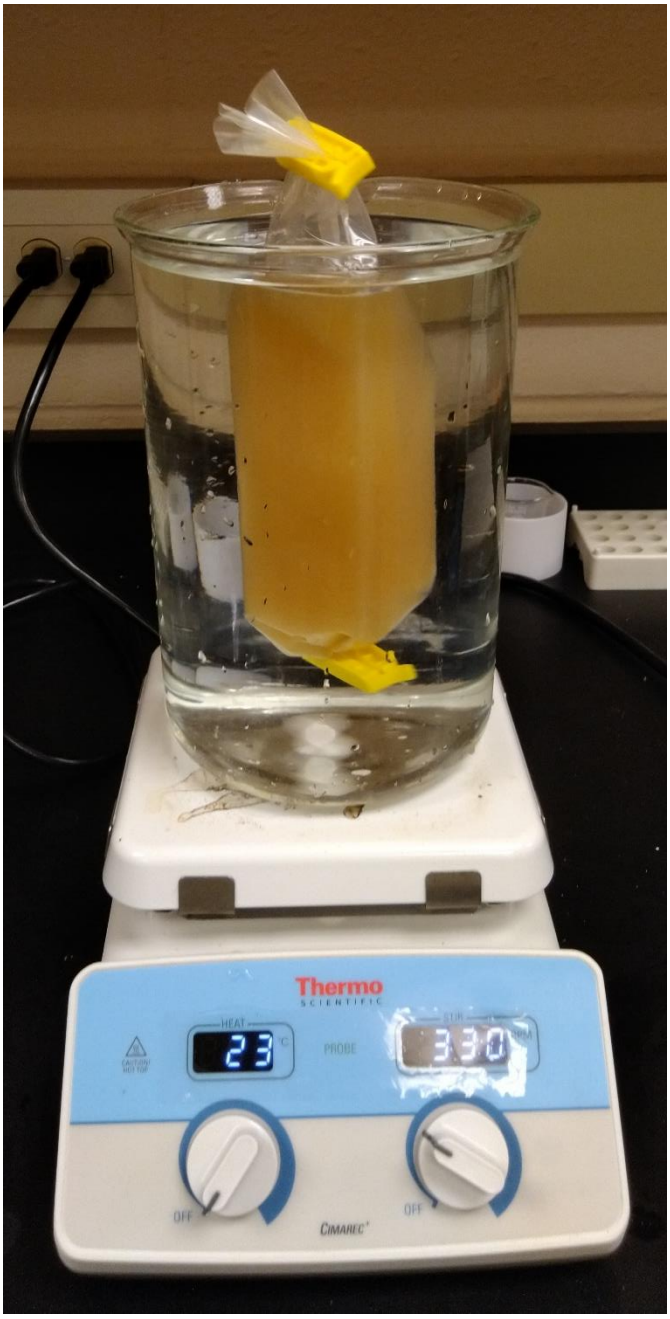


Fig. 3.1 Dialysis process

Fig. 3.2 Liquid hydrolyzed jellyfish collagen (LHC) before dialysis

Fig. 3.3 Dialyzed liquid hydrolyzed jellyfish collagen

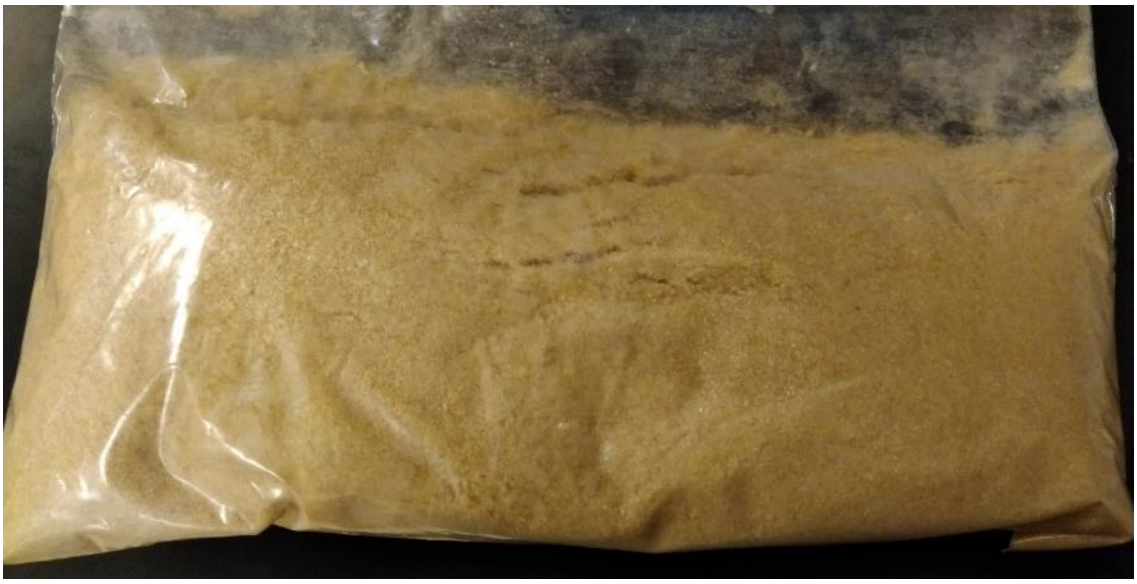


Fig. 3.4 SD jellyfish gelatin powder

3.2.3 Microencapsulation of LGRR using JG and/or MG combined with MD

3.2.3.1 Probiotic survivability

Table 3.4 shows the cell counts of LRGG before and after the drying process. In this study, LRGG cells were also microencapsulated with MG to compare the performance of JG as a microencapsulating agent. Before drying, cell counts of LRGG in the suspensions ranged between 9.04 to 9.56 Log CFU/g solids. FD powders produced with JG-MD least supported the survival of LRGG (3.63±0.37%), while FD powders produced with MG-MD best supported the survival of LRGG (82.51±10.26%). Although a large amount of LRGG cells were inactivated during drying, the surviving cell counts in all available powders processed in this study are higher than 7.6 Log CFU/g; therefore, they can still be used in the production of probiotic foods which should have a minimum of viable probiotics cell counts of 6 Log CFU/g (Ying, Sun, Sanguansri, Weerakkody, & Augustin, 2012).

Table 3.4 Cell counts (Log CFU/g solids) of *L. rhamnosus* GG in microencapsulated powders

Drying method	Microencapsulating agent	Log CFU/g solids		Cell survival (%)
		Before Drying	After Drying	
MXSD	**JG-MD**	9.30±0.07	8.73±0.04	26.69±2.62 [b]
FD	**JG-MD**	9.04±0.11	7.61±0.05	3.63±0.37 [c]
MXSD	**MG-MD**	9.19±0.13	8.41±0.12	16.44±4.60 [bc]
FD	**MG-MD**	9.56±0.04	9.47±0.06	82.51±10.26 [a]

Values are means ± SD, n=3. Cell survival rates with different letters in the same column are significantly different (P<0.05). MXSD = mixed-flow spray drying; FD = freeze drying; JG = jellyfish gelatin; MG = mammalian gelatin; MD = maltodextrin.

Even though significant differences were observed in the survival of LRGG cells in FD powders; MXSD powders showed similar survival rates of LRGG cells regardless of the microencapsulating agents used. While FD powders showed significantly ($P<0.05$) higher cell survival rates than MXSD powders when MG-MD were used as microencapsulating agents; higher cell survival rates were observed in MXSD than in FD powders when they were microencapsulated with JG-MD. These results were unexpected since FD has been known as a gentler drying process to microencapsulate probiotics. While spray drying of probiotic suspensions can thermally inactivate significant quantities and reduce the cell viability of probiotic cells due to osmotic, heat, and oxidative stresses caused by the atomization of probiotic suspensions and high temperatures of the drying air (Anekella & Orsat, 2013; Paéz et al., 2012)**.** High air temperatures used in spray drying damage the cell wall, cytoplasmic membrane, DNA and RNA of probiotic cells, which cause cell inactivation (Perdana et al., 2013). We have observed up to four times higher survival rates of LRGG cells after microencapsulation by FD compared to MXSD using fish gelatin and MD as the microencapsulating agents (Jiang, Dev Kumar, Chen, Mishra, & Mis Solval, 2020).

The results obtained in this study may be due to the presence of heat-sensitive antimicrobial peptides present in JG. While these antimicrobial peptides may be inactivated during MXSD at high temperatures; their activity remain high after FD, hence they can inactivate significant quantities of LRGG cells. It has been previously reported that that some collagen hydrolysates have antimicrobial properties, antioxidant and antihypertensive activity (Gómez-Guillén, Giménez, López-Caballero, & Montero,

2011). Guillén et al. (2010) found peptides with antimicrobial properties in squid and tuna skin gelatins.

It has been reported that gelatin is a good microencapsulating agent for probiotics because of the presence of hydrophobic amino acids residues, like alanine, proline and hydroxyproline which improves the viscosity of probiotics suspensions and decreases the heat transfer between probiotics droplets and the hot drying air during spray drying and reduces cell inactivation (Arslan, Erbas, Tontul, & Topuz, 2015). Moreover, MD (about 3kDa) can effectively protect probiotic cells from excessive dehydration stress by attaching to cell membranes and binding water molecules during MXSD; therefore, reducing mechanical stress on the probiotic cell. This makes MD a good microencapsulating agent for probiotics (Assadpour & Jafari, 2019; Semyonov et al., 2010).

