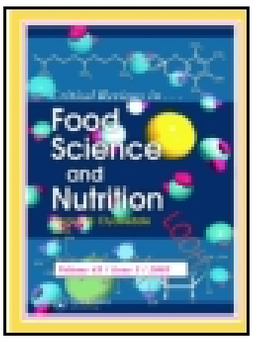

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Protective approaches and mechanisms of microencapsulation to the survival of probiotic bacteria during processing, storage and gastrointestinal digestion: a review

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Abstract:

In recent years, there is a rising interest in the number of food products containing probiotic bacteria with favorable health benefit effects. However, the viability of probiotic bacteria is

always questionable when they exposure to the harsh environment during processing, storage and gastrointestinal digestion. To overcome these problems, microencapsulation of cells is currently receiving considerable attention and has obtained valuable effects. According to the drying temperature, the commonly used technologies can be divided into two patterns: high temperature drying (spray drying, fluid bed drying) and low temperature drying (ultrasonic vacuum spray drying, spray chilling, electrospinning, supercritical technique, freeze drying, extrusion, emulsion, enzyme gelation and impinging aerosol technique). Furthermore, not only should the probiotic bacteria maintain high viability during processing, they also need to keep alive during storage and gastrointestinal digestion, where they additionally suffer from water, oxygen, heat as well as strong acid and bile conditions. This review focuses on demonstrating the effects of different microencapsulation techniques on the survival of bacteria during processing as well as protective approaches and mechanisms to the encapsulated probiotic bacteria during storage and gastrointestinal digestion that currently reported in the literature.

Keywords

microencapsulation, probiotic, protection mechanism, storage, gastrointestinal digestion

1. Introduction

In April 2016, a profound special issue named “Microbiome” was published in Science magazine (http://science.sciencemag.org/content/352/6285.toc). As demonstrated by Ash and Mueller (2016) in this issue, microbes in the human intestine play a pivotal role in shaping the development of host immune system and have long-term effects on susceptibility to several inflammatory diseases, such as allergy and autoimmunity. Probiotic bacteria, such as lactobacillus and bifidobacterium, are important part of human intestinal microbes and show great potential for rebuilding microbiotas and restoring human health. As claimed by FAO/WHO (2002), probiotics are living microorganisms which, when administered in adequate levels, confer health benefits to the host. Due to these above mentioned health benefits, applications of probiotic bacteria within food industry have already obtained highly attention. The probiotic ingredients market have been one of the prime beneficiaries of functional food industry and the probiotic market size was valued at USD 36.6 billion in 2015, with over 7% annual growth expectation in the next few years (https://www.gminsights.com/industry-analysis/probiotics-market). Due to such huge market and profit, several big famous food transnational enterprises, such as Danone (France), Nestlé S.A. (Switzerland) and Yakult Honsha Co. Ltd. (Japan), have already been actively launching their own novel competitive probiotic products to maximally amplify the market share.

To exert their biological effects on the host, sufficient number of live cells should be guaranteed during storage and gastrointestinal digestion (Dong, et al., 2013). Nowadays, there are usually two forms that probiotic bacteria present in: aqueous solution and powder. When cells are in aqueous phase, however, it is difficult to maintain their viability during storage, transport and application in food with dry matrix. While for probiotic bacteria powder, these mentioned limitations vanish. However, disadvantages still exist for the case of probiotic bacteria in powder form: low temperature is needed to keep its viability (which is noneconomic) and they are very sensitive during exposure in oxygen, acid and high temperature conditions. Although by strictly selecting bacteria strain, some oxygen or acid tolerance strain can be screened. However, the screening process is usually time consuming and costly. Furthermore, large amount of other probiotic bacteria strains with specific functions and health benefits are non-oxygen/acid resistant strain and prone to inactivate during exposure in these harsh environments. Thus, microencapsulation of these probiotic bacteria into powder form to enhance their stability during both storage and gastrointestinal digestion is proposed and obtained inspiring results.

Normally, microencapsulation technologies with low temperature (such as freeze drying (Heidebach, Först, & Kulozik, 2010), extrusion (De Prisco, Maresca, Ongeng, & Mauriello, 2015), emulsion (Shafiei, Razavilar, Javadi, & Mirzaei, 2012), etc.) are used to manufacture probiotic microcapsules. However, the extremely low temperature and osmotic stress presented in low temperature drying technology are fatal to the survival of cells (Capela, Hay, & Shah,

2006). Meanwhile, high temperature drying (such as spray drying (Rajam & Anandharamakrishnan, 2015), fluid bed drying (Poddar, et al., 2014)) is also obtained sufficient attention. However, proper protective wall materials are still needed to resist the heat, shear stress and long period of oxygen exposure during high temperature processing (Gong, et al., 2014; Hede, Bach, & Jensen, 2008). Besides, due to the sensitivity of probiotic bacteria to strong acid and bile condition during gastrointestinal digestion, the digestion properties of these encapsulating wall materials used in low or high temperature drying technologies also need to be pre-considered (Albadran, Chatzifragkou, Khutoryanskiy, & Charalampopoulos, 2015; Burgain, Gaiani, Cailliez-Grimal, Jeandel, & Scher, 2013; Meng, Stanton, Fitzgerald, Daly, & Ross, 2008).

Several reviews have already summarized the commonly used technologies and coating materials within the area of probiotic microencapsulation (Burgain, Gaiani, Linder, & Scher, 2011; Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2012; de Vos, Faas, Spasojevic, & Sikkema, 2010; Martín, Lara-Villoslada, Ruiz, & Morales, 2015; Riaz & Masud, 2013). However, they are more focused on illustrating the characteristics of different microencapsulation methods and wall materials used. For the protection mechanisms of different probiotic microcapsules prepared by low or high temperature technology, elaborate illustration is rarely discussed. In this paper, we divided the microencapsulation technology into two patterns (low temperature and high temperature drying technology) and systematically elaborated the

protection mechanisms lay behind each technology during processing, storage and gastrointestinal digestion.

2. Protective approaches and mechanisms to the survival of probiotic bacteria during microencapsulation process

Nowadays, almost all the probiotic microencapsulation technologies include a drying process. The main purpose of drying is to obtain the powder form of probiotic microcapsule and to decrease the water content of these microcapsules with the purpose of maintaining prolonged viability during storage. According to the temperatures used, two microencapsulation technologies can be divided: low and high temperature drying technologies. Low temperature drying method mainly refer to freeze drying, spray chilling, electrospinning etc. that using low temperature to produce probiotic microcapsules. While high temperature drying methods mainly refer to spray drying and fluid bed drying that using relative high temperature condition to prepare probiotic microcapsules.

2.1 High temperature drying

2.1.1 Spray drying

Spray drying is a technique that a liquid product atomized in a hot gas which instantaneously leading to the formation of powders. With the advantages of low cost, high productivity, well continuity and rapid processing, spray drying is one of the most commonly used

microencapsulation technologies within the food industry (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007). However, the high temperature, osmotic stress, dehydration and oxygen exposure conditions applied during spray drying would result in the damage of probiotic bacteria membrane substances, such as fatty acids, intracellular proteins, ribosomes, DNA and RNA etc., which eventually give rise to the death of probiotic bacteria (Teixeira, Castro, Mohácsi‐Farkas, & Kirby, 1997). In order to overcome these shortages and to improve the survival of probiotic bacteria during spray drying, several methods have been developed as discussed below.

Adjustment of spray drying parameters, such as inlet/outlet temperature, flow rate, atomizing air pressure and residence time within dryer chamber, is one of the applicable strategies for improving the survival of probiotic bacteria during spray drying. Normally, using lower inlet/outlet temperatures consists well with higher survival rates of probiotic bacteria during spray drying, which attribute to the lower degree of heat damage in cells. Ghandi, Powell, Chen, and Adhikari (2012) found that with the decrease of inlet/outlet temperature (from 200℃/65℃ to 130℃/38℃), the survival rate of *Lactococcus lactis ssp. cremoris* increased from 0.1% to 14.7%. Similar results can also be found in O'Riordan, Andrews, Buckle, and Conway (2001) who reported that relative low inlet/outlet temperature improved the viability of *Bifidobacterium PL1* during spray drying. Martín, et al. (2015) reported that compared with inlet temperature, the survival of probiotic bacteria is more dependent on the outlet temperature. Feed rate is another

