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MICROENCAPSULATION OF PROBIOTIC LACTIC ACID BACTERIUM *LACTIPLANTIBACILLUS PLANTARUM* 299v STRAIN AND APPLICATION POTENTIAL

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Abbreviations

B.	*Bifidobacterium*
CFU	Colony-forming unit
DWP	Denatured whey protein
FAO	Food and Agricultural Organization
L.	*Lactobacillus*
Lp.	*Lactiplantibacillus*
Log CFU	CFU expressed in logarithmic (base 10) unit
MD	Maltodextrin
MRPs	Maillard reaction products
MRS	Man, Rogosa, and Sharpe media
PBS	Phosphate buffer saline
RS	Resistant starch
SEM	Scanning electron microscopy
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
WHO	World Health Organization
WP	Whey protein

1. INTRODUCTION AND OBJECTIVES

Probiotics are living microorganisms that have health benefits on the host when they are administrated to a sufficient amount (FAO/WHO, 2001). They have numerous beneficial characteristics including irritable bowel syndrome controlling, endogenous or exogenous pathogens suppression, lactose tolerance improvement, colon cancer risk reduction, body weight regulation, constipation improvement, tooth decay prevention, etc. (Chen et al., 2016; Mao et al., 2018). *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) is lactic acid bacteria, which consists of various species and is recognized as potential probiotic bacteria (Eckert et al., 2017). However, in order to exert the functionality of the probiotics, they must be able to survive and multiply in the host. In the digestion system, probiotics will pass through the mouth and stomach tract and colonize in the intestine (Mao et al., 2018). Thus, during this process, there are many harsh factors such as oxygen, heat, and hydrogen peroxides that can sharply affect the viability of the probiotics (Ahmad et al., 2019; Dias et al., 2018; Mao et al., 2018; Terpou et al., 2019). Therefore, it is a great challenge to meet the generally recommended number of probiotics of more than 6 log (CFU/g) to gain their health benefits. In the last few decades, to respond this great challenge, intensive research, and developments such as the selection of new strains, improvement of oxygen, acid, heat tolerance, production of some techno-functional metabolites, encapsulation, etc. are carried out worldwide. Among these developments, microencapsulation of probiotics was proved to be one of the best directions.

Microencapsulation is a talented technology that can protect probiotics by coating them with wall materials to maintain their viability and functionality during the manufacturing, storage, and digestion process (Alfaro-Galarza et al., 2020; Arslan-Tontul & Erbas, 2017; Dimitrellou et al., 2016; Karrar et al., 2021; Kavitake et al., 2018; Liu et al., 2016; Rajam & Anandharamakrishnan, 2015; Suryabhan et al., 2019; Vanden Braber et al., 2020). Generally, spray-drying and lyophilization (or freeze-drying) are the mainly applied technologies, accomplished with other methods such as spray-chilling (Arslan-Tontul & Erbas, 2017), emulsion (Goyal et al., 2015), extrusion, etc. However, the heating process (Liao et al., 2017; Rajam & Anandharamakrishnan, 2015) in spray-drying makes it not always suitable for coating probiotics, especially for heat intolerance ones. Lyophilization (Li et al., 2019; Otero et al., 2007; Zhang et al., 2020) that sublimates the water directly from ice phase under vacuum conditions and low temperatures (10 °C-20 °C) may provide a very good alternative solution for associating the encapsulation of probiotics. Another important part of encapsulation should be the coating materials (Kavitake et al., 2018), which can be polysaccharides, proteins, lipids, and other materials. Polysaccharides such as maltodextrin (Reyes et al., 2018; Ribeiro et al., 2020) and resistant starch (Cheow et al.,

2016; Reyes et al., 2018) are naturally produced and GRAS (Generally Regarded as Safe) products and have been used as food additives in the food industry for a long time. Maltodextrin is a product produced by starch hydrolysis with high molecular weight, which has a good film-forming ability. Resistant starch is a small branch of starch, which has the properties to resist the hydrolysis by α-amylase and pullulanase in the upper gastrointestinal tracts (including the mouth, pharynx, esophagus, stomach, and duodenum) but it can be fermented by probiotics in the colon (Reyes et al., 2018). Proteins such as whey protein and denatured whey protein are widely used due to their excellent physical and chemical properties. Whey proteins (WP) are considered as an exceptional coating material due to their specific physical and chemical properties such as excellent emulsification, superb gelation, and exquisite fill-forming properties. Denatured whey proteins (DWP) are originated from WP, by managing with acid or heat as a denaturation approach, which can contribute to the specific properties of whey proteins, e.g., high tensile property, low oxygen permeability (Goyal et al., 2015; Moayyedi et al., 2018; Rajam & Anandharamakrishnan, 2015; Ying et al., 2013). Moreover, whey proteins from cheese making in the dairy industry are a main pollutant in wastewater. However, whey proteins have a high nutritional value because they are good sources of many valuable biological proteins, riboflavin, and minerals (Eckert et al., 2017). Thus, utilization of whey proteins in the production of foods especially in coating probiotics will absolutely “kill two birds with one stone”, because it helps to eliminate environmental issues as well as improve the nutritional values of foods, simultaneously (Rama et al., 2019). The polysaccharide-protein binary complexes can be made through the Maillard reaction and get Maillard reaction products (MRPs) (Fu et al., 2021; Liu et al., 2016, 2017; Mao et al., 2018). Several advantages of MRPs have been reported such as they have excellent antioxidant and emulsifying characteristics (Huang et al., 2020; Mao et al., 2018). MRPs show great potential as the delivery carrier for bioactive substances (Huang et al., 2020). In addition, they also perform prebiotic functionality because they are resistant to digestion compared to the non-glycated proteins, which means more dietary glycoconjugates are available for endogenous microbiota utilization in the distal colon (Mao et al., 2018).

Nowadays, the awareness of consumers of functional foods such as probiotics, prebiotics, synbiotics, etc., raises significantly, because of health benefits as well as expanding assortments. Unfortunately, probiotic dairy products cannot be consumed by a relatively large group who are lactose intolerants and/or have allergies to milk proteins. In this case, plant-based food matrices such as fruit juice, jams, vegetable juice, etc. can serve as a very good alternatives for carrying probiotics (Rodríguez et al., 2021). Fruits are known as fresh, nutritious, health-improving, as well as disease-avoiding foods, owing to their nutritional and functional properties. Although, these

foods are seasonal-specific in nature, modern food technologies make them accessible in plentiful forms for consumers at any time throughout the year (Chakkaravarthi & Aravind, 2021). Among those fruit products, fruit juice is one of the most favoured forms of consumption by consumers (Di Cagno et al., 2016; Dias et al., 2018; Yang et al., 2018), however, during the process fruit juice may lose on nutritional value mainly bioactive compounds. In addition, these juices also contain a sufficient amounts of sugars including glucose, fructose, sucrose (Ruiz Rodríguez et al., 2021), which may lead to over intake of carbohydrates. Reducing the sugar content in these fruit juices may assist in the elimination of the related diseases such as diabetes and obesity, which are highly correlated to excessive consumption of carbohydrates (Ishii et al., 2017). To compensate the loss of nutritional compounds and facilitate the processed fruit juices still as functional food, probiotics can be fortified into it to fulfil the function (Chakkaravarthi & Aravind, 2021). Unlike the traditional physical reduction technology of the sugar content in many soft drinks, coffee drinks, etc., the use of lactic acid bacteria to reduce the sugars in juices is an attractive method, because it does not only affect the limited taste of the juice, but it also increases the nutritional value by the production of many health beneficial intermediates such as vitamins, short chain fatty acids, etc. (Ishii et al., 2017). The main advantage of this concept is multipurpose that can be realized in one food product, and these may require new types of probiotic preparation, thus topics of my Ph.D. were based on.