3.2.3.2 Moisture content and water activity of LRGG powders

LRGG powders with significantly ($P<0.05$) higher moisture contents were produced by MXSD than by FD (Table 3.5). Moreover, LRGG powders microencapsulated with MG-MD showed higher moisture content and water activity values than those microencapsulated with JG-MD. Gelatins have the ability to bind water molecules with exposed hydrophilic groups on the surface *via* hydrogen bonds, this characteristic is especially useful during the gelling process (Baguley & McDonald, 2015).

Table 3.5 Moisture contents and water activity values of microencapsulated *L. rhamnosus* GG powders

Drying method	Microencapsulating agents	Moisture (g/100 g, wet basis)	a_w
MXSD	**JG-MD**	2.82 ± 0.06^{b}	0.13 ± 0.00^{c}
FD	**JG-MD**	0.77 ± 0.42^{c}	0.03 ± 0.00^{d}
MXSD	**MG-MD**	4.20 ± 0.13^{a}	0.27 ± 0.01^{a}
FD	**MG-MD**	3.01 ± 0.14^{b}	0.15 ± 0.01^{b}

Values are means ± standard deviation (SD), n=3. Values followed by different letters in the same column are significantly different ($P<0.05$). See Table 3.4 for definition of MXSD, FD, JG, MG, and MD.

Moisture content and a_w are important quality indicators of probiotics powders. Higher moisture content and a_w tend to correlate with poor cell survivability and shorter shelf life. Chávez and Ledeboer (2007) have suggested a moisture content below five (g/100g) is required to ensure the long-term stability of probiotics powders. In this study, the moisture content of all LRGG powders was below 4.5 (g/100g) which indicated that they might be stable during dry storage. LRGG powders produced with MXSD using MG-MD had the highest moisture content (4.20±0.13 g/100 g), while the moisture content of LRGG powders produced by FD using JG-MD showed the lowest (0.77±0.42 g/100 g). A similar trend was obtained with a_w values. All LRGG powders microencapsulated with JG-MD had significantly (P<0.05) lower a_w values than powders containing MG-MD. All of LRGG powders were microbiologically stable because they showed a_w values below 0.6 (Quek, Chok, & Swedlund, 2007). Probiotic powders should have a_w values between 0.15 and 0.3 to prevent caking and recrystallization of wall materials (Avila-Reyes, Garcia-Suarez, Jiménez, San Martín-Gonzalez, & Bello-Perez, 2014).

3.2.3.3 Color

Darker (lower L* values) LRGG powders were produced by FD than by MXSD (Table 3.6). Moreover, FD powders showed significantly (P<0.05) higher redness (higher a* values) and yellowness (higher b* values) than MXSD powders.

Table 3.6 Color values of microencapsulated *L. rhamnosus* GG powders

Drying method	Microencapsulating agent	L*	a*	b*	hue	Chroma
MXSD	**JG-MD**	59.82 ± 0.35^{b}	1.02 ± 0.08^{b}	12.59 ± 0.14^{c}	85.35 ± 0.41^{b}	12.64 ± 0.13^{c}
FD	**JG-MD**	50.58 ± 0.12^{d}	2.23 ± 0.05^{a}	15.13 ± 0.30^{b}	81.61 ± 0.08^{d}	15.29 ± 0.31^{b}
MXSD	**MG-MD**	61.77 ± 0.41^{a}	0.66 ± 0.04^{c}	10.41 ± 0.05^{d}	86.36 ± 0.20^{a}	10.43 ± 0.05^{d}
FD	**MG-MD**	56.60 ± 0.61^{c}	2.33 ± 0.03^{a}	17.11 ± 0.10^{a}	82.25 ± 0.11^{c}	17.27 ± 0.10^{a}

Values are means ± standard deviation (SD), n=3. Values followed by different letters in the same column are significantly different ($P<0.05$). See Table 3.4 for definition of MXSD, FD, JG, MG, and MD.

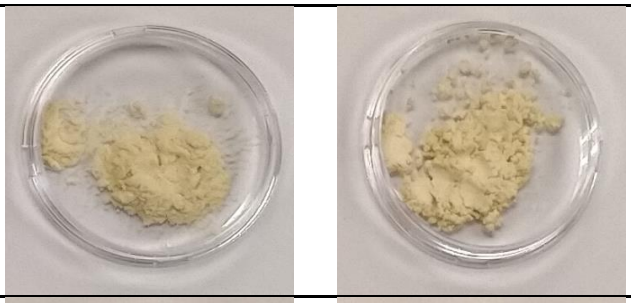
Drying method	Microencapsulating agents	
	JG-MD	MG-MD
MXSD		
FD		

Fig. 3.5 Microencapsulated *L. rhamnosus* GG powders. MXSD = mixed-flow spray drying; FD = freeze drying; JG = cannonball jellyfish gelatin; MG = mammalian gelatin; MD = maltodextrin.

Interestingly, this may be an indication that the higher drying temperatures used in MXSD may had reduced some pigments and heat sensitive compounds in LRGG powders. While hue angle values for all LRGG powders were between 80 and 90 (indicating yellowish powders); MXSD powders showed higher hue angle values than powders produced by FD. Meanwhile, the chroma (color saturation) of the FD powders were higher than MXSD powders. These results suggested that drying conditions greatly influenced the color of the LRGG powders. It was also reported that color of microencapsulated probiotics powders was affected by drying conditions (temperature of drying air, feed flow rate and atomization) and properties of encapsulant (concentration, composition and viscosity) (Costa et al., 2015).

3.2.3.4 Particle size distribution

Freeze-dried LRGG powders showed significantly (P<0.05) higher mean particle sizes (D_{50}) than MXSD powders (Table 3.7). The particle size distribution of FD powders mainly depends on the grinding conditions (Karam, Petit, Zimmer, Djantou, & Scher, 2016). In this study the grinding conditions of FD samples were kept constant, hence the differences in particle sizes may be only attributed to the characteristics of the materials. Spray drying allows the production of small particles homogenously distributed. However, it has been reported that properties of the liquid suspensions, atomization conditions, inlet drying air temperatures and particle residence times in the dryer chamber affect the particle size of the spray dried powders (Jiang et al., 2020; Pinto, Kemp, Bermingham, Hartwig, & Bisten, 2014). Intriguingly, LRGG powders microencapsulated with JG-MD showed smaller particle sizes than those microencapsulated with MG-MD. This may indicate that probiotic suspensions produced with JG-MD had a lower viscosity

than those produced with MG-MD which allowed the production of smaller droplets inside the spray dryer. Moreover, MXSD powders with smaller particle sizes showed higher cell survival. Similar observation have been reported by our group (Mis-Solval et al., 2019). The span value of FD powders microencapsulated with MG-MD (4.20±0.25) was the highest in all the treatments. And there was no significant difference of span values among the other three LRGG powders. All the span values of the four powders were above 3. It has been suggested that span values below 2 indicated homogenously distributed spray dried powders, while span values above 2 suggests particle agglomeration (Cal & Sollohub, 2010; Tonon, Grosso, & Hubinger, 2011). It has been previously reported that MXSD produces powders with high particle agglomeration. A positive correlation between span value and survival rate of the probiotics in the powders was recently reported by our group (Mis-Solval et al., 2019). The appearance, dispersibility and mouthfeel of food powders is affected by their particle size (Sharma, Kadam, Chadha, Wilson, & Gupta, 2013; Zhao, Sun, Torley, Wang, & Niu, 2008).

Table 3.7 Particle size distribution values of microencapsulated *L. rhamnosus* GG powders

Drying method	Microencapsulating agent	D_{10}(μm)	D_{50}(μm)	D_{90}(μm)	Span
MXSD	**JG-MD**	2.02 ± 0.27^{b}	7.86 ± 0.10^{d}	27.48 ± 0.79^{c}	3.24 ± 0.10^{b}
FD	**JG-MD**	2.37 ± 0.01^{b}	26.87 ± 0.62^{b}	83.15 ± 0.98^{b}	3.01 ± 0.04^{b}
MXSD	**MG-MD**	1.30 ± 0.02^{c}	10.26 ± 0.23^{c}	15.29 ± 0.17^{c}	3.19 ± 0.03^{b}
FD	**MG-MD**	8.43 ± 0.43^{a}	50.06 ± 0.19^{a}	218.57 ± 11.17^{a}	4.20 ± 0.25^{a}

Values are means ± standard deviation (SD), n=3. Values followed by different letters in the same column are significantly different ($P<0.05$). Dx = xth percentile particle size, MXSD = mixed-flow spray drying; FD = freeze drying; JG = jellyfish gelatin; MG = mammalian gelatin.

3.2.3.5 Shelf life

Cell counts and survival rates of LRGG powders after 15 days storage are presented in Table 3.8. After 15 days of storage at room temperature (~20°C) and relative humidity (%) of 50-55 and 75%, LRGG survival rates in all prepared powders were below 0.001%, except for MXSD powders microencapsulated with MG-MD. Moreover, cell counts of LRGG after 15 days of storage were lower than cell counts of LRGG microencapsulated with whey protein isolate and MD *via* spray drying after storing two weeks (> 10^5 CFU/g) reported by Ying et al. (2012).