important factor that strongly affects the variation of outlet temperature. Under the same inlet temperature condition, the higher of feed rate, the lower of outlet temperature and the corresponding survival rate would thus be improved. However, it is noteworthy that although bacterial survival is higher at lower drying temperature, the residual moisture contents within spray dried microcapsule powders would also increase. This situation would lead to several adverse effects: on one hand, powders will stick to the cyclone and collecting flask which will increase the difficulty of operation and decrease the yield, on the other hand, high moisture content within probiotic microcapsules will accelerate the death of cells during storage. Compared with inlet/outlet temperature, atomizing air pressure also affects the viability of cells during spray drying, although the extent of its influence is relatively less than that of temperature. As reported by Xueyong Zhou, Dong, Gao, and Yu (2008), when the inlet temperature and outlet temperature both maintained at constant value, the higher of the atomizing air pressure, the lower of the survival rate of cells. Two possible reasons may lay behind of this situation: 1) with the increase of air pressure, the size of sprayed droplets decreased which leading to the exposure of inside probiotic bacteria to the hot air (X Zhou, Chen, & Yu, 2004); 2) increasing the atomizing air pressure would result in high shear rates and shear stress which leading to the damage of cells during atomizing (TO & ETZEL, 1997). Resultant residence time is another influencing factor that affects the viability of probiotic bacteria during spray drying. The less of the resultant residence time of probiotic bacteria

exposed in the drying chamber, the less possibility for them to suffer from heat damage. The resultant residence time is mainly controlled by the aspirator: the higher of the aspirator setting value, the less of the exposure time. As reported by O'Riordan, et al. (2001), with the aspirator power setting level increased from 0 to 12, the survival rate of spray dried cells increased from 11.26% to 29.62%.

Addition of different kinds of wall materials also plays an important role in protecting probiotic bacteria from the adverse conditions during spray drying. Nowadays, the most frequently used wall materials (all natural, inexpensive, biocompatible and generally regarded as safe) in spray drying for microencapsulation of probiotic bacteria are carbohydrates (starch, maltodextrin, pectin, pullulan, gum arabic etc.) and proteins (whey protein, caseinate, soy protein etc.). Spray drying of *Lactobacillus zeae LB1* in water without any wall material was reported lost all the viability during spray drying (Liu, et al., 2016a). In the early stage, single polymer formula has been used to encapsulate probiotic cells using spray drying, such as modified starch (O'Riordan, et al., 2001), gum arabic (Desmond, Ross, O'Callaghan, Fitzgerald, & Stanton, 2002), gelatin (Lian, Hsiao, & Chou, 2003) and whey protein isolate (Picot & Lacroix, 2004). More recently, binary systems using protein and/or polysaccharide have been widely applied, such as maltodextrin mixed with gum arabic (Su, Lin, & Chen, 2007), β-cyclodextrin mixed with gum arabic (Zhao, Sun, Torley, Wang, & Niu, 2008), skim milk mixed with either starch or whey protein (Paéz, et al., 2012) and reconstituted skim milk mixed with oligofructose

(Fritzen-Freire, et al., 2012). Properly selected binary systems have resulted in higher encapsulation efficiencies and/or higher survival rates, superior *in vitro* gastrointestinal digestion and storage properties as compared to single component system (Tanzina, Avik, Ruhul, Riedl, & Lacroix, 2013). For instance, Liu, et al. (2016b) encapsulated *L. zeae* LB1 in a matrix of sodium caseinate and gum arabic or gum ghatti through spray drying; the viability of cells during gastrointestinal digestion and storage was greatly improved with increasing sodium caseinate content in the encapsulating materials. On the surface of probiotic bacteria, there are several macromolecules located, which enable probiotic bacteria interact with carbohydrates and proteins through electrostatic, hydrophobic interactions (Abd El-Salam & El-Shibiny, 2015). As for wall materials of proteins, normally they exhibits well film forming property and thus can form a protective coating on the cell wall during spray drying, which prevent cellular injury by stabilizing cell membrane constituents. As for wall materials of carbohydrates, commonly they presents well solubility, low viscosity at high concentration, high glass transition temperature and rapid drying property, which enable them suitable as coating materials during spray drying.

Recently, low melting point fat (LMF) was also found to be a potential protective agent to protect probiotic bacteria from heat damage during spray drying. When the temperature increase above its melting point, the LMF rapidly transforms from solid to liquid phase, during which it absorbs considerable thermal energy. As reported by Liu, et al. (2015), with the addition of LMF in microcapsules from 0% to 50% (w/w), the viability of *Lactobacillus zeae LB1* increased from

16% to 63%, which may attributed to the solid-to liquid phase transition of LMF during spray drying that absorbed part of the thermal energy transferred into the powder particles. However, this protection effect of LMF may strain dependent, as there was no improvement when spray drying *Lactobacillus reuteri S64 and K67* (Liu, et al., 2015).

2.1.2 Fluid bed drying

Fluid bed drying technology is another method using relative high temperature condition to prepare probiotic microcapsules, whereas compared with spray drying, a limited number of studies have reported using fluid bed to dry probiotic contained microcapsules. In this technology, the core material, which contains the probiotic bacteria, should first be in a solid form and suspends in hot air flow. Biopolymer solution is then sprayed on the surface of these bioactive cores during which water evaporates immediately and the biopolymer adheres tightly on the surface of cells contained cores. The advantages of fluid bed drying technology are low cost, easy to scale up (as it has already been widely used in the industry) and can provide multi-coating layers with different function properties. However, this technology is difficult to master and takes long time of processing which prone to inactivate the probiotic bacteria (Hede, et al., 2008).

Adjustments of atomizing air pressure, processing temperature and time are generally applicable strategies for improving the survival of probiotic bacteria during fluid bed drying. Both too high or low atomizing air pressure and too long or short processing time

will cause damage of probiotic bacteria during fluid bed drying, which can attribute to the alteration in mechanical stress and relative humidity in final products. When over-drying of cells, cellular proteins could be damaged due to the elimination of the whole cellular water; while for short processing time, excessive high water content would exist within the final microcapsules and the resulting degradation of the acidification activity will cause severe membrane damage and cell death. Besides, high mechanical stress applied on the cells during fluid bed drying due to high atomizing air pressure will also cause the damage of probiotic bacteria. As reported by Stummer, et al. (2012), after exorbitantly adjusting either atomizing air pressure (from 1.5 bar to either 1 bar, 2.5 bar or 3.5 bar) or processing time (from 15min to either 7min or 30min), significant decrease in the viability of *Enterococcus faecium M74* during fluid bed drying can be observed which can contribute to the too high or low water activities within probiotic bacteria microcapsules. The temperature during fluid bed drying is found to be another important factor that has a dramatic effect on post drying cell viability. Generally, with the processing temperature increasing, it will cause the collapse of the cellular redox system which resulting in a remarkable adverse impact on the degree of membrane damage of dried cells. As reported by Semyonov, Ramon, Kovacs, Friedlander, and Shimoni (2012), a temperature difference of + 15℃ decreased survival rate of *Lactobacillus paracasei* at 250-fold during fluidized bed drying.

Generally, addition of protective coating materials is found to be another effective way to protect probiotic bacteria during fluid bed drying. The mostly used protectants herein are various sugars, such as trehalose, sorbitol, glucose and sucrose. Drying will cause various types of cell damage, mainly due to the changes in the physical state of membrane lipids and in the structure of sensitive proteins leading to the decrease in bacterial viability. With the addition of sugars, the hydroxyl groups presented in carbohydrates has the ability of replacing the water which originally interacting with the cell membrane and thus stabilizing the cell membrane lipids and proteins from damage. Schell and Beermann (2014) demonstrated that with the absence of protective carbohydrates, the viability of *Lactobacillus reuteri DSM 20016* was only 16.95±2.35% during fluid bed drying, however, after drying with trehalose and sorbitol as thermos-protectants, highest survival rates of 42.85±4.62 and 42.69±4.84% were obtained. Similar results can also be found in Strasser, Neureiter, Geppl, Braun, and Danner (2009) who reported that addition of either trehalose or sucrose showed the highest similar viabilities of 36.9±2.8 and 36.4±6.8%, respectively.

2.2 Low temperature drying

Due to the general thermal damage of probiotic bacteria occurred in high temperature drying (spray drying and fluid bed drying), relative low temperature drying technology (ultrasonic vacuum spray drying, spray chilling, electrospinning, supercritical technology etc.)

and extreme low temperature drying technology (freeze drying) are also widely researched and obtained highly attention.