Objectives

The main goal of my Ph.D. research is the development and characterization of encapsulated probiotics preparation as well as for application in the production of probiotic apple juice.

The specific objectives were the following:

- **Formulation of probiotic microcapsules**
  - Effect of coating materials
    - polysaccharides (maltodextrin, resistant starch)
    - proteins (whey protein, denatured whey protein)
    - Maillard reaction products of maltodextrin and whey protein (MRPs)
  - Effect of ratios of core-to-wall and the different wall materials
- **Characterization of probiotic microcapsules**
    - cell number and bulk density
    - morphology
    - viability change during long-term storage at different temperatures
    - tolerance to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)
- **Application of microcapsules in production of probiotic apple juice**
    - effect of production method: fermentation and fortification
    - pH changes during long-term storage at different temperatures
    - viability change during long-term storage at different temperatures

2. LITERATURE REVIEW

2.1 Probiotics

2.1.1 Definition of probiotics

The modern history of probiotics starts from the 1900s when groundbreaking work was carried out by Russian scientist Elie Metchnikoff at the Pasteur Institute in Paris. Meanwhile, Louis Pasteur identified the responsibility of the microorganisms during the fermentation process, whereas Metchnikoff first one who figured out the potential effects of microbes on human health. In 1953, the term probiotic was introduced by German scientist Werner Kollath to appoint "active substances that are essential for a healthy development of life" (Olson & Aryana, 2022). In fact, "probiotic" word was originated from the Latin language *pro* and the Greek language *βιοσ* words and it is literally meaning for "the life". This definition is a relatively new concept for bacteria that are associated with beneficial effects for humans and animals. Later, with the fast development of science and further scientific research, World Health Organization (WHO) and Food Agricultural Organization (FAO) had defined probiotics as "live microorganisms, when administrated in sufficient amounts, will have a beneficial effect on the health of the host" (FAO/WHO, 2001). This amount varies from one area to another, but basically, it is generally accepted that the limit dose of the probiotic's product is 10^6 CFU/g or 10^7 CFU/mL (Mao et al., 2018).

2.1.2 Types of probiotics

Microorganisms used as probiotics are mainly *Lactobacilli* (*Lactobacillus acidophilus, Lacticaseibacillus casei, Lactiplantibacillus plantarum, Limosilactobacillus reuteri, Lacticaseibacillus rhamnosus, Ligilactobacillus salivarius*); *Bifidobacteria* (*Bifidobacterium breve, B. longum, B. lactis*), *Bacillus* (*B. subtilis, B. cereus var. toyoi*), and *Enterococcus* (*E. faecium*), among others. Among these, the strains of *Lactobacillus* and *Bifidobacterium* are commonly used (Dimitrellou et al., 2016; Li et al., 2019; Mao et al., 2018). The main reasons are these two species are considered generally recognized as safe (GRAS) and are the most dominant microorganisms in the human intestinal microbiota (Rajam & Subramanian, 2022). They have been applied in various types of foods such as dairy products, plant-based foods, beverages, sausages, etc., because they not only improve the quality, flavour, and taste of fermented foods, but they also have a positive function in improving human health conditions. Additionally, probiotics produce various beneficial compounds, including antimicrobials, lactic acid, hydrogen peroxide, and a variety of bacteriocins. Moreover, probiotics have the ability to interact with the host microflora and compete with microbial pathogens, bacterial, viral, and fungal (Chen et al., 2016; B. Yue et al., 2020).

2.1.2.1 The genus of *Lactobacillus*

Genus *Lactobacillus* takes the largest domain of lactic acid bacteria (LAB). However, the term 'lactic acid bacteria' does not demonstrate a phyletic class, but rather the metabolic capabilities of this heterogeneous bacterial group, the most significant of which is the capacity to ferment sugars primarily into lactic acid (Frakolaki et al., 2021). Apart from lactic acid, LAB metabolism generates various substances such as diacetyl, acetoin, and 2-3 butanediol from citrate, and a variety of bioactive peptides and volatile compounds from the catabolism of proteins and amino acids (Mayo et al., 2010). The major LAB used in food industries is *Lactobacillus* species, *Lactococcus* species, and *Streptococcus* species. They have been used for food fermentation and are able to serve a function by acting as potential health benefits providers since ancient times. They are GRAS and are able to produce lactic acid, butyric acid, a substance that has been demonstrated that has a positive effect on health in different aspects (Xiao et al., 2021). *Lactobacillus* species are the fundamental component of human and animal microbiota, such as the digestive and female genital systems. *Lactobacillus* species are Gram-positive, facultatively anaerobic, rod-shaped microorganisms. The optimum temperature and pH for their growth are in the ranges from 35 °C to 40 °C and from pH 4.5 to pH 6.4, respectively (Chen et al., 2016; Rama et al., 2019). They are able to utilize different types of sugars in the fermentation process and produce lactic acid as the primary metabolite. Based on the specificity of metabolic pathways, they can be classified into different groups such as obligate homofermentative (*L. acidophilus*, *L. gasseri,* etc.), obligate heterofermentative (*Ll. fermentum*, *Ll. reuteri*, etc.), and facultative heterofermentative (*Lp. plantarum*, *Lc. casei*, etc.) (Vinderola et al., 2019). The former group of species mainly produces lactic acid while the last two groups of species are able to accumulate various fermented end-products, such as lactic, acetic, formic acids, ethanol and carbon dioxide (Claesson et al., 2007; Pot et al., 1994). Among these *Lactobacillus* species, *Lp. plantarum* is a widely distributed and versatile lactic acid bacterium and has been widely used for the development of therapeutic and functional food. It represents part of the microbiota of many foods and feeds, including dairy, meat, fish, vegetable fermented products (e.g., must, sauerkraut, pickled vegetables, sourdoughs), and silage (Costa et al., 2019; Palomino et al., 2015; Ruiz et al., 2021; Song et al., 2016); it is also a natural inhabitant of the human and animal mucosa (oral cavity, gastrointestinal tract, vagina, etc.).