Moisture content and a_w values of LRGG powders increased significantly after 15 days of storage at room temperature. At 50-55% RH, MXSD powders microencapsulated with MG-MD showed an increase in moisture of 0.33 g/100 g; while MXSD powders microencapsulated with JG-MD increased their moisture content by 14.96 g/100 g. In general, LRGG powders microencapsulated with JG-MD showed higher moisture contents than powders microencapsulated with MG-MD. It was noted that the samples (especially those prepared with JG-MD) were highly hygroscopic and difficult to manipulate because of their high moisture content (as seen in Fig. 3.6 and 3.7). Therefore, the determinations of water activity values of those samples may have not been accurate. A new shelf life study is recommended at lower relative humidity conditions and adequate packaging materials.

Table 3.8 Cell counts (Log CFU/g solids) of *L. rhamnosus* GG in microencapsulated powders after 15 days storage

Drying method	Microencapsulating agent	50%-55% RH		75% RH	
		Log CFU/g solids	Cell survival (%)	Log CFU/g solids	Cell survival (%)
MXSD	**JG-MD**	<4	<0.001	<4	<0.001
FD	**JG-MD**	<4	<0.001	<4	<0.001
MXSD	**MG-MD**	5.88±0.06	0.05±0.01	6.25±0.07	0.11±0.02
FD	**MG-MD**	<4	<0.001	<4	<0.001

Values are means ± standard deviation (SD), n=3. Values followed by different letters in the same column are significantly different (P<0.05). See Table 3.4 for definition of MXSD, FD, JG, MG, and MD.

Table 3.9 Moisture contents and water activity values of microencapsulated *L. rhamnosus* GG powders after 15 days storage

Drying method	Microencapsulating agent	50%-55% RH		75% RH	
		Moisture (g/100 g)	a_w	Moisture (g/100 g)	a_w
MXSD	**JG-MD**	17.78±0.35[a]	0.50±0.01[a]	17.73±0.38[a]	0.54±0.00[b]
FD	**JG-MD**	13.69±0.44[b]	0.49±0.00[ab]	15.29±0.40[b]	0.50±0.00[c]
MXSD	**MG-MD**	4.53±0.55[d]	0.47±0.01[b]	16.95±0.45[a]	0.56±0.00[a]
FD	**MG-MD**	8.01±0.42[c]	0.49±0.02[ab]	16.65±0.45[a]	0.56±0.00[a]

Values are means ± standard deviation (SD), n=3. Values followed by different letters in the same column are significantly different ($P<0.05$). See Table 3.4 for definition of MXSD, FD, JG, MG, and MD.

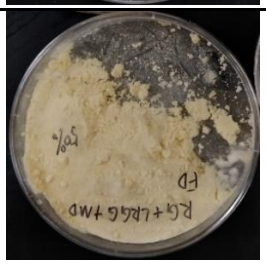
Drying method	Microencapsulating agents	
	JG-MD	MG-MD
MXSD		
FD		

Fig. 3.6 *L. rhamnosus* GG powders after 15 days storage under RH=50-55%. MXSD = mixed-flow spray drying; FD = freeze drying; JG = cannonball jellyfish gelatin; MG = mammalian gelatin; MD = maltodextrin.

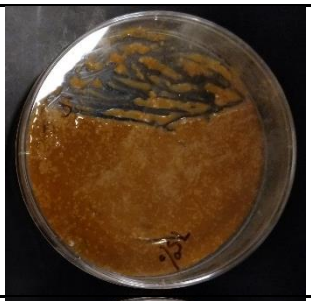
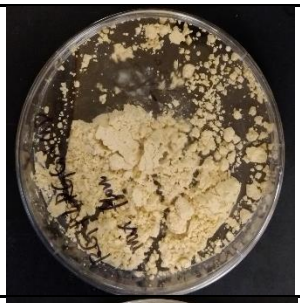
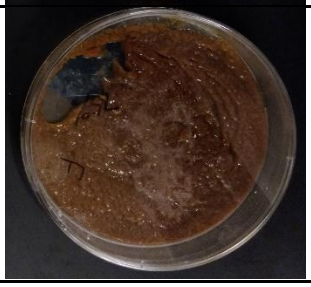
Drying method	Microencapsulating agents	
	JG-MD	MG-MD
MXSD		
FD		

Fig. 3.7 *L. rhamnosus* GG powders after 15 days storage under RH=75%. MXSD = mixed-flow spray drying; FD = freeze drying; JG = cannonball jellyfish gelatin; MG = mammalian gelatin; MD = maltodextrin.

LRGG powders microencapsulated with JG-MD showed similar moisture contents when they were stored at 50-55 and 75% RH conditions, while LRGG powders microencapsulated with MG-MD had lower moisture contents and a_w values when they were stored under 50-55% RH than at 75% RH. These results may indicate that LRGG powders prepared with JG-MD were more hygroscopic than LRGG powders prepared with MG-MD. The gain in moisture may had significantly reduced the cell counts in LRGG powders after 15 days of storage. The results may indicate that LRGG powders should be stored at RH lower than 50% to prevent moisture absorption; especially those powders microencapsulated with JG-MD. It was reported that vacuum storage was better than nitrogen and air for probiotics powders (Chávez & Ledeboer, 2007). And the viability of probiotic bacteria in powders is inversely related to temperature during storage (Tripathi & Giri, 2014). For long-term storage, Bruno and Shah (2003) recommended temperature of −18 °C for bifidobacteria.

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CHAPTER 4

THE EFFECT OF ULTRA-HIGH-PRESSURE HOMOGENIZATION (UHPH) OF SOYMILK AND SKIM MILK WHEN USED AS ENCAPSULATING MATERIALS ON THE SURVIVABILITY OF *LACTOBACILLUS PLANTARUM* NRRL B-1927 AFTER DRYING

4.1 Materials and Methods

4.1.1 Materials

Organic unsweetened soymilk (SOY) was obtained from Eden Foods (Clinton, MI, USA), fat free skim milk (SKIM) from local supermarket (Kroger, Cincinnati, OH, USA), MRS broth and MRS agar from Thermo Fisher Scientific (Waltham, MA, USA), Butterfield's phosphate buffer from Hardy Diagnostics (Santa Maria, CA, USA), Lyophilized culture of *Lactobacillus plantarum* (NRRL B-1927 aka. ATCC 10241) from the US. Department of Agriculture – Agricultural Research Service (USDA-ARS) Culture collection (Peoria, IL, USA). All other chemicals were analytical grade and were obtained from Sigma Aldrich (St. Louis, MO, USA).

4.1.2 Preparation of probiotic cultures

Culture stocks of *L. plantarum* NRRL B-1927 (LP) were prepared by following the recommended conditions from the USDA-ARS and kept in 20% glycerol (w/v) at -30°C (USDA-ARS, 2019). A loop of LP culture stock was transferred into 9 mL MRS broth in a culture tube which were then capped and incubated at 37 °C for 48 h. The activated cultures were then inoculated into 3 L of MRS broth and incubated for another

48 h at 37 ℃. Afterwards, the LP cultures were kept for up to 24 h at 4 ℃ and centrifuged by a refrigerated ultracentrifuge (Sorvall RC-6 plus, Thermo Fisher Scientific, Waltham, MA, USA) with rotor (Fiberlite F10-6x500, Thermo Fisher Scientific, Waltham, MA, USA) at 5000 × *g* for 15 min at 4 ℃. The pellet was collected and re-suspended in 50 mL of 0.1% peptone water and centrifuged at 5000 × *g* for 10 min at 4 ℃. The new pellet was collected and used for preparation of probiotic suspensions.

4.1.3 Preparation of LP suspensions

Suspensions containing viable cells of LP were prepared by following the conditions previously reported by our group (Mis-Solval, Jiang, Yuan, Joo, & Cavender, 2019). Briefly, 1 L of SOY and/or SKIM was ultra-high-pressure homogenized (UHPH) at 150 and/or 300MPa using a dual-intensifier continuous high-pressure homogenizing system (Stansted nm-gen 7900, Stansted Fluid Power, Stansted, England) that was fitted with a stainless steel metering valve (Model 60vrmm4882, Autoclave Engineers, Fluid Components, Erie, PA, USA) at the outlet and modified to feed from a 6 L vessel that was pressurized with compressed air at approximately 550 kPa. The flow rate was kept at 1 ± 0.25 L/min *via* adjusting the metering valve. After UHPH treatment, samples were immediately cooled to below 20 ℃ before suspending the LP pellets to produce LP suspensions (~10^9 CFU/g solids). Alternatively, LP suspensions were also prepared with non-UHPH treated SOY and SKIM. The suspensions were kept at 4℃ for up to 48 h before drying for the need of complete chilling and transition between processing locations.

4.1.4 Drying of LP suspensions

The LP suspension were spray dried under concurrent (CCSD) and/or mixed-flow (MXSD) conditions using a pilot-scale spray dryer (Anhydro, PSD 52, Denmark) with similar parameters previously reported by our team (Mis-Solval et al., 2019). In both CCSD and MXSD, inlet air temperature was set at 140°C, while outlet air temperature was kept at 80±1°C by adjusting the feed flow rate between 1.5 – 2.0 L/h. Then, LP powders were collected and kept in a desiccator. Concurrently, LP suspensions were frozen at -18°C for 12 h and freeze dried (FD) at −55 °C for 96 h using a pilot-scale lyophilizer (Virtis, the Virtis Company, Gardiner, NY, USA). Then, FD samples were powdered using a high-performance blender (Vitamix 7500, Olmsted Township, OH, USA). Probiotic powders were kept in a desiccator under room temperature and characterized within five days after the drying procedure.