2.2.1 Ultrasonic vacuum spray drying

As presented above, due to the simultaneous dehydration, thermal and oxygen stresses applied, the use of spray drying to produce probiotic microcapsules would result in high bacterial mortality. Thus, theoretically, if one can minimize the thermal and oxidative stresses during the spray drying process, effective improvement in cell survival rate would obtain. Recently, a new drying method based on this hypothesis called ultrasonic vacuum spray drying (Fig. 1) was developed and obtained significant improvement in the cell viability (Semyonov, Ramon, & Shimoni, 2011). This technology is performed through two stages: vacuum spray drying of the solution followed by nitrogen environment fluidized bed drying of the powder. Feed solution is first dispersed by a special designed ultrasonic atomizer which can operate in a vacuum environment and fall free with low velocities into vacuum chamber. The temperature of the drops does not exceed 20 – 30 ℃ and majority of the free water was evaporated during this vacuum stage. Finally, a nitrogen environment fluidized bed is continually applied on the powders until the required water activity is obtained. Using this technology, the heating intensity is largely reduced and the vacuum in the drier chamber significantly reduces the temperature of the microcapsules, the oxidative stress, as well as the residence time of particles in the drying chamber. Semyonov, et al. (2011) used maltodextrin combined with trehalose to encapsulate

Lactobacillus paracasei LMG P-21380 using ultrasonic vacuum spray drying and found that a glassy state of the particles was rapidly formed by using this technology and highest survival of probiotic cells after processing around 70% was obtained with formula of maltodextrin: trehalose = 1:1 (w/w). Apart from the favorable low temperature and oxygen condition originally provided by this technique, maltodextrin-trehalose embedding matrix can also increase the survival by maintaining the probiotic cells membrane integrity during drying and storage as well as promoting the stabilizing effect of the bacteria's protein.

2.2.2 Spray chilling

Spray chilling, also known as spray cooling and spray congealing, is similar to spray drying with respect to the production of fine droplets. However, spray chilling technology consists of making a solution dispersion or emulsion containing the active ingredient and a molten carrier (lipid), which is then atomized into a chamber where cold air or liquid nitrogen is injected (Okuro, de Matos Junior, & Favaro-Trindade, 2013). Due to its low cost, low temperature using and easy to scale up nature, spray chilling is a convenient technology for the encapsulation of food ingredients. However, some technological disadvantages still exist, such as low encapsulation efficiency and the possibility of expulsion of the active ingredient during storage. For microencapsulation of probiotic bacteria, another important favorable factor is the using of lipid matrix. As the lipid matrix can not only well encapsulate the cells with high viability by spray chilling, it also prolongs the viability during storage by blocking the cells from exposure to

water and stressors, such as H^+ ions. Besides, after ingesting into the gastrointestinal tract, the lipid matrix can also easily digested by lipases within intestine, which causes the microorganisms to be released near their site of action to exert their health function (Favaro-Trindade, Heinemann, & Pedroso, 2011). D. d. L. Pedroso, Thomazini, Heinemann, and Favaro-Trindade (2012) used an interesterified fat with palm and palm kernel, which has melting point of 47.5 °C, to encapsulate *B. lactis* BI-01 and *L. acidophilus* LAC-04 and found that there was no lost in cell viability in both probiotic bacteria. Similar results can also be found in D. Pedroso, Dogenski, Thomazini, Heinemann, and Favaro-Trindade (2013) who used cocoa butter (melting point of 36.5 °C) instead to encapsulate the same probiotic bacteria.

2.2.3 Electrospinning

Electrospinning is a process that produces continuous polymer fibers with diameters in the submicrometer range through the action of an external electric field imposed on a polymer solution or melt leading to the formation of continuous fibers (Fig. 2). Normally, this technique is used in biomedical area, as the surface area of the electrospum fibers mimic the extracellular matrix and can be used in a variety of applications such as scaffolds for tissue engineering or drug delivery devices (López-Rubio, Sanchez, Sanz, & Lagaron, 2009). Recently, due to the advantages of room temperature process, possible of large scale production, efficient encapsulation and enhanced stability of cells achieved by the use of food grade polymers and biopolymers, several studies have already used electrospinning technique to encapsulate

probiotic bacteria. For instance, López-Rubio, Sanchez, Wilkanowicz, Sanz, and Lagaron (2012) used a protein based matrix (whey protein concentrate) and a carbohydrate based matrix (pullulan) to encapsulate *Bifidobacterium animalis* subsp *lactic* Bb12 through electrospinning and found that whey protein concentrate based submicro- and microcapsules proved a greater improvement in cell viability when compared to pullulan based structures. Fung, Yuen, and Liong (2011) used soluble dietary fiber (SDF) from certain agricultural waste streams-okara (soybean solid waste), oil palm trunk (OPT), and oil palm frond (OPF) to encapsulate *L. acidophilus* FTDC 8933 by electrospinning technique and found high bacterial survivability (78.6–90%) after electrospinning and retained viability at refrigeration temperatures during the 21 day of storage. Although the numbers of applications of electrospinning and its potentials in the food systems (laboratory scale) have been recognized in the recent years; however, the industrial scale application of this technology in the food industry as a delivery vehicle is still limited and needs to be extended. The main reason why the potentialities of nanofibers have not been widely adopted in the food industry can be attributed to the prevalence of studies on synthetic polymers (which are not acceptable in food applications) rather than on food grade biopolymers (Ghorani & Tucker, 2015). Thus, exploration of new food grade wall materials to encapsulate probiotic bacteria using electrospinning can be a potential area for all food researchers.

2.2.4 Supercritical technology

Most of the probiotic microencapsulation methods involve exposure of the cells to water condition which may compromise survival of encapsulated cells as they are sensitive to moisture. Supercritical fluids are attractive alternative solvents which describe the state of a material above its critical point at which its vapour/liquid phase equilibrium can exist. Above these conditions, the liquid-gas phase transition disappears and the properties, such as diffusion coefficient and density, continuously change with variation in pressure or temperature (Moolman, et al., 2006). Supercritical processes give micro- or even nano-particles with narrow size distribution, and can also be used to achieve microencapsulation and surface coating (Fages, Lochard, Letourneau, Sauceau, & Rodier, 2004). Supercritical carbon dioxide ($scCO_2$) is one of the most commonly used supercritical fluid due to its environmental compatibility and low reactivity and low critical parameters. In biotechnological area, $scCO_2$ is commonly used to micronize of drugs, encapsulate of sensitive actives for controlled drug release and impregnate of biomaterial scaffolds with pharmaceutical actives (Moolman, et al., 2006). In probiotic microencapsulation area, supercritical technique was also applied, although not so general compared with that in biotechnological area. Normally, in supercritical technique, the probiotic cells are first immobilized during the process of interpolymer complex formation in $scCO_2$ and then the probiotic microcapsule was obtained by gasify the $scCO_2$ through depressurizing. Thantsha, Cloete, Moolman, and Labuschagne (2009) used polyvinylpyrrolidone (PVP) and vinyl

acetate-crotonic acid copolymer (VA-CA) to encapsulate *B. longum* Bb-46 using this technique. These two polymers are both plasticizable in $scCO_2$ and can form an interpolymer complex through hydrogen bonding between the carboxylic acid groups of the PA-CA and the carbonyl group of the PVP. Results showed that the live cell concentration in final probiotic microcapsule could maintain as high as 10^{12} CFU/g. Besides, the interpolymer complex displayed pH-responsive release properties, with litter to no release in SGF and substantial release in SIF. Although supercritical technology holds the potential for large scale manufacturing, it should keep in mind that the high investment requirements relating to the high pressure operation and the feasibility of this techniques in food industry need to be justified by an economic evaluation (Chen, Wang, Liu, & Gong, 2017).

2.2.5 Freeze drying

Freeze drying has typically been used to manufacture commercial probiotic bacteria powders for decades. The mechanism behind is based on sublimation, where the cells are first frozen at extreme low temperature and then dried through sublimation under high vacuum (Chalat Santivarangkna, Kulozik, & Foerst, 2007). Compared with spray drying, the processing conditions applied in freeze drying is milder and thus the survival rate of probiotic bacteria during freeze drying is normally higher (Wang, Yu, & Chou, 2004). However, during freezing at low temperature, cellular inactivation still occurs (Mills, Stanton, Fitzgerald, & Ross, 2011). First, formation of extracellular ice crystals and resulting increase in osmolality will cause the

damage of cells (Talwalkar & Kailasapathy, 2003). Besides, as soon as the ice crystals formed, the bound water from probiotic bacteria is removed, which results in the damage of surface proteins, cell membrane and cell wall. As bound water interacts with biological macromolecules that present on the cell wall and cell membrane through several weak bonding, it plays an important role in stabilizing the integrity of cell structure and function. Consequently, remove of bound water during freeze drying can lead to destabilization of the structural integrity of these cellular components, resulting in the damage or loss of their functions (Brennan, Wanismail, Johnson, & Ray, 1986; Meng, et al., 2008). As reported by Sheu, Marshall, and Heymann (1993) that only 4% of *L. delbrueckii ssp. bulgaricus L2* survived during freeze drying using water without any protective materials. According to the features of using low temperature drying method to prepare probiotic microcapsules, two different freeze drying patterns can be divided: direct and indirect freeze drying.