2.1.2.2 The genus of *Bifidobacterium*

Species belonging to the *Bifidobacterium* genus are also Gram-positive, obligatory anaerobic, and branched rod-shaped bacterium. The optimum temperature for growth is 37 °C to 42 °C, and the

optimum pH value is pH 6.5 to pH 7.0 (Chen et al., 2016; Morales & Ruiz, 2016). They normally appear with the characteristic “bifid”, with branches or Y/V-shaped rods. Although *Bifidobacterium* can produce lactic acid simultaneously with other metabolites such as acetic acid with equal or even more amounts (3:2), they will not produce carbon dioxide, propionic acid, or butyric acid during fermentation (Rajam & Subramanian, 2022). This happened though the fructose-6-phosphate phosphoketolase pathway differed from the homofermentative or heterofermentative that has been found in the case of lactic acid bacteria.

2.1.3 Functionality of probiotics

Different scientific research (Chen et al., 2016; Mack et al., 2021; Rama et al., 2019) have shown high potential health advantages or physiological benefits to the host of probiotics by producing specific enzymes, generating antibacterial metabolites, gaining adhesion and colonization ability, and consisting anti-mutagen properties. The specific performances are providing nutritional function, regulating intestinal microbiota, weakening irritable bowel syndrome, reducing lactose intolerance illness, decreasing cholesterol levels, improving oral and dental health, relieving constipation, regulating body weight, and facilitating immunity.

2.1.3.1 Nutrition

Probiotics are good resources that can produce essential nutritional compounds during life activity (Reyes et al., 2018), which is significant to the host in some cases, especially some nutritional compounds that the host cannot synthesize and must obtain exogenously. Some probiotics such as members of *Bifidobacterium* and *Lactobacillus* genera can synthesize some vitamins especially vitamin B-s and vitamin K-s. The *Bifidobacterium* species are reported to be able to synthesize some essential amino acids such as valine and threonine. Threonine is an essential amino acid for animals and the limiting amino acid in swine and poultry diets, which plays a vital role in the modulation of nutritional metabolism, macromolecular biosynthesis, and gut homeostasis (Tang et al., 2021). Additionally, threonine is not only an important component of gastrointestinal mucin, but also acts as a nutritional modulator that influences the intestinal immune system via complex signaling networks, particularly mitogen-activated protein kinase and the target of the rapamycin signal pathway (Tang et al., 2021). Probiotics can synthesize both fundamental and specialized enzymes that can promote the decomposition and absorption of food (Chen et al., 2016; Eckert et al., 2017).

2.1.3.2 Regulation of intestinal microbiota

Probiotics can regulate the intestinal microbiota either by direct or indirect methods. On the one hand, in a direct way, the intake of probiotics can change the content or the inherent structure of the intestinal microbiota by inhibiting of the growth/activities of pathogen bacteria as well as promoting beneficial bacteria (Rama et al., 2019). The mechanism of direct regulation is that probiotics can secrete antibacterial substances by competing for nutrients and intestinal epithelial adhesion sites, inhibiting, or directly eliminating intestinal pathogenic microorganisms. According to Sgouras and co-workers (2004), the intake of *Bifidobacterium* BB-12 strain can significantly increase the cell number of *Bifidobacteria* and *Lactobacillus* in the intestinal tract, and significantly reduce the number of some potential pathogenic bacteria. On the other hand, probiotics can affect the gut microbiota and the metabolic profile of the host, thereby promoting the regulation of colonic cell proliferation and clearance of harmful substances in the intestinal tract (Vitali et al., 2012). This involves various antibacterial mechanisms and substances. Probiotics such as *Bacillus, Lactobacillus* can produce short-chain fatty acids including acetic acid and propionic acid to lower the pH of the intestine to inhibit the growth of pathogenic microorganisms and promote the growth of favourable microorganisms avoid gastrointestinal and extraintestinal disorders (Chen et al., 2016; Li et al., 2019). Besides, bacteriocins can damage the formation of the cytoplasmic membranes, and microcins can disturb the synthetic pathways of macromolecular resulting in the clearance of pathogenic bacteria in the intestinal tract (Yue et al., 2020).

2.1.3.3 Decrease in the risk of irritable bowel syndrome

Irritable bowel syndrome (IBS) is a sort of intestinal dysfunction disease, characterized by symptoms with persistent or intermittent abdominal pain or discomfort correlated to uneasy defecation and often accompanied by bloating and/or distension (Quigley, 2016). However, the underlying pathogenesis of IBS is not completely understood (Enck et al., 2016), therefore, the treatment should concentrate on reducing the ramification of the symptoms of IBS. This group (Enck et al., 2016) also showed that the change of gastrointestinal microbiota due to acute gastrointestinal infections or the abuse of antibiotics caused the increase of incidences to have a correlation with IBS, thus the therapy focused on modulating the gastrointestinal microbiota by using probiotics is a promising and reasonable treatment of IBS (Mack et al., 2021). According to Niu and Xiao (2020), the meta-analysis of 35 randomized controlled trials led to conclude that probiotics are effective against IBS, with regard to improvement in overall symptoms as well as abdominal pain, bloating, and flatulence. Additionally, evidence of the effectiveness of the use of

combinations of probiotics in the treatment of IBS was found. Niu and Xiao (2020) also confirmed that multi-strain probiotics have a better possibility to weaken IBS symptoms than a single strain.

2.1.3.4 Reduce lactose intolerance illness

The prevalence of lactose intolerance is quite high in the world, especially in Asian and African countries. Basically, 57% to 65% of the population is facing this clinical syndrome (Catanzaro et al., 2021) that is due to the inability to digest lactose caused by the loss or decreasing the activity of the lactose-phlorizin hydrolase (β-galactosidase) enzyme in the intestine. This change results in the increase of the osmotic pressure of the small intestine and the lactose is fermented by microbiota in the colon. The lactose will reach the colon without absorption in the small intestine, and there it will be fermented by bacteria releasing short-chain fatty acids (SCFAs) and hydrogen gas. As a result, it may cause abdominal pain, flatulence, diarrhoea, and sometimes nausea and vomiting (Zuppa et al., 2016; Catanzaro et al., 2021). Hence, except to use the traditional method to avoid the consumption of lactose-containing foods as a therapeutic option, intaking live probiotics is another choice. Some probiotics such as *Lactobacillus spp.* and *Bifidobacterium spp.* can increase the production of β-galactosidase, thus it is rational to use probiotics in the treatment of lactose intolerance illness (Zuppa et al., 2016).