4.1.5 Enumeration of *L. plantarum* NRRL B-1927 cultures

Plate counts of viable cells were determined in both LP suspensions and powders right before and immediately after the drying process, respectively. 100 µL of LP suspensions or 1 g of LP powders were serially diluted in sterile Butterfield's phosphate buffer and 100 µL of each dilution was pour plated on 100 mm diameter MRS agar plates in triplicate. Then, the inoculated plates were put in Ziploc bags (S.C. Johnson & Son, USA) and incubated for 40 h at 37°C under aerobic conditions before cell colonies were counted. For ease of comparison, results were expressed as Log of colony forming units (CFU) per gram of dried solids (Log CFU/g). Cell survival rate of the probiotic cells was calculated using Eq. (1)

$$Cell\ survival\ (\%) = \frac{POWD}{SUSP} * 100 \quad (1)$$

where, POWD = cell counts (CFU/g solids) in LP powders after drying; SUSP= cell counts (CFU/g solids) in LP suspensions before drying.

4.1.6 Physical properties of LP powders

4.1.6.1 Moisture content and water activity (a_w)

Moisture content (g/100 g) of the LP suspensions and powders were measured using a moisture analyzer (HR73 Halogen Moisture Analyzer, Mettler-Toledo GmbH, Greifensee, Switzerland). Moreover, the a_w values of LP powders were determined by a water activity meter (AquaLabSeries 3 TE, Decagon Devices, Inc., Pullman, WA, USA).

4.1.6.2 Particle Size Distribution

Particle size distribution of the LP powders was acquired with a particle size analyzer (Model PSA 1190, Anton Paar GmbH, Graz, Austria). Powders were illuminated with three lasers from different angles, the whole light scatter pattern was collected and used to calculate the particle size distribution by the system with modified Michelson interferometer method. The results were reported for D_{10}, D_{50}, and D_{90} which are the volume diameter of the particles at 10%, 50%, and 90% cumulative volume respectively and the Span value (spread of particles) was calculated by following the method referred to Mis Solval, Bankston, Bechtel, and Sathivel (2016).

4.1.6.3 Scanning electron microscopy

SEM micrographs of the probiotic powders were obtained using a method previously reported by Donhowe, Flores, Kerr, Wicker, and Kong (2014). LP powders were sputter-coated with gold, before being analyzed with a scanning electron microscope (1450 EP,

Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) with an acceleration potential of 10 kV to observe the particle morphologies. The powders were systematically observed at different magnifications.

4.1.7 Statistical analysis

Means and standard deviations (SD) of test results were reported. A three-way analysis of Variance (ANOVA) (UHPH level, encapsulants, and drying methods), and post-hoc Tukey's studentized range tests ($\alpha = 0.05$) were employed to determine the statistical significance of observed differences among means by RStudio statistical software version 1.2.5033 (RStudio, Inc. Boston, MA, USA).

Table 4.1 Nutritional values of EDEN, EDENSOY, ORGANIC SOYMILK, UNSWEETENED (per 100 mL) from USDA FoodData (provided by food brand owners) (U.S. Department of Agriculture (USDA) & Agricultural Research Service, 2018)

Component	Amount	Component	Amount
Energy	50 kcal	Potassium, K	192 mg
Protein	5 g	Sodium, Na	2 mg
Total lipid	2.5 g	Zinc, Zn	0.5 mg
Carbohydrate, by difference	2.08 g	Copper, Cu	0.083 mg
Fiber, total dietary	0.4 g	Riboflavin	0.043 mg
Sugars, total including NLEA	0.83 g	Niacin	0.333 mg
Calcium, Ca	17 mg	Vitamin B-6	0.067 mg
Iron, Fe	0.75 mg	Vitamin K (phylloquinone)	6.7 µg
Magnesium, Mg	25 mg	Fatty acids, total saturated	0.42 g
Phosphorus, P	62 mg		

Table 4.2 Nutritional values of KROGER, FAT FREE SKIM MILK (per 100 ml) from USDA FoodData (provided by food brand owners) (U.S. Department of Agriculture (USDA) & Agricultural Research Service, 2018)

Component	Amount	Component	Amount
Energy	33 kcal	Potassium, K	162 mg
Protein	3.33 g	Sodium, Na	50 mg
Total lipid	0 g	Vit. C, total ascorbic acid	1 mg
Carbohydrate, by difference	5 g	Vitamin A, IU	208 IU
Sugars, total including NLEA	4.58 g	Vitamin D (D2 + D3), IU	42 IU
Calcium, Ca	125 mg	Cholesterol	2 mg

4.2 Results and discussion

4.2.1 Probiotic survivability

Cell counts of LP in suspensions prepared with UHPH-treated SOY and/or SKIM at different pressures (0, 150, and 300 MPa) and in LP powders is shown in Table 4.3. Cell counts in LP suspensions ranged between 8.81 to 9.50 Log CFU/g solids. Moreover, all the LP suspensions prepared with UHPH-treated SOY and SKIM had cell counts higher than 10^9 CFU/g solids. Higher Log reductions of LP cells was observed in powders microencapsulated with non-UHPH treated SOY and SKIM compared to the powders microencapsulated with UHPH-treated SOY and SKIM.

Among all treatments, powders microencapsulated with non-UHPH-treated SOY *via* CCSD showed the highest log reduction (1.41±0.07), while powders microencapsulated with 150-MPa-treated SKIM *via* FD showed the lowest Log reduction (0.01±0.02). Although a significant amount of LP cells was inactivated after drying, the cell counts in all LP powders were higher than 7.9 Log CFU/g solids. So, they can still be utilized to produce probiotic foods with a recommended minimum number of viable probiotics cells of 6 Log CFU/g (Ying, Sun, Sanguansri, Weerakkody, & Augustin, 2012).

Table 4.3 Cell counts (Log (CFU/g solids)) of *L. plantarum* NRRL B-1927 in suspensions and powders.

UHPH	Drying method	Encapsulant	Log CFU/g solids		Log reduction
			Before drying	After drying	
0-MPa	CCSD	SOY	9.50±0.06	8.09 ± 0.07	1.41±0.07
		SKIM	8.81±0.08	8.27 ± 0.06	0.54±0.06
	MXSD	SOY	8.94±0.17	8.05 ± 0.08	0.89±0.08
		SKIM	9.23±0.16	7.95 ± 0.04	1.28±0.04
	FD	SOY	8.94±0.17	8.52 ± 0.08	0.43±0.08
		SKIM	9.23±0.16	8.58 ± 0.11	0.65±0.11
150-MPa	CCSD	SOY	9.20±0.07	8.42±0.11	0.78±0.11
		SKIM	9.07±0.05	8.76±0.05	0.31±0.05
	MXSD	SOY	9.20±0.07	9.03±0.02	0.17±0.02
		SKIM	9.07±0.05	8.87±0.07	0.20±0.07
	FD	SOY	9.20±0.07	8.86±0.02	0.34±0.02
		SKIM	9.07±0.05	9.06±0.02	0.01±0.02
300-MPa	CCSD	SOY	9.07±0.07	8.59±0.11	0.48±0.11
		SKIM	9.28±0.14	8.19±0.16	1.09±0.16
	MXSD	SOY	9.07±0.07	8.99±0.02	0.08±0.02
		SKIM	9.28±0.14	8.28±0.08	1.00±0.08
	FD	SOY	9.24±0.03	9.12±0.05	0.12±0.05
		SKIM	9.28±0.14	9.18±0.04	0.10±0.04

Values are Means ± Standard Deviation (SD). n = 3. CCSD = concurrent spray drying, MXSD = mixed-flow spray drying; FD = freeze drying; SOY= soymilk; SKIM= skim milk

4.2.2 Effect of UHPH treatment on cell survival (%)

After CCSD

Higher cell survival (%) was observed in LP powders microencapsulated with SKIM than with SOY treated at 0 and 150-MPa, *via* CCSD (Fig. 4.1 A and B). Moreover, LP powders microencapsulated with 300-MPa-treated SOY had a significantly (P<0.05) higher cell survival than those prepared with 150-MPa-treated and non-UHPH-treated SOY, respectively. LP powders prepared with 300-MPa-treated SOY showed more a tenfold increase in cell survival (%) (34.01±8.24) than those produced with non-UHPH-treated SOY (3.09±0.56), while the cell survival (%) of LP in powders microencapsulated with 150-MPa-treated SOY was 16.82±4.53 (Fig. 4.1 A).

Meanwhile, LP powders prepared with 150-MPa-treated SKIM (49.21±5.31) showed significantly (P<0.05) higher cell survival (%) than those prepared with non-treated (29.08±4.23) and 300-MPa-treated SKIM (8.43±2.96) *via* CCSD (Fig. 4.1 B), respectively. These results indicated that cell survival of LP after microencapsulation by CCSD can be improved by using UHPH treated SOY and SKIM.

After MXSD

For powders produced *via* MXSD, similar trends were observed with CCSD (Fig. 4.1 A and B). LP powders microencapsulated with SOY had higher cell survival than those microencapsulated with SKIM after MXSD. When SOY was used as a microencapsulating agent, higher cell survival cell survival (%) was obtained in LP powders microencapsulated with 300-MPa-treated SOY than in LP powders produced with 150-MPa-treated and non-UHPH-treated SOY (Fig. 4.1 A). The cell survival (%) in

LP powders prepared with 300-MPa-treated SOY was 83.72 ± 3.11, almost 8 times higher than that of LP powders prepared with non-UHPH-treated SOY.