2.2.5.1 Direct freeze drying

In direct freeze drying, probiotic bacteria were first mixed with cryoprotectants solution and then directly placed in a freeze drier to remove the water. These protectants used can generally be divided into two categories: low molecular weight and high molecular weight materials.

i Low molecular weight protectants

Low molecular weight protectants mainly refer to various sugars, such as glucose, lactose, mannose, trehalose, sorbitol etc. For active cellular membrane, the phospholipids are hydrated by

various water molecules. During drying, the water is removed which resulting in the increase of van der Waal's interactions between hydrocarbon chains. During this process, increased compressive stresses will cause the phospholipids undergo a phase transition (from liquid crystalline phase to gel phase), during which the leakage of cellular membrane occurred (Wolfe, 1987). Due to the water replacement effect of sugars, it is generally accepted that they can depress the phase transition of cellular membranes during drying (Fig. 3). In order to provide such protective effect, specific interactions between phospholipids and sugars should properly occur. As water locates around the polar headgroups between the glycerol backbone and the fatty acid residues, sugars can interact with the phospholipids through the hydrogen bond between hydroxyl groups of sugars and phosphate group at the surface of the bilayer (C Santivarangkna, et al., 2008). Trehalose is considered more effective than other sugars in protecting probiotic bacteria during freeze drying. As reported by Luzardo, et al. (2000), for water molecules that tightly bound to carbonyls, only trehalose, rather than sucrose, can displace them. Besides, several extra protection effects of sugars were found and were slightly differed from one another. For example, beyond the water replacement effect, sorbitol can also prevent lipid oxidation due to its anti-oxidant properties (Linders, Wolkers, Hoekstra, & van't Riet, 1997) and owning to the formation of a glassy matrix of trehalose during freeze drying, its characteristic high viscosity and low mobility properties guarantee the integrity of protein functions in probiotic bacteria during drying (BELL & HAGEMAN, 1996).

ii High molecular weight protectants

High molecular weight protectants mainly refer to polysaccharides and proteins. Due to their relative rigid structure, these materials alone can not easily interact with the cell wall or membrane. However, they prefer to adsorb on the surface of microorganisms with forming a viscous layer, which can inhibit the growing rate of ice by increasing the solution viscosity and keeping the amorphous structure of ice in the close proximity of cell (Ana S. Carvalho, et al., 2004b). Gwak, et al. (2015) reported that ten percentage of soy powder showed a strong protective effect upon viability of *Lactobacillus brevis WK12* and *Lactococcus lactis WK11* (showing 1.85×10^{11} CFU/mL and 1.89×10^{11} CFU/mL, respectively) during freeze drying. Rajam, Karthik, Parthasarathi, Joseph, and Anandharamakrishnan (2012) used native/denatured whey protein with sodium alginate to encapsulate *Lactobacillus plantarum (mtcc 5422)* and the results showed that survival of cells during freeze drying was significantly higher than that during spray drying. Others using freeze drying method with wall materials of whole milk, skim milk, gelatin, maltodextrin, xanthan gum etc. to encapsulate probiotic bacteria can also be found (Ana S. Carvalho, et al., 2004a; Champagne, Mondou, Raymond, & Roy, 1996; Poddar, et al., 2014; TO, et al., 1997) and showed effective protections.

2.2.5.2 Indirect freeze drying

In indirect freeze drying, probiotic bacteria were first restrained within polymer matrix and then placed in a freeze drier to remove water. There are mainly four patterns of indirect freeze drying namely extrusion, emulsion, enzyme gelation methods and impinging aerosol technique.

i Extrusion

In extrusion method, a hydrocolloid solution contain probiotic bacteria is first prepared and dripped through a syringe needle or nozzle. The formed droplets are allowed to fall into a hardening solution to form beads and finally freeze dried. Due to its simplicity, low cost, gentle conditions and high cell viability, extrusion technology is one of the most popular methods that widely used to encapsulate probiotic bacteria. The mainly disadvantage of this technology is that it is difficult to scale up due to the slow formation of beads. Alginate is the most favorable material that used in this technology. Alginate consists of two different units: D-mannuronic (M) and L-guluronic acids (G). The G units have a bucked shape and when present in divalent cations condition, such as Ca^{2+}, Sr^{2+} or Br^{2+}, two G-units align side by side resulting in the formation of cross-linking structure. When using extrusion technique encapsulating probiotic bacteria, an alginate solution is first mixed with a cell suspension and then drip into a solution containing divalent cations. The droplets would form gel spheres instantaneously which entrapping the probiotic bacteria in a three dimensional structure (typical structure of alginate beads can

be found in Fig. 4). For example, De Prisco, et al. (2015) used 2% (w/w) alginate hardened in 0.5 mol/L $CaCl_2$ solution to encapsulate *Lactobacillus reuteri DSM 17938* and found that nearly all the bacteria survived during the extrusion process. Similar results can also be found elsewhere (Krasaekoopt, Bhandari, & Deeth, 2004; K.-Y. Lee & Heo, 2000; Muthukumarasamy, Allan‐Wojtas, & Holley, 2006). The nozzle size, G unit content, viscosity of alginate solution, and the distance between the syringe and the hardening solution were reported to affect the size of beads. Normally, small nozzle size, high unit content and high viscosity of alginate solution (high alginate concentration) decrease the size of beads and thus weaken the protection effect to encapsulated cells.

ii Emulsion

Apart from extrusion method, alginate beads can also be prepared by emulsion method through external or internal gelation and followed by freeze drying. In the type of external gelation, alginate solution is first mixed with probiotic bacteria and suspended in an oil bath containing emulsifier to form W/O emulsion. The emulsion is then broken by adding $CaCl_2$ solution and the formed beads can be collected by centrifugation. In the type of internal gelation, alginate solution contains $CaCO_3$ is first prepared instead. A W/O emulsion is then prepared using the similar method in that of external gelation, however, the gel is finally formed by adding an organic acid into the emulsion (organic

acid will penetrate into the water phase and react with $CaCO_3$ leading to the release of calcium ions which react with alginate to form the gel structure).

Other materials, such as carrageenan and sodium carboxymethyl cellulose (NaCMC), can also be used to prepare probiotic microcapsules through emulsion method by external gelation. The operating procedure is similar to that of alginate, however, the gelatin mechanism varies. For carrageenan, it needs to first dissolve at relative high temperature (60-90℃) and then adding probiotic bacteria into carrageenan solution at 40--45℃. Gelation occurs through cooling to room temperature and after the formation of beads, K^+ is added to induce and stabilize the gel structure (Miles, Morris, & Carroll, 1984). For NaCMC, Al^{3+} are used to form crosslinking structure and due to its gastric acid resistance and intestinal solubility properties, NaCMC is suitable to utilization in probiotic bacteria delivery (Chitprasert, Sudsai, & Rodklongtan, 2012).

iii Enzyme gelation

Milk proteins can be gelled by enzymes to form water insoluble probiotic bacteria microcapsules. In enzyme gelation method, enzyme, cell and milk protein mixtures are first prepared and adding into oil phase to form W/O emulsion. Gelation of protein is then triggered by temperature alteration or stirring for long time and followed by freeze drying (typical structure of enzyme gelled protein microcapsules is shown in Fig. 5). Due to the mild processing condition, viability of probiotic during enzyme gelation process is

relatively very high. For enzymes, rennet and transglutaminase (TGase) are normally used and whey protein, casein and caseinate are their typical target objects. Rennet is a proteolytic enzyme complex mainly consisting of chymosin, which is capable of cleaving the κ-casein molecule protruding from the surface of the casein micelles in milk. When a sufficient level of κ-casein hydrolysation is achieved, the lower net negative charge and the higher hydrophobicity of the para-k-casein lead to an aggregation of the casein micelles. Through raising the temperature above 18℃, non-covalent cross-links are then progressively formed between chains of flocculating micelles leading to the formation of gel structure (Heidebach, Först, & Kulozik, 2009a). TGase is a transferase that forms both inter- and intra-molecular isopeptide bonds in and between many proteins by cross-linking of the amino acid residues of protein bound glutamine and lysine. For microcapsules produced using TGase, long time of stirring is needed to obtain gel structure with sufficient strength. There are three kind of TGase reaction mechanisms that inducing in the modification of protein. Cross-linking of proteins is the most dominant reaction for TGase, resulting in the formation of high molecular weight of proteins. Besides, in the presence of primary amines, TGase can cross-link the amines to the glutamines of a protein (acyl-transfer reaction). In the absence of lysine residues or other primary amines, water will react as a nucleophile, resulting in deamination of glutamines (DeJong & Koppelman, 2002; Heidebach, et al., 2009b). For example, Heidebach, et al.