2.1.3.5 Decrease in the risk of colorectal cancer

Colorectal cancer (CRC) is one of the most lethal cancers of humans in the world. The mortality of it takes third place after lung cancer in males and breast cancer in females (Rafter, 2003; Rahbar Saadat et al., 2020; Y. Yue et al., 2020). Scientific research has proved that CRC was caused by genetic and environmental factors jointly, which include aging, gender, inflammatory bowel diseases, diet, smoking, and frequency of physical activity (Saadat et al., 2020). Since the diet has been proven to contribute to the risk of colorectal cancer (Rafter, 2003), thus, a diet containing probiotics is a good combination to reduce CRC risks. In mechanism, when probiotics colonize the human intestine, they can promote the growth of themselves and the other beneficial bacteria, inhibit the growth of pathogenic bacteria, and speed up the peristalsis of the intestine. It not only prevents the production of carcinogens but also significantly reduces the time of carcinogens in the intestine. Based on the research of Saadat and co-workers (2020), extracellular polymers (EPSs) of *Kluyveromyces marxianus* and *Pichia kudriavzevii* have a promising application in the initiation, progression, metastasis, and chemical resistance of colorectal cancer signalling pathways as targeted fundamental components and they have none or less influence on normal cells. Another research carried out by Yue and co-workers (2020) revealed that *Lp. plantarum* YYC-3 strain has

a high possibility in the field of prevention CRC for those who are high-risk individuals.

2.1.3.6 Role in the control of cholesterol level

Cholesterol is a vital compound related to life phenomena, which is not only involved in the formation of the cell wall but also works as the original material in the synthesis of bile salts, vitamin D, and hormones steroids (Chen et al., 2016). However, high cholesterol in the blood increases the risk of heart disease, which may form clogged arteries. Besides, the correlation between cholesterol content and blood pressure is high, and the application of LAB is promising to reduce the risk of both factors (Jitpakdee et al., 2021). Studies have shown the characteristics of lowering the cholesterol level by cholesterol dehydrogenase/isomerase produced by LAB. The *Lp. plantarum* 49 strain and *Lp. plantarum* 201 strain reduced total cholesterol levels in rats (da Costa et al., 2019). Sui and co-workers (2021) isolated five *Lactiplantibacillus plantarum* strains from tangerine vinegar, and *in vitro* evaluated for cholesterol-lowering properties. They found that strain NF4 showed the highest cholesterol-reducing rate 55.8% (Sui et al., 2021).

2.1.3.7 Improvement of oral and dental health

Oral pathogens are one of the most common bacterial infections in humans (Natanzi et al., 2020). They can affect oral and dental health by forming biofilm in the oral cavity (Messora et al., 2021). Although, keeping good hygiene of the oral and teeth together with chemical methods as a complementary method can effectively maintain the health of the oral cavity. However, they cannot prevent the recolonization of the oral pathogenic bacteria, thus, enduring using chemical compounds may have more disadvantages than advantages (Messora et al., 2021). All these reasons make the development of more healthy and less aggressive protecting agents for oral and dental health become a priority. Probiotics have been reported that they have antimicrobial and immune response modulation abilities together with the ability to adhere to epithelial cells to inhibit the binding and growth of pathogenic bacteria, which make them a promising therapeutic method in the prevention of oral and dental infectious illness (Karbalaei et al., 2021; Messora et al., 2021). Natanzi and co-workers (2020) indicated that oral and dental health can be improved by probiotics, and the survival of HGECs (Human Gingival Epithelial Cells) can be improved by *Lp. plantarum* and *Lig. salivarius* in the presence of *Streptococcus mutans* pathogen. Renye and Steinberg (2021) reported that thermophilin 110 is a kind of bacteriocin naturally produced by *Streptococcus thermophilus* B59671 strain and it can inhibit the growth of the oral pathogen *Streptococcus* UA159 mutans. Meanwhile, health-associated commensal streptococci were inhibited by thermophilin 110 at high level, whereas it will not lead to significant oral dysbiosis.

Furthermore, concentrated thermophilin 110 has the possibility to inhibit the growth of oral streptococci to prevent the formation of caries as an antimicrobial compound.

2.1.3.8 Relieve constipation

Constipation is a universal troublesome gastrointestinal disease whose etiology and pathophysiology still stay poorly comprehended (Lu et al., 2021; Zhao & Yu, 2016), typified by a series of symptoms, including difficulty in bowel movements, infrequent, or incomplete defecation, defecation with dry and hard stools, as well as bloating and abdominal pain (Araújo et al., 2021). However, an increasing amount of evidence illustrated that the microbiota of the gastrointestinal can alleviate constipation and constipation-related symptoms (Botelho et al., 2020; Zhao & Yu, 2016). Some microorganisms such as *Lactobacillus* and *Bifidobacterium* species have been validated/proven to relieve constipation. These microorganisms do not only act on the integrity of membranes in the gastrointestinal tract, but also can modify the gastrointestinal microbiota, which does benefit in the increasing production of short-chain fatty acids, and as a result, decreasing the pH in the colon, promoting intestinal peristalsis (Araújo et al., 2021; Lu et al., 2021). Botelho and co-workers (2020) carried out a randomized, double-blind, placebo-controlled clinical trial on constipation for 30 days with 35 individuals, who were randomly separated into two groups: the control capsule (CC) and the probiotic capsule (PC) groups. The results indicated that the bacteria are generally increased in patients with constipation and can be reduced by the modulated gut microbiota through capsule form multispecies probiotics. As a result, it will benefit the individual.

2.1.3.9 Regulation of body weight

Obesity has been an expanding epidemic since the last century and may cause serious health problems, including cardiovascular diseases, diabetes Type-II, and numerous types of cancer. Further, the giant cost of economic and social resources for obesity and related comorbidities has already threatened the safety of the world healthcare system. As the cause of obesity, except for the reason that WHO mentioned, i.e., the imbalance of the energy between intake and expend, genetic, neural, and endocrine aspects have also been proved as factors causing obesity. Evidence illustrates that the bacteria in the gastrointestinal tract, normally referred to as gut microbiota, can affect the absorption of nutrition and consumption of energy. However, it is different when compared to an obese person with a lean individual. Hence, this information may be used as a therapy by modulating the gut microbiota, through a diet with probiotics, to work as a treatment for obesity (Rouxinol-Dias et al., 2016). In fact, myriad research has been done on the effect of specific probiotics on the organisms and the significant contribution to the treatment of obesity.

The most frequently used species are *Lactobacillus spp.*, *Bifidobacterium spp.*, and *Enterococcus spp.* (Raoult, 2009). Rouxinol-Dias and co-workers (2016) reported that the effect of the probiotic on body weight is species and strain specific. In their investigation, meanwhile *L. gasseri* BNR17 reduces weight gain compared to controls, whereas *L. gasseri* L66-5 promoted weight gain, and *L. rhamnosus* GGMCC is the only one that had a positive effect on weight loss in humans. Hence, the probiotics diet can be used as a treatment for obesity, and both the species and strain are being considered. In other words, probiotic diet products should refer to strain to ensure the effectiveness of the treatment of obesity.