Meanwhile, LP powders produced with 150-MPa-treated SKIM (63.60 ± 10.49) showed significantly (P<0.05) higher cell survival (%) than those prepared with non-UHPH-treated (5.21 ± 0.55) and 300-MPa-treated SKIM (10.12 ± 1.74) (Fig. 4.1 B). This indicates that the cell survival (%) in powders prepared with 150-MPa-treated SKIM was increased twelvefold compared to the powders prepared with non-UHPH-treated SKIM (Fig. 4.1 B). As in the previous case of CCSD, using UHPH-treated SOY and SKIM as microencapsulating agents improved the survivability of LP after MXSD.

After FD

LP powders produced *via* FD and microencapsulated with 300-MPa-treated SOY showed higher cell survival (%) than those powders prepared with 150-MPa-treated and non-UHPH-treated SOY after FD, respectively (Fig. 4.1 A). In contrast, LP powders prepared with 150-MPa-treated SKIM had significantly (P<0.05) higher cell survival (%) than LP powders microencapsulated with 300-MPa-treated and non-UHPH-treated SKIM after FD, respectively (Fig. 4.1 B). After FD, LP powders microencapsulated with non-UHPH-treated SOY showed higher cell survival (%) than LP powders prepared with non-UHPH-treated SKIM; while LP powders microencapsulated with 150-MPa-treated SKIM showed significantly (P<0.05) higher cell survival (%) than LP powders prepared with 150-MPa-treated SOY. Even more, no differences in cell survival (%) was observed in LP powders prepared with 300-MPa-treated SOY and SKIM (Fig. 4.2 A, B and C).

These results support the initial hypothesis that UHPH treatment change the three-dimensional structure of proteins/peptides present in SOY and SKIM. These UHPH-

modified proteins may protect probiotic cells from severe drying conditions better than unmodified proteins. Moreover, the observed results also suggested that the same UHPH pressure level can affect differently the properties of the microencapsulating materials. In this study SOY required higher UHPH pressure levels to modify its components and achieve higher survivability of LP after drying than SKIM. In the case of SKIM, once the UHPH treatment goes beyond certain level (higher than 150 MPa), it is believed that its properties are excessive modified that the ability to protect LP from drying conditions decreases. This may be explained with the difference in composition and structure of proteins found SOY and SKIM. As we can learn from Table 4.1 and Table 4.2, the SOY contained more proteins and lipids than SKIM, while SKIM had more carbohydrates. The structure of soy proteins in SOY are different to the structures of casein and whey proteins in SKIM.

Similar results were previously reported by our group where higher cell survival rates in probiotics powders microencapsulated with UHPH-treated encapsulants compared to those microencapsulated with non-UHPH-treated encapsulants (Mis-Solval et al., 2019). We have previously reported that under the same microencapsulating condition, whey protein isolate has a better ability to protect probiotic cells than soy protein isolate (Mis-Solval et al., 2019).

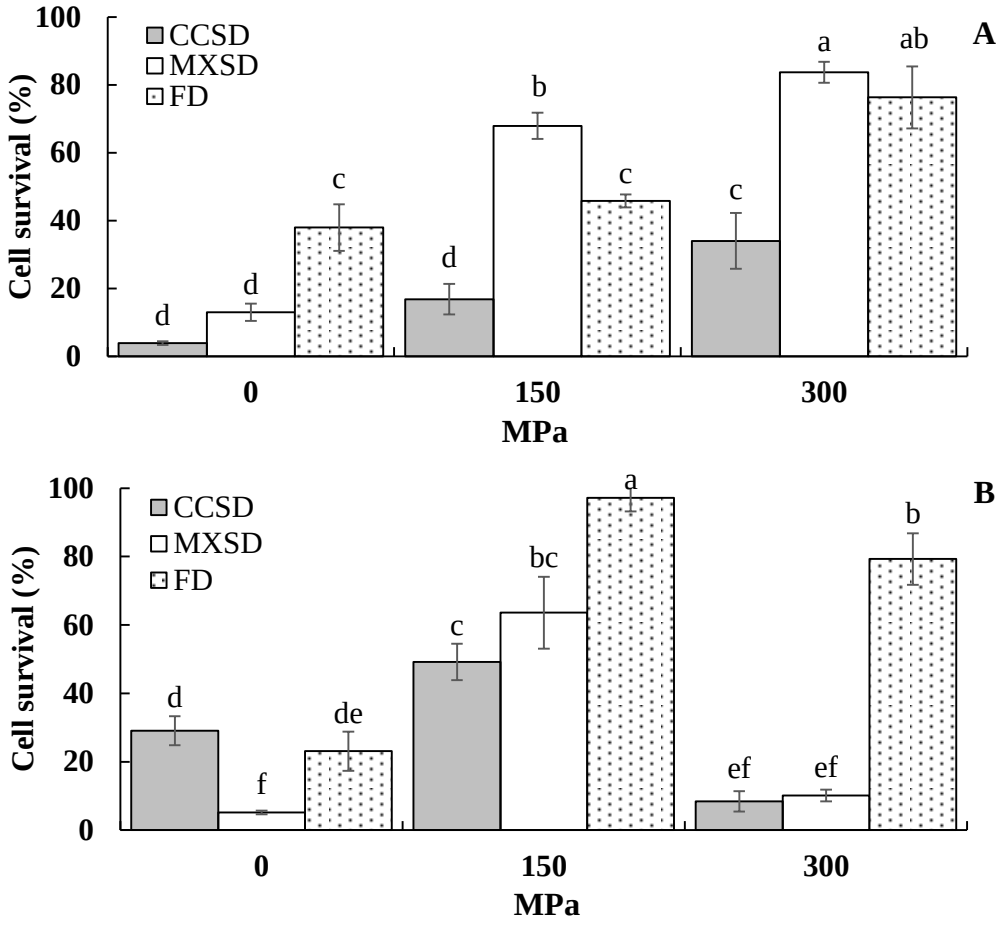

Fig. 4.1 Cell survival (%) of *L. plantarum* NRRL-1927 microencapsulated in UHPH-treated (A) SOY and/or (B) SKIM at 0, 150, and/or 300 MPa after CCSD, MXSD, and/or FD. Values with different lowercase (a, b, c, d) letters in the same graph are significantly different ($P<0.05$).

It has been previously reported that treating liquid milks at high UHPH pressure levels improves the binding efficiency of various compounds, like α-tocopherol acetate, triclosan, curcumin, vitamins, or other nutritional hydrophobic compounds (Benzaria, Maresca, Taieb, & Dumay, 2013; Chevalier-Lucia, Blayo, Gràcia-Julià, Picart-Palmade, & Dumay, 2011; Roach, Dunlap, & Harte, 2009). However, protein micelle aggregation may be observed in liquid milk treated at pressures higher than 200 MPa (Hayes & Kelly, 2003), which may had influenced the ability of 300-MPa-treated SKIM to protect probiotics cells during drying. High UHPH pressure levels can also affect the denaturation/aggregation of whey proteins (globular proteins) (Trujillo, Roig-Sagués, Zamora, & Ferragut, 2016). Cruz et al. (2007) reported a partial denaturation of soymilk proteins when soymilk was treated at UHPH pressures of 200 MPa and the resulting UHPH-treated soymilk was more stable than non-UHPH-treated and ultra-high temperature treated soymilks. Higher protein denaturation of soymilk proteins has been reported with increased pressures (Ferragut et al., 2015; Sidhu & Singh, 2016). UHPH treatment can promote the exposure of hydrophobic residues on the surface of soy protein isolate and the interaction between soy protein isolate and phosphatidylcholine in emulsions (Li et al., 2019).

4.2.3 Effect of drying method on cell survival

Non-UHPH-treated encapsulants

LP powders microencapsulated with non-UHPH-treated SOY showed significantly (P<0.05) higher cell survival (%) after FD (37.94±6.87) than MXSD (12.97±2.55) and CCSD (3.90±0.56), respectively (Fig. 4.2 A). Meanwhile, LP powders prepared with non-UHPH-treated SKIM showed higher cell survival (%) after CCSD

(29.08±4.23) compared to FD (23.1±5.72), and MXSD (5.21±0.55). Overall, higher cell survival (%) was observed in LP powders prepared with SOY than those prepared with SKIM after FD.

150-MPa-treated encapsulants

Higher cell survival (%) (69.94±3.89) was observed after MXSD than FD (45.79±1.91) and CCSD (16.82±4.53) in LP powders microencapsulated with 150-MPa-treated SOY (Fig. 4.2 B). Moreover, higher cell survival (%) was observed in LP powders microencapsulated with 150-MPa-treated SKIM after FD and MXSD. LP powders prepared with 150-MPa-treated SKIM showed almost a twofold increase in cell survival (%) after FD (97.14±3.96) compared to CCSD (49.21±5.31). Interestingly, cell survival rates in MXSD powders prepared with 150-MPa-treaed SOY and SKIM reached more than 63%, which were much higher than cell survival rates in LP powders microencapsulated with non-UHPH treated encapsulants.