(2009b) used TGase gelled caseinate microcapsules to encapsulate *Lactobacillus paracasei ssp. paracasei F19* and *Bifidobacterium lactis Bb12* and high survival rates of 70% and 93% were obtained respectively. Later, Heidebach, et al. (2009a) used rennet gelled casein to encapsulate the same probiotic bacteria mentioned above and found no loss in the number of living probiotic bacteria.

iv Impinging aerosol technique

Impinging aerosol technique is a relatively new probiotic encapsulation method (Bhandari, 2015). Similar to extrusion and emulsion method, cross-linking of sodium alginate solution using calcium chloride to form probiotic beads was also applied in impinging aerosol technique. However, impinging aerosol technique is a continuous encapsulation process which holds the potential for large scale manufacture (Fig. 6). In impinging aerosol technique, an aerosol of mixture of encapsulating material and probiotics is sprayed at the top of chamber into a fine mist of hardening solution sprayed from the bottom, resulting in an immediate gelation of the microdroplets, which can be collected at the base of chamber (Krasaekoopt, 2013). Due to this special spray technique, the average diameter of produced water insoluble cross-linked microbeads was normally less than that by traditional extrusion method (Sohail, Turner, Coombes, Bostrom, & Bhandari, 2011). However, this process also has some disadvantages including the loss of materials by adhesion to walls of the spray chamber and broad

particle size distribution due to aggregation of some microcapsules. Sohail, Turner, Prabawati, Coombes, and Bhandari (2012) used alginate and calcium chloride cross-linking solution to encapsulate *Lactobacillus rhamnosus* GG and *Lactobacillus acidophilus* NCFM by impinging aerosol technique and found that there is no loss in the viability of cells during process. Furthermore, Sohail, et al. (2011) further coated the above mentioned probiotic microbeads with chitosan and compared with the macrobeads prepared using traditional extrusion method. The results showed that the alginate mirobeads prepared by impinging aerosol technique was as effective as that of extrusion technique to protect probiotics in the presence of high acid and bile salts respectively.

3. Protective approaches and mechanisms to the survival of probiotic bacteria during storage

After processing, probiotic microcapsules need to further storage until it is consumed. In order to exert its health benefits, high viability of live probiotic bacteria is needed until the end of storage and then ingested by consumers. There are already many studies on stability of microencapsulated probiotic bacteria during storage, however, due to the different probiotic bacterial strains, storage conditions, coating materials and microencapsulation methods used, it is difficult to directly compare the bacterial survival data during storage among these studies. However, some common regularities still can be found. The storage temperature and water

activity, glass transition temperature, formula and structure of wall materials are the main factors that influence stability of microencapsulated bacteria during storage.

Storage at low temperature has always been proven effective in maintaining the survival of microencapsulated probiotic bacteria for long time (Albadran, et al., 2015; Boza, Barbin, & Scamparini, 2004; Heidebach, et al., 2010). For instance, Jalali, et al. (2012) reported that after encapsulating *Lactobacillus paracasei* subsp. *tolerance* (DSM 20258) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (DSM 20081) by freeze drying, a marked decline in the number of viable cells in capsules was observed in all samples stored at 23°C compared to those stored at 4°C. However, the effect of temperature to the viability of probiotic bacteria during storage is not always turned to be the lower the better. As reported by Kailasapathy and Sureeta (2004), the survival of *Lactobacillus acidophilus* CSCC 2409 and *Bifidobacterium infantis* CSCC 1912 encapsulated with whey proteins was lower than that encapsulated with alginate after six weeks of storage at -20 ℃, which could attribute to the denaturation of whey proteins during storage at freezing temperature and thus reducing its effectiveness as a protectant.

Water activity and moisture content are two important factors that affect the stability of encapsulated probiotic bacteria during storage. As water activity defines the free water in the product which is available for microbes' metabolic purpose, in terms of determining the viability of encapsulated probiotic bacteria during storage, water activity is more important than the moisture content (Roos & Roos, 1995). In general, food have an a_w level in the range of 0.1 for

very dry food to 0.99 for moist fresh foods (Vesterlund, Salminen, & Salminen, 2012). Probiotic bacteria stored at low water activity is normally turned out to be favorable to maintain the high viability of cells during storage. For instance, Kurtmann, Carlsen, Skibsted, and Risbo (2009) reported that water activity of 0.11 was capable of providing higher viability of freeze-dried *Lactobacillus acidophilus* (La-5) than a_w of 0.23 and 0.43 during 10 weeks of storage at 20 ℃. However, over drying of microcapsules also turned out to be harmful to the survival of probiotic bacteria during storage. As reported by Zayed and Roos (2004), the survival of freeze dried powder stored at water activity of 0.00 showed a higher death rate compared with that stored at water activity of 0.11 and 0.33. This high death rates caused by over drying may be related to the elimination of the three fraction of cellular water (free water, intermediate water and structural water) with the consequent damage of cellular protein (Mellor, 1978).

Phase state of probiotic microcapsule matrix also plays an important role to the storage stability of encapsulated probiotics. In glassy state, molecular mobility is extremely slow due to the high viscosity of the matrix, which can stabilize probiotic microcapsules by retarding molecular mobility and slowing down the reaction rate (Laine, Kylli, Heinonen, & Jouppila, 2008). Thus, the survival rate of encapsulated bacterial cells would be largely improved when stored in a matrix that is in its glassy state (Fig. 7). The ability of sugars to form glasses, which can hence raising the T_g of microcapsule matrix, is thought to be an important factor for the stabilization of probiotic bacteria during storage (Meng, et al., 2008). For example, Miller,

Anderson, and de Pablo (1998) reported that the protective capacity of sugars follows the order of glucose < sucrose < maltose < trehalose, which corresponding to the order of their T_gs (36, 67, 91 and 107℃, respectively). Meng, et al. (2008) reported that the survival of *Lactobacillus rhamnosus GG* microencapsulated in trehalose, lactose/trehalose and lactose/maltose using freeze drying were the highest, which can also attribute to their high T_gs. However, protection extent of sugar is also strain dependent. As A. Sofia Carvalho, et al. (2002) reported that compared with trehalose, sorbitol was the most effect protectant for *Lactobacillus plantarum*, even through the glass formation ability of trehalose is better than sorbitol. Other high molecular weight coating materials with different T_g values can also affect the stability of probiotic microcapsules. As reported by Semyonov, et al. (2012), the viability of *Lactobacillus paracasei LMG P-21380* coated by maltodextrin DE3 and trehalose is significantly higher than that of maltodextrin DE17 either at 4, 25 or 37℃, which can attribute to the higher T_g of maltodextrin DE3 that increase the gap between T_g of probiotic microcapsule and storage temperature and thus significantly reduced the rate of physical, chemical and biological changes. However, it showed be noted that some polymers may also thermodynamic incompatible and mixture of them would lead to the formation of phase separation structure within the microcapsule. For these microcapsules with phase separation structure, two or more T_gs can be detected. As long as one polymer in the mixtures is not in its stable glassy state (unstable rubbery state), it will have an adverse effect on the viability of cells during storage. As reported by Liu, et al. (2016a), there

are two glass transition regions displayed in sodium caseinate-pullulan (NCPU) particles which suggested the occurrence of a phase separation between NaCas and pullulan. The higher T_g value represents the glass transition of NaCas-rich phase, as it agrees well with those found in sodium caseinate only particles. Correspondingly, the lower T_g value represents the glass transition of a pullulan-rich phase as pullulan typically has a lower T_g value compared with that of sodium caseinate. When storage at a_ws of 0.54 and 0.76, even though the NaCas-rich phase in the NCPU particles was still in its glass state, the storage property of the probiotic particles was severely compromised. This result suggests that as long as one polymer in the mixtures is in its unstable rubbery state, it could accelerate the deterioration of storage properties, especially when the structural integrity of the matrix was affected by phase separation between the two polymers. The residual moisture content of the microcapsules can also affect the T_g of matrix. Due to the plasticization effect of water which would increase the molecular mobility of polymer chains, increasing in residual moisture content normally consists well with an decrease in T_g. Our data showed that with the RVP increased from 11% to 76%, the T_gs of NaCas microcapsule decreased from 105 ℃ to 60 ℃ corresponding to a huge lost in viability of microencapsulated probiotic bacteria during 8 weeks of storage (Liu, et al., 2017).