2.1.3.10 Facilitation of immunity

The mechanism of facilitation or enhancement of the immunity of the host is the influence of probiotics on the composition and functions of gut epithelial and immune cells (Ashaolu, 2020). It is well known that probiotics can enhance the immunity of the host by protecting against pathogens in the gastrointestinal tract. The mechanisms typically include the production of antimicrobial compounds, competitive prohibition for adhesion points and nutritional sources, development of the function of the gastrointestinal barrier, and immunomodulation (Ashaolu, 2020). Antimicrobial substances such as organic acids, bacteriocins, etc., can inhibit the growth of $Gram^+$ and $Gram^-$ bacteria by pore formation or cell wall synthesis inhibition (Hassan et al., 2012), together with inhibition of the transport of nutrients (De Keersmaecker et al., 2006) to targeted destroy the pathogenic cells. The competitive exclusion mechanism contains adhesion points and nutritional sources between a probiotic bacterium and a pathogenic microorganism. Probiotics can achieve this target by secreting antimicrobials, disrupting of the receptor of pathogens by probiotics enzymes, production of receptor analogues, and secreting biosurfactants to inhibit the growth of pathogens (Oelschlaeger, 2010; Wan et al., 2019). Additionally, Wan and co-workers in 2019 demonstrated that probiotics can maintain the intestinal barrier integrity intact due to the increased gene expression linked to tight junction signalling.

2.1.4 Factors affect the viability of probiotics

Probiotics are living microorganisms and can exert their functionality when they are alive. Viability is the crucial indicator to ensure the effectiveness of the functionality of probiotics, which is also the most important indicator in the selection of the probiotic product whether it is qualified or not. However, during the process of manufacturing, transport, storage, and digestion, probiotics are always affected by risk factors, e.g., oxygen, heat during processing, transport, and storage, other ingredients in the food system, and gastrointestinal juice, bile salts, enzymes, etc., during the

digestion process. In addition, much evidence also demonstrated that postbiotics have a positive effect on the viability of probiotics and strengthen the intestinal microbiome (Klemashevich et al., 2014; Marzec and Feleszko, 2020).

2.1.4.1 Temperature

Temperature is one of the main factors that influence the viability of probiotics during the process of production, transportation, storage, and even consumption. Probiotics can only live and grow under a certain temperature range. High temperatures may damage the cell membrane, denature the DNA and RNA structure (Rajam & Subramanian, 2022), and cause the loss of the enzyme activity of bacteria (Tao et al., 2019). Freeze-drying is preferred compared to spray-drying due to the lower temperature and has higher viability of the final probiotic microcapsules. However, the too low a temperature still damages the bacteria cells. The loss of cell viability may occur even though milder handling conditions that can be applied, especially during the freezing process (Meng et al., 2008). The inactivation of the cells depends on the cooling rate during the freezing process, the greatest viability loss occurs during the slow cooling stage. Due to the formation of ice crystals, the freezing phase induces intense stress and causes damage to the cell wall. The formation of extracellular ice crystals increases the extracellular osmotic pressure, and the cells begin to dehydrate. There are two mechanisms that result in the loss of viability of the bacteria during the freeze-drying process: (i) changes in the physical state of membrane lipids and (ii) changes in the structure of sensitive proteins in the cells (Rajam & Subramanian, 2022). In addition, the fermentation and utilization process of different strains is not the same, but they should all be controlled under a certain temperature range to exert their function. Moreover, the temperature during storage is still essential, relatively lower temperatures easy maintain the viability and extend the shelf life of the probiotic products, too high storage temperature may increase the metabolism rate and cause the loss of viability (Ranadheera et al., 2010).

2.1.4.2 Oxygen

Oxygen and redox potential are another influential factors that affects the viability of probiotics during the process. The intensity of effects depends on the types of strains. Strains belonged to *Lactobacillus* genus are facultative anaerobes, so by considering the processing condition, they can be treated under aerobic or anaerobic. *Bifidobacteria* are strict anaerobes, thus they must be handled under anaerobic conditions. Therefore, oxygen concentration and oxygen permeability of the packing should be maintained at a low level to ensure low viability loss. Hence, microencapsulation of probiotics with better film-forming ability has a higher protection capability

for probiotics.

2.1.4.3 pH

The other paramount factor is pH, which not only guides the growth of probiotics, but also controls the life or death of microorganisms. Both too high and too low of the pH can inactivate the bacteria. Basically, the suitable pH for the growth of *Lactobacillus* is pH 4.5 to pH 6.0, while the optimum pH for *Bifidobacterium* is pH 6.5 to pH 7.0. However, during the digestion process in the gastrointestinal tract, high acid content together with enzymes will do harm to the probiotics and finally inactivate them. The reason may be due to too low pH (pH 2.0) that damages the cell membrane. Moreover, more energy may need to maintain the intracellular pH when in a low-pH environment (pH < 4.5), which may result in an insufficient amount of energy that is required for other significant functions and finally caused the death of the cells (Meybodi et al., 2020).

The characteristics and mechanisms of the complex digestion process (**Figure 1**) as well as the physiology of the gastrointestinal tract (**Table 1**) need to be considered when designing the microencapsulation process. Generally, there are 3 types of modes to trigger the release process of the probiotics namely degradation, disintegration, and dissolution. Since bacterial cells are too large, so traditional delivery devices cannot be used for the releasing process (Cook et al., 2012). After taking probiotics from the mouth, they passed through the oesophagus very quickly (around 10-14 seconds) and finally reached the stomach, which is the harshest environment for them. Generally, most of the probiotics lost their viability at this place due to the high-acid environment, whose pH is around pH 1 to pH 2.5 (Evans et al., 1988). However, the pH and retention during this point vary significantly from man to man depending on various factors such as types of foods consumed, time since eating, health conditions, age, etc. Gastric emptying time is an indicator that can monitor the retention time of probiotics in gastric juice. It is usually around 5 min to 2 hours and a half gastric emptying time is 80.5 min, which has been measured by using ^{13}C with a breath test (Hellmig et al., 2006). After passing through the stomach, the probiotics enter the small intestine, which can be divided into the proximal and distal small intestines. The pHs of the two parts of the small intestine are pH 6.15 to pH 7.35 and pH 6.80 to pH 7.88, respectively, showing an increase from proximal to distal small intestines. The retention time of the two parts of the small intestine is 3.2±1.6 hours. After passing through the small intestine, the probiotics will reach the large intestine, where the pHs are in the range of pH 5.26 to pH 6.72 in the ascending large intestine, and pH 5.20 to pH 7.02 in the descending the large intestines, respectively. The retention time during large intestine is greatly different, with the range from 6 to 32 hours (Cook et al., 2012). The probiotic bacteria will exert their functionality in the colon.