300-MPa-treated encapsulants

When LP powders were microencapsulated with 300-MPa-treated SOY, similar cell survival rates (%) were observed after FD (76.31±9.17) compared to MXSD (83.72±3.11) (Fig. 4.2 C). Meanwhile, LP powders prepared with 300-MPa-treated SKIM showed significantly (P<0.05) higher cell survival (%) after FD (79.29±7.52) than MXSD (10.12±1.74) and CCSD (8.43±2.96), respectively. FD has been known as a gentler drying process to microencapsulate probiotics without thermal inactivation of probiotics cells, while spray drying of probiotic suspensions can thermally inactivate significant quantities and reduce the viability of probiotic cells due to osmotic, heat, and oxidative stresses caused by the atomization of probiotic suspensions and high

temperatures of the drying air (Anekella & Orsat, 2013; Paéz et al., 2012). High temperatures used in spray drying can damage the cell wall, cytoplasmic membrane, DNA and RNA of probiotic cells, which cause cell inactivation (Perdana et al., 2013). Due to different contact configurations between the hot drying air and atomized droplets, the effect of MXSD and CCSD on the cell survival rate are different. In this study, MXSD yielded higher cell survival than CCSD and the cell survivability of LP was tremendously improved in powders microencapsulated with UHPH-treated encapsulants after MXSD and CCSD.

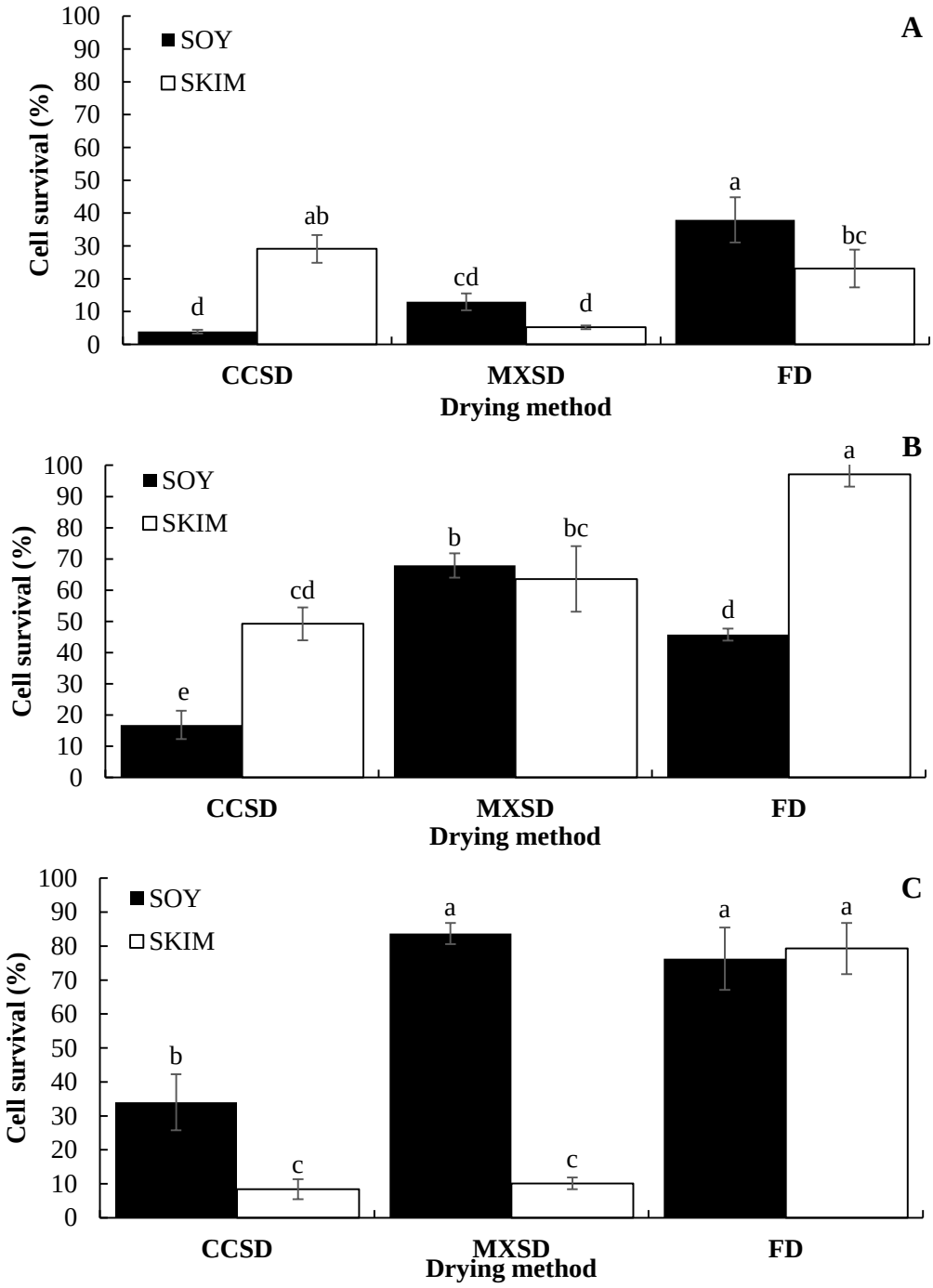

Fig. 4.2 Cell survivability (%) of *L. plantarum* NRRL-1927 microencapsulated in UHPH-treated SOY and/or SKIM at 0 (A), 150 (B), and/or 300 (C) MPa after CCSD, MXSD, and/or FD. Values with different lowercase (a, b, c, d) letters in the same graph are significantly different ($P<0.05$)

4.2.4 Moisture Content and a_w of LP powders

LP powders produced with UHPH-treated SOY and SKIM had significantly (P<0.05) lower moisture content and a_w values than those microencapsulated with non-UHPH-treated SOY and SKIM (Table 4.4). Moreover, drier powders were obtained *via* FD compared to CCSD and MXSD. LP powders dried under FD and microencapsulated with 150-MPa-treated SOY and SKIM had the lowest moisture contents and a_w values. In most cases, LP powders prepared with SKIM had higher moisture and a_w values than LP powders microencapsulated with SOY. The differences in moisture content between powders produced with SOY and SKIM may be attributed to their different contents of protein, sugars, and lipids. In general, sugars are highly hygroscopic, which may increase the moisture content of LP powders. In this study, SKIM had higher sugar content than SOY.

Higher moisture content and a_w normally correlates with poor cell survivability and shorter shelf life of probiotic powders. Chávez and Ledeboer (2007) reported that a moisture content below five (g/100g) is required to ensure the long-term stability of probiotics powders. In this study, except for FD powders produced with non-UHPH-treated SKIM, the moisture contents of all powders were below 4.2 (g/100g) which indicated shelf stability. Besides, a_w values of all powders were below 0.3, except for LP powders dried under MXSD and microencapsulated with non-UHPH-treated and 150-MPa-treated SKIM. The a_w values below 0.6 indicated microbiologically stable (Quek, Chok, & Swedlund, 2007). And probiotic powders with a_w values between 0.15 and 0.3 can prevent caking and recrystallization of wall materials (Avila-Reyes, Garcia-Suarez, Jiménez, San Martín-Gonzalez, & Bello-Perez, 2014).

Table 4.4 Moisture content and a_w values of *L. plantarum* NRRL B-1927 powders

Drying method	Encap.	Non-UHPH (0 MPa)		UHPH (150MPa)		UHPH (300MPa)	
		Moisture (g/100 g, w. b.)	**a_w**	**Moisture (g/100 g, w. b.)**	**a_w**	**Moisture (g/100 g, w. b.)**	**a_w**
CCSD	**SOY**	$3.51\pm0.13^{c, A}$	$0.28\pm0.01^{b, A}$	$2.44\pm0.05^{b, B}$	$0.26\pm0.02^{b, A}$	$2.36\pm0.07^{b, B}$	$0.20\pm0.01^{c, B}$
	SKIM	$3.27\pm0.08^{c, A}$	$0.28\pm0.01^{bc, A}$	$2.17\pm0.14^{c, C}$	$0.24\pm0.00^{b, B}$	$2.46\pm0.07^{b, B}$	$0.23\pm0.00^{b, C}$
MXSD	**SOY**	$2.50\pm0.14^{e, A}$	$0.24\pm0.01^{c, A}$	$2.13\pm0.07^{c, B}$	$0.23\pm0.01^{b, A}$	$1.56\pm0.03^{c, C}$	$0.13\pm0.01^{e, B}$
	SKIM	$4.16\pm0.04^{b, A}$	$0.33\pm0.02^{a, A}$	$3.38\pm0.03^{a, B}$	$0.32\pm0.02^{a, A}$	$3.23\pm0.05^{a, C}$	$0.25\pm0.00^{a, B}$
FD	**SOY**	$2.88\pm0.06^{d, A}$	$0.13\pm0.02^{d, B}$	$0.77\pm0.11^{d, C}$	$0.10\pm0.02^{c, C}$	$1.31\pm0.07^{c, B}$	$0.26\pm0.00^{a, A}$
	SKIM	$6.69\pm0.06^{a, A}$	$0.13\pm0.01^{d, B}$	$0.88\pm0.04^{d, C}$	$0.06\pm0.01^{c, C}$	$1.36\pm0.19^{c, B}$	$0.17\pm0.00^{d, A}$

Values are Means ± Standard Deviation (SD). Moisture and water activity (a_w) followed by different lowercase (a, b, c, d) letters in the same column are significantly different ($P<0.05$). Moisture and a_w followed by different uppercase (A, B, C) letters in the same row for a given measure (moisture or water activity (aw) are significantly different ($P< 0.05$). See Table 4.3 for definition of SOY, SKIM, CCSD, MXSD, FD.