4. Protective approaches and mechanisms to the survival of probiotic bacteria during gastrointestinal digestion

As shown in Fig. 8, after microencapsulation process, there are mainly three kinds of microcapsule structure that used to protect probiotic bacteria: matrix, cross-linking and external coating structure. In matrix structure, the polymers constitute the coating wall by hydrogen bond, Van der Waal's force, hydrophobic force etc., which is the typical structure of products prepared by spray drying, ultrasonic vacuum spray drying, spray chilling, electrospinning, supercritical technology and direct freeze drying. For cross-linking structure, the polymer chains are normally cross linked by ions or enzymes, which is the typical structure of products prepared by indirect freeze drying (extrusion, emulsion, enzyme gelation and impinging aerosol technique). Microcapsules with these two structures also can be further enhanced by coating fat, enteric material or polymers with opposite charges on their surface through fluid bed drying or extrusion/emulsion method.

4.1 Matrix structure

In order to resist the gastrointestinal digestion, matrix structure formed by polysaccharide and proteins is commonly used. During gastrointestinal digestion, proteins act as buffering agents and polysaccharides provide a physical barrier which protects encapsulated cells from acid and bile condition. Milk proteins, such as skim milk and whey protein, are the most commonly used materials to encapsulate probiotic bacteria both during spray drying and freeze

drying. For example, Dimitrellou, et al. (2016) reported that without encapsulation, *Lactobacillus casei* lost viability of 1.46 and 4.03 log CFU/g at pH values 3 and 2, however, after encapsulating with skim milk using spray drying, reduction of only 0.36 and 1.63 log CFU/g were found at pH values 3 and 2, respectively. De Castro-Cislaghi, Silva, Fritzen-Freire, Lorenz, and Sant'Anna (2012) encapsulated *Bifidobacterium Bb-12* with whey protein by spray drying and found that after encapsulation, there was only 0.73 log CFU/g lost in viability during acidic condition (pH 2.0) for 3h, whereas in the free cells there was 1.51 log CFU/g lost. The protective effect of protein also can be further enhanced by denaturation. After denaturation, the compactly folded protein molecules are modified into an unfolded state, which allows protein-protein interactions, disulphide cross-linking and hydrogen-bonding. These interactions make the denatured protein stiffer, stronger and more stretchable. As a result, denatured protein own stronger and insoluble film forming properties which restrict the release and exposure of cells in simulated gastric environment. As reported by Rajam, et al. (2012), in both simulated acidic and bile conditions, denatured whey protein encapsulated *Lactobacillus plantarum (mtcc 5422)* showed better stability than undenatured whey protein. Other studies using gelatin, soluble starch and gum arabic to encapsulate probiotic bacteria were also reported and found effective protection function during gastrointestinal digestion (Desmond, et al., 2002; Lian, et al., 2003). Enteric materials, such as cellulose acetate phthalate (CAP) and shellac, can also be used. As these materials are insoluble at low pH condition and can dissolve quickly at intestinal pH

condition, they own the ability of inhibiting encapsulated probiotic bacteria exposing to stomach fluid and releasing into the intestinal (Buch, Penning, Wächtersbach, Maskos, & Langguth, 2009; Burgain, et al., 2011; Stummer, et al., 2010).

Apart from the buffering and physical barrier effects provided by protein and polysaccharide, interactions between protein and polysaccharide, such as hydrogen bonds, ware also reported to protect cells during gastrointestinal digestion. Liu, et al. (2016a) spray dried gum arabic/gum ghatti with sodium caseinate to encapsulate *Lactobacillus zeae LB1* and after exposing 2 h at pH 2.0, both significant higher survival rates were obtained compared with that of sodium caseinate alone. This result can be attributed to the carboxyl groups carried by gum arabic and gum ghatti molecules that forming strong hydrogen bonds with the amide and carboxyl groups of sodium caseinate molecules, which strengthened the structure of microcapsules and protected the cells from the harsh environment. Compared with gum ghatti containing microparticles, the survival of probiotic bacteria in gum arabic containing microparticles was slightly higher. This result could be attributed to the higher carboxyl groups in gum arabic compared to that in gum ghatti that leading to a stronger hydrogen bonding in sodium caseinate – gum arabic matrix compared with sodium caseinate gum ghatti matrix (Phillips, Williams, Phillips, & Williams, 2000).

4.2 Cross-linking structure

For microcapsules with cross-linking structure, physical obstructing formed by cross-linking of polymer chains is the main protection mechanism for cells during gastrointestinal digestion. According to the mechanism of cross-linking, their structures can be divided into two types: cross-linked by divalent cations (such as alginate, xanthan and carrageenan) and gelled by enzymes (such as caseinate, whey protein and gelatin).

Microcapsules formed by cross-linking of alginate chains is the most popular used method to encapsulate probiotic bacteria. In the presence of divalent cations (such as Ca^{2+}), alginate will form gel with “egg box” structure. Although probiotic bacteria can be well encapsulated into the alginate beads with high viability, the structure of the cross-linking polymers formed by divalent cations are turned out to be porous, which cause the easy entry and exit of H^{+} and other detrimental substances leading to the damage of cells. Besides, due to the susceptibility towards acidic condition of stomach, alginate beads prone to lost their chemical stability and crack during passing through gastrointestinal tract. In order to overcome these disadvantages, addition of bulking agents were usually added. Corn starch is commonly used as one of the bulking agents in alginate beads. With the addition of corn starch, pores between cross-linked alginate chains can be filled leading to the enhance in integrity and strength of beads structure and thus retarding the entrance of harmful substance. Sabikhi, Babu, Thompkinson, and Kapila (2010) reported that after adding native corn starch into alginate beads the survival of *Lactobacillus acidophilus LA1*

increased by approximately 3 log both in SGF with pHs at 1.0, 1.5 and 2.0 and in SIF with bile concentrations at 1.0, 1.5 and 2.0%. Besides, prebiotic resistant starch is also used as bulking agent. Compared with corn starch, the prebiotic effect of resistant starch which could metabolized by probiotic bacteria provides extra energy for probiotic bacteria to survive in the acidic conditions. However, excess addition of resistant starch was also proved to have an adverse effect on the survival rate of cells at pH 2.0 which leading to the disruption of alginate gel matrix and resulting in the rapid exposure of encapsulated probiotic bacteria into acidic conditions (And & Kailasapathy, 2005). The effect of alginate-starch beads to protect probiotic bacteria in gastrointestinal digestion was also strain dependent. As reported by Sultana, et al. (2000) that immobilization of *Lactobacillus acidophilus 2409* and *Bifidobacterium infantis 1912* does not enhance the bacterial survivals both in SGF with pHs at 2.0, 3.0 and 4.0 and in SIF with bile concentrations at 1.0, and 2.0%. Expect from alginate beads, Xanthan gum crosslinked by Ca^{2+} (Ding & Shah, 2009a), carrageenan crosslinked by K^{+} (Miles, et al., 1984) and NaCMC crosslinked by AL^{3+} (Chitprasert, et al., 2012) also showed the potential for improving the survival of probiotics during their passage through the gastrointestinal tract.

For enzyme induced gelation, caseinate and whey protein crosslinked by rennet and TGase are generally applied. The protein gelation matrix of microcapsules could attenuate the diffusion of H^{+} as well as create and maintain a relative higher local pH microenvironment during gastrointestinal digestion, which thus improves the stability of encapsulated probiotic bacteria.

Heidebach, et al. (2010) used rennet gelled casein as the matrix to encapsulated *Lactobacillus F19* and *Bifidobacterium Bb12* and found that after 90 min of digestion at pH 2.5, both two encapsulated probiotic bacteria showed an increase of 0.8 and 2.8 log CFU/g respectively in viability compared with that of the free cells (Heidebach, et al., 2009a). Later, even higher survival rates of these two probiotic bacteria exposed in SGF were obtained by encapsulating cells in TGase gelled caseinate, which could attributed to the covalent network built by TGase induced crosslinking compared to the non-covalently aggregated gels built by the rennet reaction, leading to capsules own higher resistance against low pH condition (Heidebach, et al., 2009b).

4.3 External coating structure

Apart from adding bulking agents into alginate beads, external coating using coating materials (such as chitosan, whey protein and poly-l-lysine) is another way to enhance the stability of microencapsulated probiotic bacteria during gastrointestinal digestion.

Chitosan is a natural, linear cationic polysaccharide. The positively charged carboxylic acid groups in chitosan can form a membrane on the surface of alginate beads through electrostatic interaction with negatively charged carboxylic acid groups in alginate, which inhibits the leakage of entrapped materials from the capsules and further provide a protective layer to resist harmful substances (Chávarri, et al., 2010; Lin, et al., 2008). For example, Koo, Cho, Huh, Baek, and PARK (2001) used chitosan coated alginate microcapsule to encapsulate *Lactobacillus casei YIT*

9018 and found that the survival rate is significantly higher than that of free cells at 22℃. The protection effect of low molecular chitosan is better than that of high molecular chitosan during gastrointestinal digestion, as it diffuses faster into the alginate matrix, leading to the formation of beads with higher density and strength (Krasaekoopt, Bhandari, & Deeth, 2006). For example, J. Lee, Cha, and Park (2004) reported that after exposing chitosan coated alginate beads to SGF for 180 min, the viability of cells in microcapsules coated with low molecular weight chitosan (3.18 log CFU/mL) is higher than that of high molecular weight (2.75 log CFU/mL).