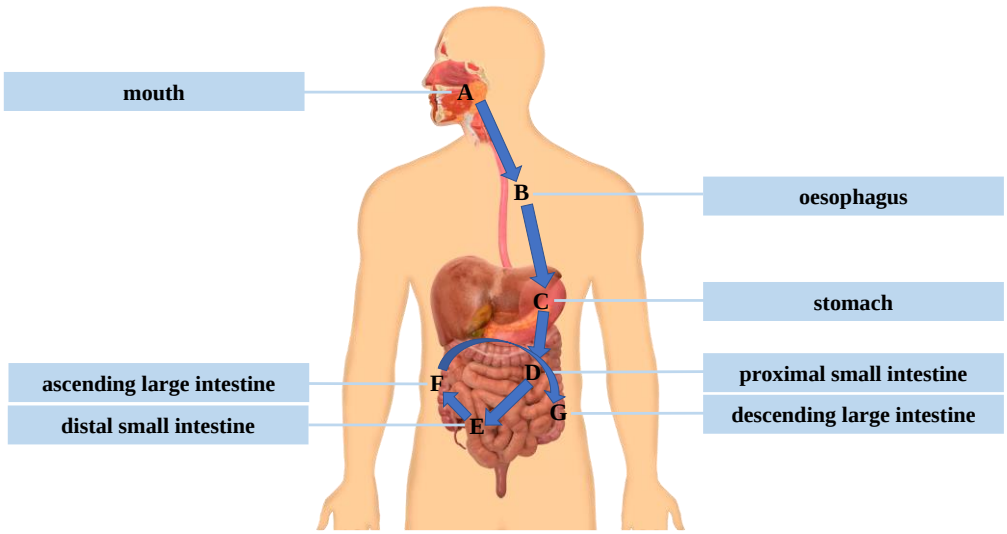

Figure 1. Scheme of digestion process of human beings (Cook et al., 2012)

The difference alphabets refer to the places and organs that take part in the digestion process. A: mouth; B: oesophagus; C: stomach; D: proximal small intestine; E: distal small intestine; F: ascending small intestine; G: descending large intestine.

Table 1. pH and retention time at the different part during the digestion process (Cook et al., 2012)

Mark	Region	pH	Retention Time
A	mouth	5.60-7.90	2-5 sec
B	oesophagus	~7.00	10-14 sec
C	stomach	1.00-2.50	161 min
D	proximal small intestine	6.15-7.35	
E	distal small intestine	6.80-7.88	3.2 ± 1.6 h
F	ascending small intestine	5.26-6.72	
G	descending large intestine	5.20-7.02	variable

2.1.4.4 Enzymes and bile salt

Enzymes and bile salt contents are two main factors that can affect the viability of probiotics during the digestion process, which includes salivary enzymes, lysozyme, pepsin, pancreatic amylase, lipase, and bile salts (Terpou et al., 2019). These compounds can decrease the viability of the probiotics targeted by damaging the cell membrane or inactive them directly. It is known that microencapsulation can avoid or delay the contact of probiotics with enzymes and bile salt to some extent (Apiwattanasiri et al., 2022; Meybodi et al., 2020) and enzymes and bile salt are abundant at a certain time after eating (Cook et al., 2012). Hence, microencapsulation of the probiotics or consuming the probiotic products at the right time are both suggested methods to avoid direct

contact between probiotics and enzymes and bile salts to maintain high viability of probiotics.

2.1.4.5 Postbiotics

The definition of the postbiotic is the "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" (Salminen et al., 2021). It has been proved that postbiotics have a positive effect on the viability of the probiotics. The mechanisms may be due to the probiotics together with postbiotics can have a better physiological condition (Hua et al., 2022). Hua and co-workers (2022) did research on the effects of postbiotics on the functionality of edible coating probiotics, and on the stability of the probiotic during simulated digestion. Their results indicated that the sample with postbiotics-enforced coating shows much better protection (with only minimal reduction after the gastric digestion) than the case of the probiotic *Lp. plantarum* 299v alone. The probable explanation of this phenomenon may be due to the probiotic bacteria *Lp. plantarum* 299v coated in materials together with their metabolites (the postbiotics) maintaining better physiological conditions, thus they are more resistant against the low pH and pepsin during the digestion process.

2.2 Microencapsulation technology

Microencapsulation technology can be described as a technology to encapsulate solid, liquid, or gaseous substances with tiny size capsules (microsphere), thus it can ensure activity and functionality in hazardous environments (Zhu et al., 2021). Finally, it can protect the substance by limiting its interaction with other parts of the system or with the external environment. Microencapsulation technology can provide alternative answers to maintain the integrity of probiotics when they go through the harsh environment of the digestive system to reach the target. The main goal of microencapsulation is to enhance the stability of the core substance, control the release of the core at the target point, facilitate transportation, and promote storage stability.

There are several strategies that can be applied to the microencapsulation process of probiotics and other bioactive substances. Based on the final state of the microcapsules, the microencapsulation techniques can be classified into three main groups:

- gel-forming technique: extrusion, emulsion,
- powder-forming technique: spray-drying, freeze-drying (lyophilization),
- other types of techniques: electrospinning, spray cooling.

2.2.1 Gel-forming microencapsulation technique

Gel-forming or microgel technique is the most commonly used microencapsulation technology for

probiotics. Probiotic microgels are formed by ionotropic cross-linking polymer solution that contains probiotics in the presence of proper oppositely charged monovalent or divalent ionic solution. The successful formation of microgels is largely determined by factors such as the gelling ability of polymers, reaction time, ionic concentration, temperature, etc. (Elvan et al., 2022; Farias et al., 2019; Gentile, 2020).

2.2.1.1 Extrusion

Extrusion is the most traditional and the most commonly studied method due to its simplicity, low cost, and mild formulation conditions that guarantee high cell viability (Martín et al., 2015). The process was carried out by extruding the cell suspension that was prepared by mixing probiotics with hydrophilic colloids through a capillary (e.g., needle or nozzle) into a hardening solution to form the probiotic microgels (**Figure 2**). The size of the microcapsules was determined by the diameter of the capillary, which is generally 2-4 mm. However, the size of the microcapsules will affect the taste quality of the food. Generally, the size of the probiotics is 1-4 μm, when the size of the microcapsules is smaller than 10 μm, there is little influence on the taste quality of the food, hence this is a disadvantage of the extrusion technology (De Prisco & Mauriello, 2016). Moreover, the microcapsules formed by extrusion present the ball shape and the shape determines the flowability in the food, which means microcapsules produced by extrusion have good flowability.