4.2.5 Particle size distribution of LP powders

Particle size distribution of LP powders is shown in Table 4.5. The appearance, dispersibility, solubility and mouthfeel of food powders is affected by their particle size (Sharma, Kadam, Chadha, Wilson, & Gupta, 2013; Zhao, Sun, Torley, Wang, & Niu, 2008). In this study, the mean particle size (D_{50}) of spray-dried powders ranged from 8.73 ± 0.08 µm (for powders microencapsulated with non-UHPH-treated SKIM *via* MXSD) to 21.03 ± 0.14 µm (for powders prepared with non-UHPH-treated SOY *via* CCSD). Significantly (P<0.05) higher mean particle sizes (D_{50}) were observed for LP powders dried by FD compared to MXSD and CCSD. Karam, Petit, Zimmer, Djantou, and Scher (2016) reported that grinding conditions can affect the particle sizes of FD powders. In this study the grinding conditions of freeze-dried samples were kept constant, hence the differences in particle sizes among FD powders may be the result of the intrinsic characteristics of the microencapsulating materials. Furthermore, LP powders prepared *via* MXSD showed significantly (P<0.05) smaller mean particle sizes (D_{50}) than those produced *via* CCSD. Spray drying allows a homogenous distribution of small particles. It has been reported that during the spray drying of liquid foods, atomization conditions, properties of the liquid suspensions (*e.g.* viscosity, pseudoplastic properties, solid content, etc.), inlet and outlet air temperatures, as well as the particle residence times inside the dryer chamber may affect the particle size of the powders (Jiang, Dev Kumar, Chen, Mishra, & Mis Solval, 2020; Pinto, Kemp, Bermingham, Hartwig, & Bisten, 2014).

Under the same drying conditions, LP powders microencapsulated with non-UHPH and/or 150-MPa-treated SKIM showed smaller particle sizes (D_{10}, D_{50}, D_{90}) than LP powders produced with non-UHPH-treated and 150-MPa-treated SOY. Furthermore, LP powders microencapsulated with 300-MPa-treated SOY showed significantly (P<0.05) smaller mean

particle sizes (D_{50}) than those microencapsulated produced with non-UHPH and/or 150-MPa-treated SOY when they were produced *via* MXSD and CCSD. Under CCSD conditions, LP powders microencapsulated with 150-MPa-treated SKIM had smaller mean particle sizes (D_{50}) than those microencapsulated with non-UHPH and 300-MPa-treated SKIM. At MXSD conditions, LP powders microencapsulated with 150-MPa-treated SKIM showed smaller particle sizes than those produced with non-UHPH and 300-MPa SKIM.

Powders with smaller particle sizes might result from smaller droplets produced by the spray dryer's atomizer due to the lower viscosity of the liquid suspensions such as the one prepared with 300-MPa-treated SOY. As mentioned previously, the components in SOY and SKIM are different, especially in terms of protein, lipid, and carbohydrate content. SKIM had less protein and lipid but more carbohydrate than SOY, which may had caused the particle size distribution difference between LP powders prepared with SOY and SKIM. UHPH modified viscosity and the structure of proteins of SOY and SKIM, which affected particle size distribution of the LP powders.

The span value of LP powders ranged from 1.90 ± 0.04 μm (for those microencapsulated with non-UHPH-treated SOY *via* CCSD) to 33.03 ± 0.06 μm (for those microencapsulated with 300-MPa-treated SKIM *via* MXSD). All the span values of powders prepared with SOY were below 3, except for MXSD powders produced with 300-MPa-treated SOY. Furthermore, span values were found to be higher in powders produced with SKIM than with SOY under the same drying method. Besides, the span values of MXSD powders were significantly (P<0.05) higher than CCSD powders, which indicated particle agglomeration in MXSD powders. In general, powders produced with 300-MPa-treated SOY and/or SKIM had higher span values than powders produced with non-UHPH and 150-MPa-treated SOY and/or SKIM. It has been

suggested that span values below 2 indicated homogenously distributed spray dried powders, and span values above 2 suggested particle agglomeration in spray dried powder (Cal & Sollohub, 2010; Tonon, Grosso, & Hubinger, 2011).

Table 4.5 Particle size distribution values of dried probiotic powders containing *L. plantarum* NRRL B-1927.

UHPH	Drying method	Encap.	Particle size distribution values			
			D_{10}(μm)	D_{50}(μm)	D_{90}(μm)	Span
0 – MPa	CCSD	SOY	3.44±0.34$^{c, A}$	21.03±0.14$^{c, A}$	43.45±0.63$^{c, A}$	1.90±0.04$^{d, B}$
		SKIM	1.71±0.02$^{d, B}$	14.05±0.16$^{d, B}$	32.36±0.17$^{de, B}$	2.18±0.01$^{c, B}$
	MXSD	SOY	1.56±0.01$^{d, A}$	12.27±0.36$^{e, A}$	36.38±0.73$^{d, A}$	2.84±0.03$^{b, B}$
		SKIM	1.27±0.09$^{d, A}$	8.73±0.08$^{f, C}$	31.56±0.58$^{e, B}$	3.47±0.06$^{a, B}$
	FD	SOY	39.98±0.50$^{a, A}$	138.51±1.03$^{a, A}$	336.48±3.95$^{a, A}$	2.14±0.02$^{c, C}$
		SKIM	8.55±0.17$^{b, C}$	62.04±0.32$^{b, C}$	229.12±0.27$^{b, C}$	3.56±0.02$^{a, A}$
150-MPa	CCSD	SOY	2.57±0.54$^{c, AB}$	18.14±0.40$^{c, B}$	37.65±1.51$^{c, B}$	1.93±0.06$^{e, B}$
		SKIM	1.76±0.03$^{cd, B}$	13.66±0.07$^{d, C}$	28.68±0.27$^{e, C}$	1.97±0.01$^{e, C}$
	MXSD	SOY	1.57±0.04$^{d, A}$	11.71±0.79$^{e, A}$	35.32±1.45$^{cd, A}$	2.89±0.08$^{c, B}$
		SKIM	1.41±0.04$^{d, A}$	9.40±0.03$^{f, A}$	32.78±0.41$^{de, B}$	3.34±0.04$^{b, B}$
	FD	SOY	30.88±0.47$^{a, B}$	114.40±0.29$^{a, B}$	314.13±2.47$^{a, C}$	2.48±0.01$^{d, B}$
		SKIM	10.15±0.15$^{b, B}$	66.12±0.36$^{b, B}$	239.73±2.37$^{b, B}$	3.47±0.02$^{a, B}$
300-MPa	CCSD	SOY	1.81±0.01$^{c, B}$	15.38±0.22$^{c, C}$	38.70±0.74$^{d, B}$	2.40±0.03$^{d, A}$
		SKIM	1.89±0.01$^{c, A}$	15.76±0.12$^{c, A}$	39.95±0.79$^{d, A}$	2.42±0.03$^{d, A}$
	MXSD	SOY	1.24±0.03$^{d, B}$	8.73±0.11$^{d, B}$	29.40±0.36$^{e, B}$	3.23±0.06$^{b, A}$
		SKIM	1.37±0.01$^{d, A}$	9.03±0.04$^{d, B}$	299.49±1.78$^{b, A}$	33.03±0.06$^{a, A}$
	FD	SOY	24.75±0.11$^{a, C}$	114.29±1.21$^{a, B}$	326.08±4.78$^{a, B}$	2.64±0.02$^{c, A}$
		SKIM	13.34±0.28$^{b, A}$	84.88±0.42$^{b, A}$	281.71±1.99$^{c, A}$	3.16±0.01$^{b, C}$

Values are Means ± SD of triplicate determinations. Values followed by different lowercase (a, b, c, d) letters in the same column for powders produced with encapsulants treated at the same UHPH pressures are significantly different (P<0.05). Values followed by different uppercase (A, B, C) letters for powders produced with the same encapsulant treated at different UHPH pressures for a given measure (D_{10}, D_{50}, D_{90}, span) are significantly different ((P<0.05). See Table 4.3 for definition of CCSD, MXSD, FD. Dx = xth percentile particle size.

4.2.6 Powder morphology of LP powders

The SEM micrographs of LP powders were shown in Fig. 4.3 and Fig. 4.4. The spray dried LP powders presented spherical particle with various sizes which were typical spray dried powders' morphology (Alves, Messaoud, Desobry, Costa, & Rodrigues, 2016). Actually, the morphology of spray dried powders are highly related to the contact configuration between drying air and atomized droplets, drying air temperature, the type and concentration of encapsulants (Alves et al., 2016; Du et al., 2014). For the powders produced *via* CCSD and MXSD, the SEM micrographs revealed that LP powders microencapsulated with SOY had a higher degree of shrinkage and agglomeration than those microencapsulated with SKIM. Particles may shrink, inflate, agglomerate, distort or fracture depending on the composition, rheological properties, porosity degree of the particle skin or crust formed during spray drying (Walton, 2000). Moreover, more agglomeration was observed in LP powders produced *via* MXSD than CCSD. This confirms our previous observations with particle size distribution of the LP powders. While LP powders produced *via* FD were non-spherical, flakes-like with spikes and sharp edges, and with more agglomeration observed in powders produced with SOY compared to those microencapsulated with SKIM. This might because that the FD powders were milled after freeze-drying the probiotic suspensions. Higher particle agglomeration observed in LP powders microencapsulated with SOY. It is believed that particle agglomeration allowed higher survival rates of probiotic cells. Besides, the particle size distribution results discussed in the previous section was confirmed with the observations in the SEM micrographs.