Besides, poly-L-lysine and whey protein can also be used to coat alginate beads. Poly-L-lysine is a naturally occurring homo-polyamino acid and has the ability to react with the carboxyl of alginate (Ying Ma, et al., 2013). Cui, Goh, Kim, Choi, and Lee (2000) reported that the survival rates of *Bifidobacterium bifidum* encapsulated in poly- L -lysine coated alginate beads were significantly increased in SGF with pH 1.5 (around 10^8 CFU/g) compared with that of free cells. Whey protein can also provide a layer around the microcapsule which cannot be degraded in the stomach (Ding & Shah, 2009b; Gbassi, et al., 2009). As reported by Gbassi, et al. (2009), after encapsulating *Lactobacillus plantarum 229v, CIP A159 and 800* in alginate only beads, the survival of these cells exposed in SGF for 90 min were 2.19, 1.89 and 1.65 log CFU/g, however, with coating of whey protein, the survival rate of cells increased to 7.76, 6.67 and 5.81 log CFU/g, respectively.

External coating structure can also be provided by fluid bed drying with coating materials such as shellac and wax. Shellac is a natural anionic polymer and due to its acid resistant property, coating of shellac could provides distinct acid resistance to the capsules and thus improves the bacterial survival during gastrointestinal transit. Schell, et al. (2014) encapsulated *Lactobacillus reuteri DSM 20016* with sweet whey powder and dietary shellac by a two-step fluidized bed granulation and top spray coating technique and found that after exposing to gastrointestinal digestion, shellac coated cells indicated significant increase in survival rate (76.74%) compared with that of granulated only and free samples (25.00 and 17.74%, respectively). However, the solubility of shellac in intestinal fluid is very low which limits its application as an enteric coating material. To improve this shortage, a modified method with adding sodium alginate, hydroxypropyl methylcellulose and polyvinylpyrrolidone as additional water-soluble polymer and glycerol, glyceryl triacetate as plasticizers were applied (Stummer, et al., 2010). Semyonov, et al. (2012) also developed a microencapsulation process using wax and ethylcellulose to coat *Lactobacillus paracasei* loaded trehalose-meltodextrin probiotic microcapsules. The wax layer served as a precoating, which can provide protection against moisture penetration during storage, and ethylcellulose, a food-grade enteric coating, was chosen to provide enhanced protective effect during pass through the gastrointestinal tract. The results showed that after exposure to acid condition with pH

2.0 for 1 h, the survival rate of coated cell coated with wax and ethylcellulose was 2000-fold higher than that of free cells.

5.Concluding remarks

Microencapsulation is a useful tool to improve the survival of probiotic bacteria during processing, storage and gastrointestinal digestion. Different mechanisms, such as cross-linking, hydrogen bonding, electrostatic interaction etc., can be used to form microcapsules and protect encapsulated probiotic bacteria from harsh environment. The coating materials used nowadays are mainly polysaccharides and proteins, however, only small range of species were tested. For different protective purposes and application areas, different coating materials and interaction mechanisms within polymer matrix should be chosen. Further researches should thus be carried out to find more appropriate food grade coating materials. Besides, only few *in vivo* studies can be found that tested the real gastrointestinal digestion property of probiotic microcapsules. Although the results showed promising perspectives, it is still far from enough to ensure the obtainment of claimed protective and targeted release effects in humans or animals. Finally, creation of new designed probiotic microcapsules based on novel coating materials and microencapsulation technologies, as well as the use of clinical trials to examine efficacy in human or animal models are still scarce and deserve further in depth researches.

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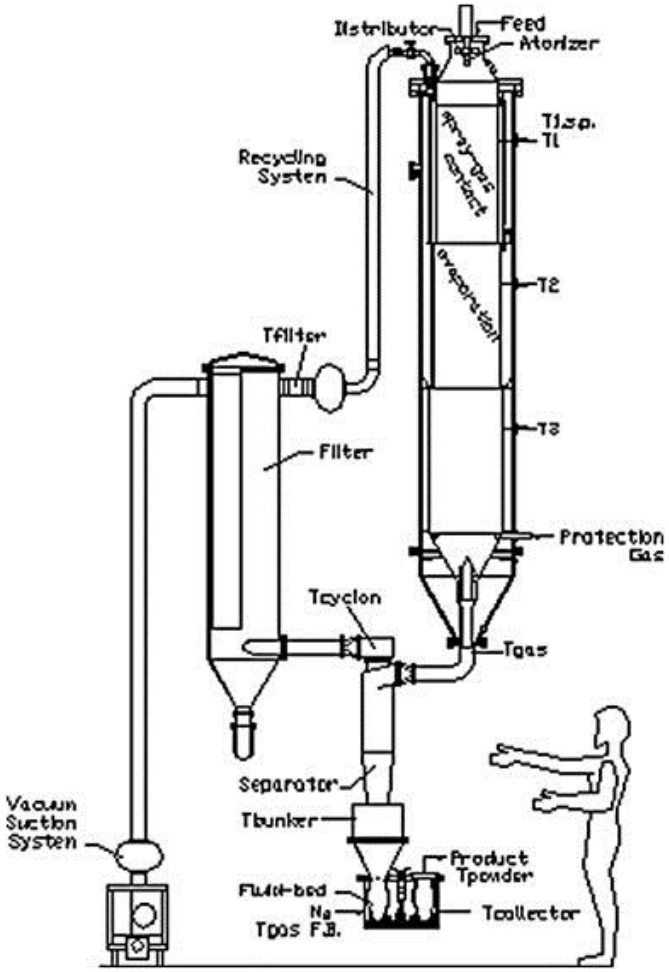

Fig. 1 Schematic representation of ultrasonic vacuum spray dryer. (Semyonov, Ramon, & Shimoni, 2011)

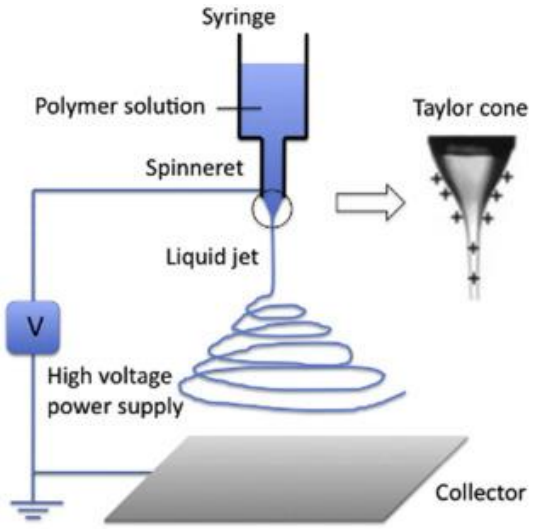

Fig. 2 Schematic representation of electrospinning. (Ghorani and Tucker 2015)

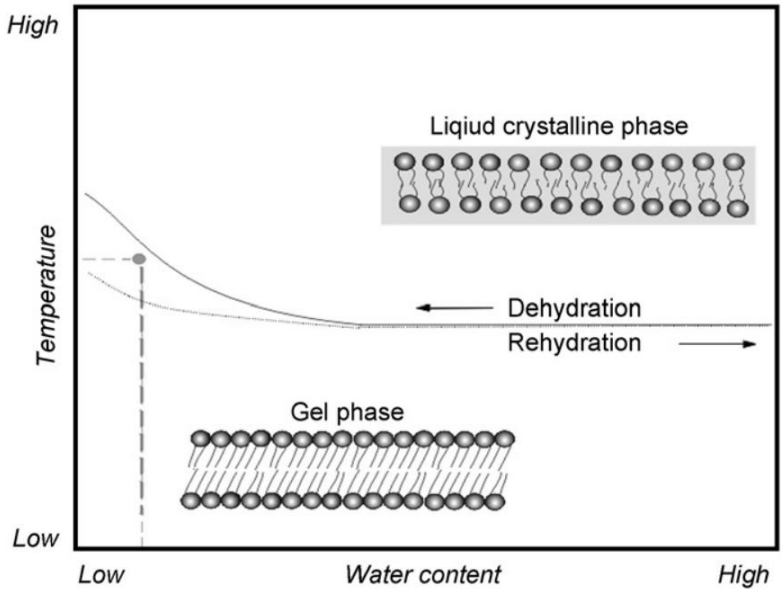

Fig. 3. A simplified phase diagram of a phospholipid associated with drying and rehydration processes. The solid line shows a liquid crystalline – gel phase transition. The dotted line shows the depression of membrane phase transition (T_m) when cells are dried in the presence of a sugar. During drying at a given temperature until cells reach a relatively low moisture content (closed circle), cell membrane normally transforms to gel phase. However, at this same point, cell membrane may remain in liquid phase in the presence of a sugar. (Santivarangkna, Higl et al. 2008)