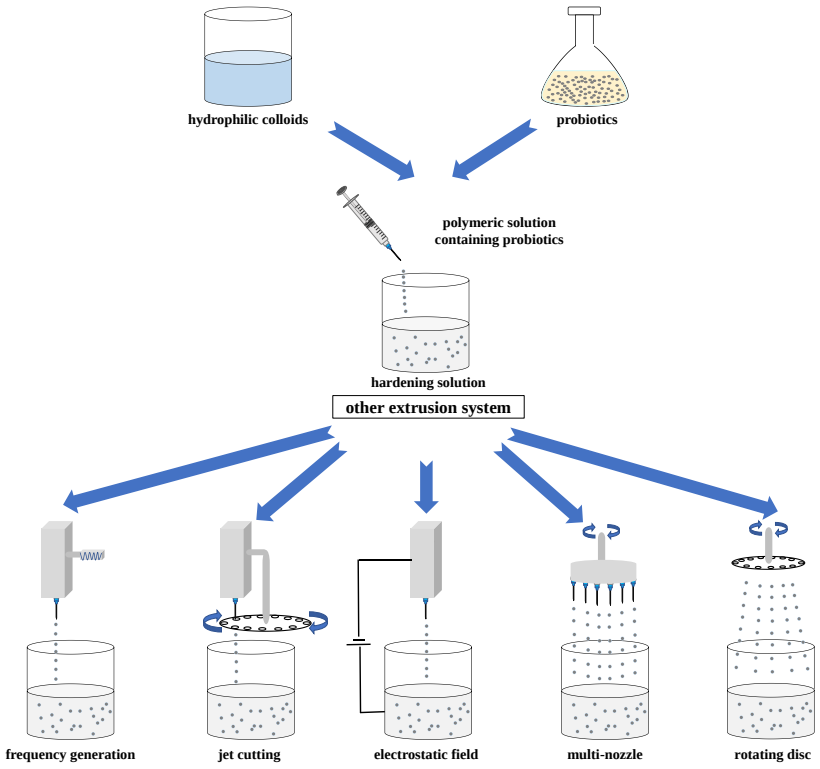

Figure 2. Scheme of extrusion technology (Martín et al., 2015)

The process of extrusion was carried out by extruding the cell suspension that was prepared by mixing probiotics with hydrophilic colloids through a capillary into a hardening solution to form the probiotic microgels. It can later be extended to frequency generation, jet cutting, electrostatic field, multi-nozzle, and rotating disc.

Prilling is a controlled method of droplet formulation by extrusion. This is realized by the pulsation of the jet or the vibration of the capillary (**Figure 2**). The use of co-axial flow or electrostatic field is another technique for the forming of droplets. This is realized due to the electrostatic forces that can disrupt the liquid surface at the needle tip, hence forming a charged stream of tiny droplets, when an electrostatic field is applied. Besides, the size of the droplet can be adjusted by varying the applied potential. To realize the large production purpose, a multi-nozzle or a rotating disc method can be applied (Martín et al., 2015).

2.2.1.2 Emulsion

Emulsion or emulsification (**Figure 3**) is another widely used technology for the gel-forming microencapsulation technique of probiotics. In this technique, the discontinuous phase (polymer suspension) is added to the continuous phase (oil) (Cook et al., 2012; Martín et al., 2015), and then, the mixture is homogenized to generate water-in-oil (W/O) emulsion. Based on the ionic gelation mechanism, the emulsion method can be distinguished into two diverse types: internal and external emulsion techniques. In the external emulsion technique, when the ionic solution is added to the emulsion, the ions (calcium carbonate or any other insoluble calcite crystals solution) diffused into the discontinuous phase (sodium alginate) and the polymer cross-linking occurs when the sodium ions are exchanged from the guluronic acids by divalent cations (Ca^{2+}) (Cook et al., 2012; H. Zhang et al., 2016). In the internal emulsion technique, insoluble calcite crystals (calcium carbonate) are added to the discontinuous phase solution (sodium alginate) to form an emulsion. Then the organic acid (acetic acid) is added into the emulsion gently, H^+ penetrates the surfactant layer at the water-oil interface, decreasing the pH of the sodium alginate solution, triggering the release of calcium ions from insoluble calcium crystals, resulting in the exchange of sodium ions with calcium ions from guluronic acids, forms the gelled microspheres from inner part (Cook et al., 2012; H. Zhang et al., 2016).

Emulsion technique have numbers of advantages such as

- do not need specific equipment,
- easy to process,
- easy to control the emulsion process,
- high loading yield of probiotics,

- mild process conditions,
- and the size of the microcapsules are relatively small, the diameter is usually 20 μm -2 mm (Cook et al., 2012; Martín et al., 2015; H. Zhang et al., 2016).

These characteristics make this technique possible to become an industrial-scale method for production. The main drawback of it, however, should be the need for a large amount of emulsifier (oil) and the oil-removing process from the microcapsules. These factors may result in a high cost for large industry applications using the emulsion techniques.