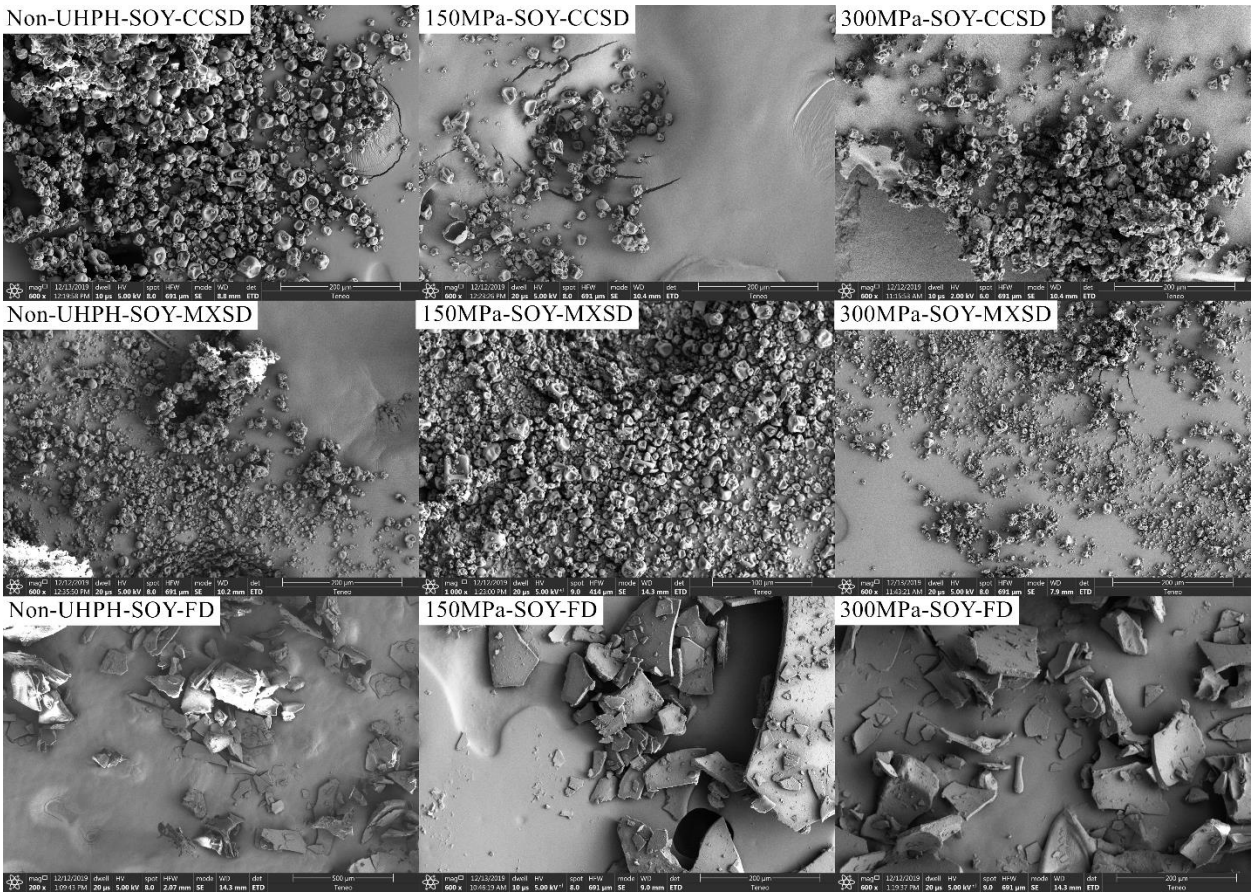

Fig. 4.3 SEM micrographs of *L. plantarum* NRRL B-1927 powders microencapsulated with SOY *via* CCSD, MXSD and FD. See Table 4.3 for definition of SOY, CCSD, MXSD, FD, UHPH.

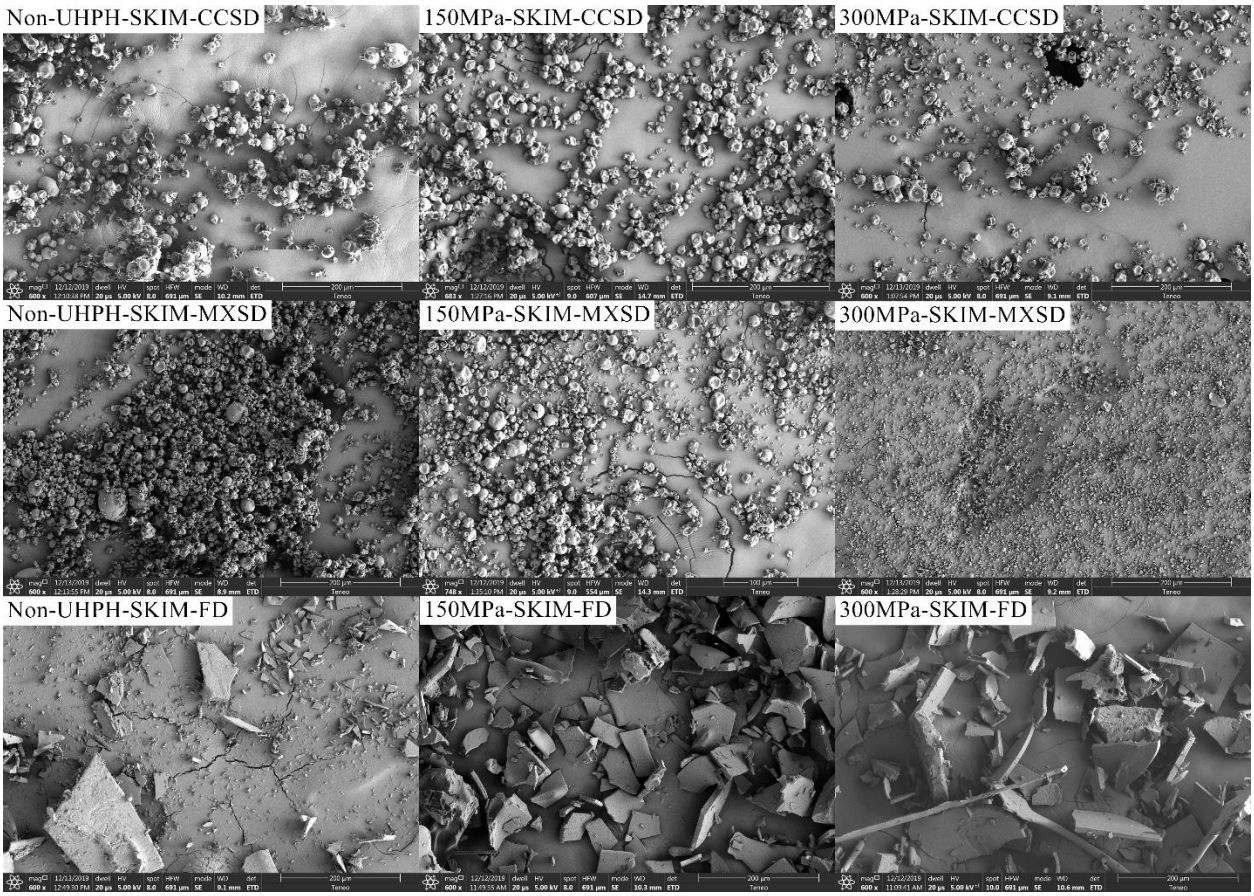

Fig. 4.4 SEM micrographs of *L. plantarum* NRRL B-1927 powders microencapsulated with SKIM *via* CCSD, MXSD, and FD. See Table 4.3 for definition of SKIM, CCSD, MXSD, FD, UHPH.

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CHAPTER 5

CONCLUSIONS

This research was divided in two parts about microencapsulating probiotics *via* spray drying and/or freeze drying. The results obtained in both sections indicated that spray drying is a feasible method to microencapsulate probiotics. With an approprite drying design and microencapsulating materials, spray drying can yield equal or even higher survivality of probiotics than freeze drying.

The first section of this research demonstrated that jellyfish gelatin with a low mineral content can be successfully produced from salted and dry cannonball jellyfish through a series of processes: rehydration, chopping, blending, homogenization, hydrolyzation, dialysis, freeze drying and milling, and can be combined with maltodextrin to microencapsulate LRGG together *via* MXSD and/or FD. Furthermore, an effective dialysis method was introduced to remove more than 85% of minerals in jellyfish gelatin. Higher cell survival was observed in LRGG powders after MXSD compared to FD. This might suggest the presence of heat-sensitive antimicrobial compounds in JG which were inactivated during MXSD process. After 15 days of storage at room temperature, 50-55% and 75% relative humidity conditions, LRGG powders microencapsulated with jellyfish gelatin and maltodextrin absorbed significant amounts of moisture, while the cell counts of LRGG were reduced more than 99.9%.

The second section demonstrated that the cell survival of LP after drying can be improved when powders were microencapsulated with UHPH treated soymilk and skim milk. Higher cell survival of LP was observed in powders microencapsulated with 300-MPa-treated

SOY and 150-MPa-treated SKIM. Using UHPH-treated SOY and SKIM as microencapsulating agents improved the cell survival of LP after MXSD and CCSD dramatically. In general, higher cell survival was found in powders produced *via* FD followed by MXSD and CCSD.

The results obtained in this study can be used to develop better probiotic powders with high cell viability using novel or UHPH-modified microencapsulating materials *via* spray drying, a more cost-effective drying method than freeze drying.