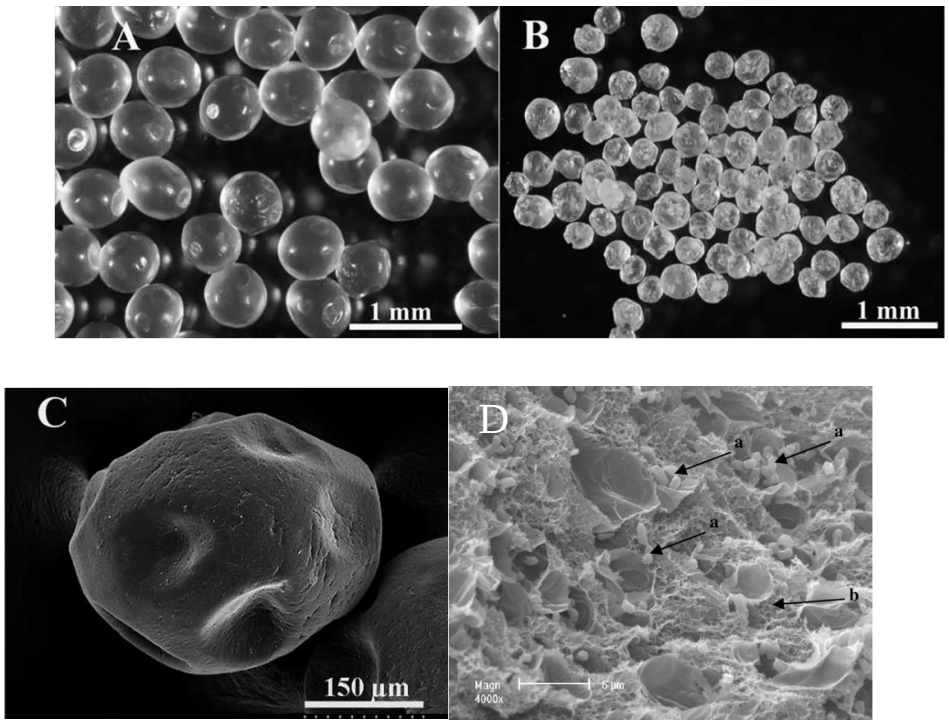

Fig. 4. Morphology and cross-section structure of alginate beads. Optical micrographs of alginate beads before (A) and after (B) drying. Scanning electron micrograph of external surface (C) and cross-section (D) structure of dried beads. Black arrows in (D): a means probiotic bacteria, b means alginate gel matrix. (Ma, Pacan et al. 2008, Gbassi, Vandamme et al. 2009)

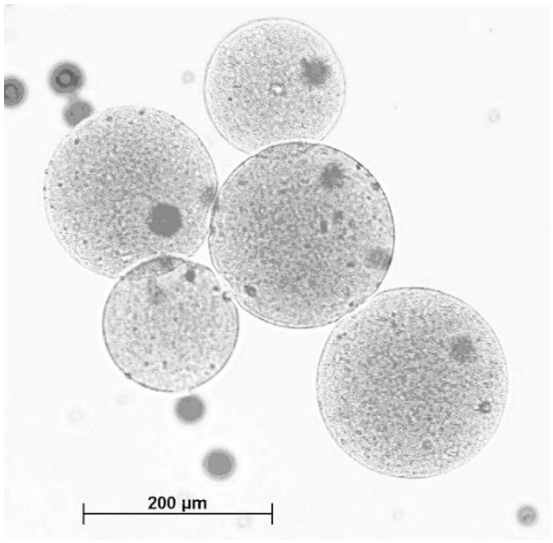

Fig. 5. Image of probiotic-containing casein microcapsules. The capsules are of spherical shape with a measured volume-base median diameter ($d_{50,3}$) of 165±23µm. (Heidebach, Först et al. 2009)

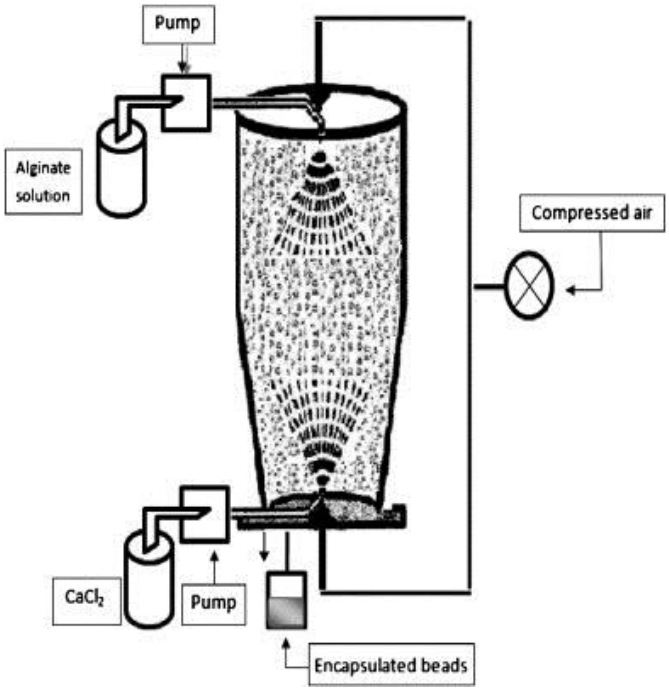

Fig. 6 Schematic representation of impinging aerosol technique. (Sohail, Turner, Coombes, Bostrom, & Bhandari, 2011)

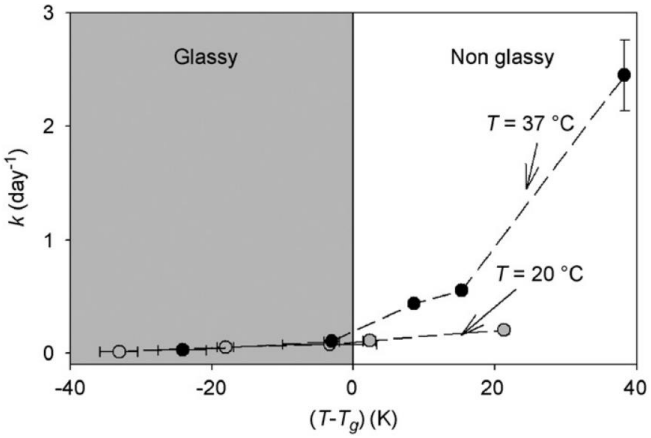

Fig. 7. The rate constants of cell inactivation (k) at different phase states of matrix. (Higl, Kurtmann et al. 2007)

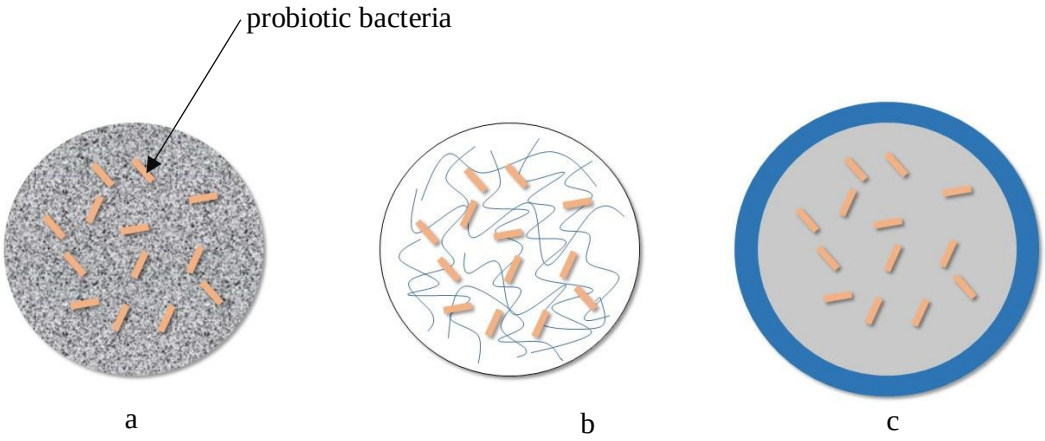

Fig. 8. Schematic representation of microcapsule structures: (a) matrix structure, (b) cross-linking structure, (c) external coating structure.