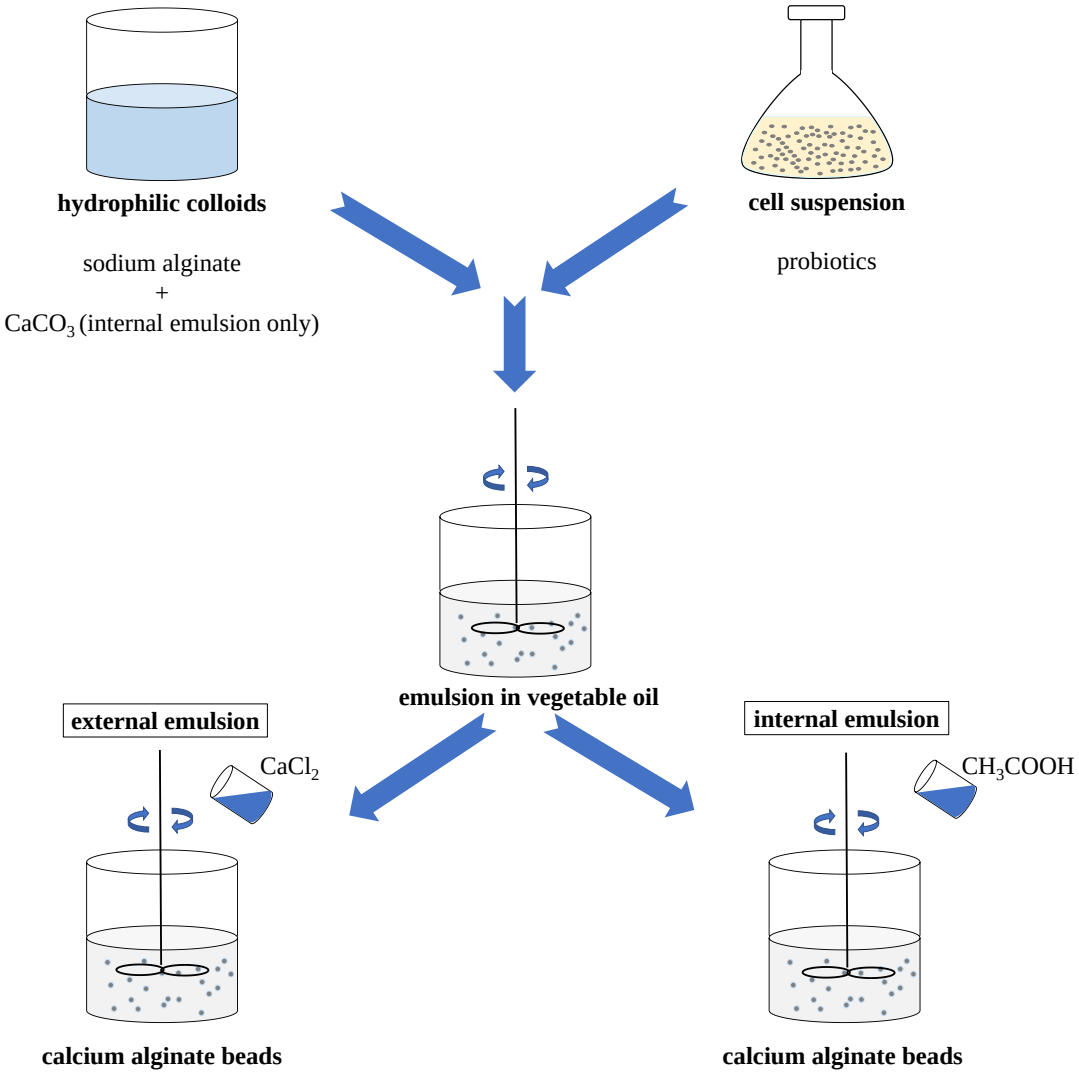

Figure 3. Scheme of emulsion technology (Martín et al., 2015)

The process of the emulsion was carried out by adding the discontinuous phase into the continuous phase. The mixture is homogenized to generate water-in-oil (W/O) emulsion. An internal or external emulsion technique was performed to form the capsules.

2.2.2 Powder-forming microencapsulation technique

2.2.2.1 Spray-drying

Spray-drying (**Figure 4**) is a commonly operated microencapsulation technique in the food and pharmaceutical industry. Compared with freeze-drying or lyophilization, the consumption of energy is around 5-10 times lower for the same amount of high-quality products (Martín et al., 2015). The mechanism of this technique is based on the atomization of an emulsion or solution and evaporates the water content from the atomized droplets by hot air and resulting in the formation of microparticles (Dias et al., 2017; Nazzaro et al., 2012). In the case of encapsulation of probiotics, it involves the atomization of a homogenized probiotic suspension and coating materials into the drying air, leading to rapid evaporation of water. After that, the microparticles or microcapsules can be collected in a cyclone, part of the air will be exhausted by a gas collection device, while the remaining air in the system has a lower temperature but a higher humidity (Dias et al., 2017). The inlet temperature, outlet temperature, product feed, and gas flow are crucial factors in this process (Martín et al., 2015).

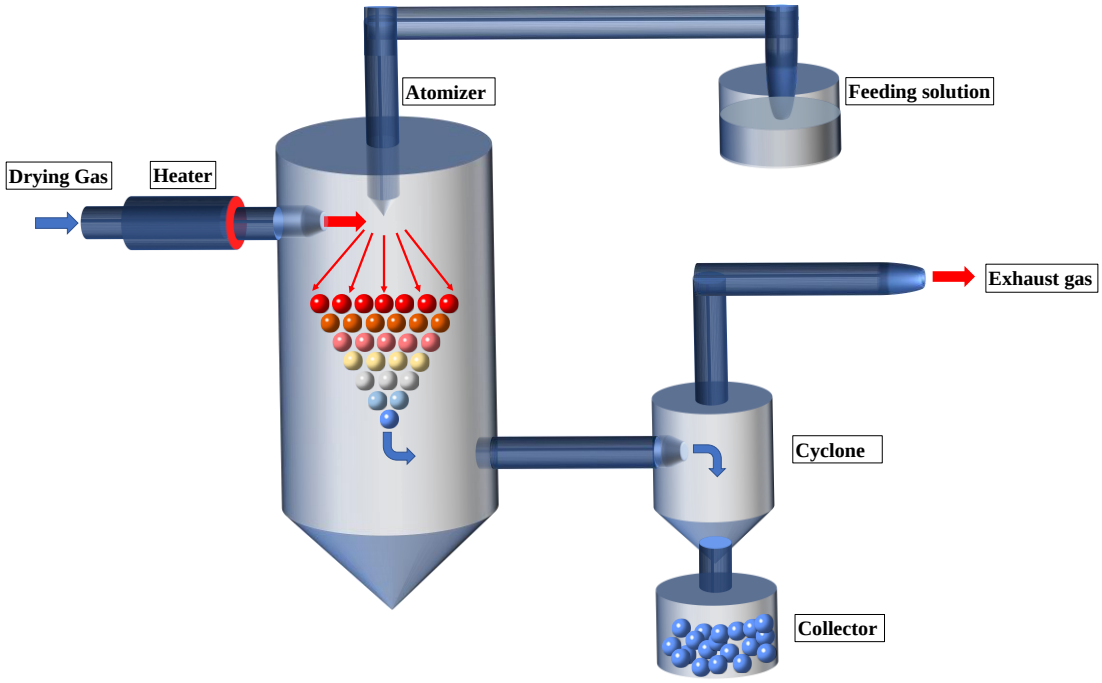

Figure 4. Scheme of spray-drying process
(adapted from Martín et al., 2015; Rajam & Subramanian, 2022)

Atomization of an emulsion or solution and evaporation of the water content from the atomized droplets by hot air resulted in the formation of microparticles. The microparticles or microcapsules were collected in a cyclone.

There are many advantages of spray-drying such as simple production process, large production scale, continuous production, high production efficiency, and low production cost, furthermore, the produced microparticles have high dispersibility, flowability, and solubility (Dias et al., 2017; Kavitake et al., 2018; Martín et al., 2015; Nazzaro et al., 2012). However, it may not only cause damage to the bacterial membrane and result in the leakage of the intracellular substance, but it also may cause the inactivation of the enzymes that bacteria need for the growing (Martín et al., 2015; Nazzaro et al., 2012). Generally, high temperatures may not be suitable for the microencapsulation of probiotics by spray-drying. However, under the same inlet temperature, the higher the flow rate results the lower the outlet temperature and thus gives a higher survival rate (Rajam & Subramanian, 2022; Vivek et al., 2023). This may indicate that the survival ability of the bacteria principally depends on the outlet temperatures.

2.2.2.2 Freeze-drying

Freeze-drying or lyophilization (**Figure 5**) emerged from laboratory curiosity to a well-organized systematic method for the preservation of biopharmaceutical products since the twentieth century (Kasper et al., 2013). Until now, freeze-drying is still the fundamental preservation method for an abundance of biopharmaceutical products. In the past, it was applied to the drying of labile pharmaceutical products such as antibiotics, however, the range of the application of this technique has already expanded to valuable biopharmaceutical products including probiotics and postbiotics. The main principle of freeze-drying is the sublimation of water from ice-phrase, and thus enhances the viability of probiotics. Hence, by using this technique, the solvents are frozen and removed through sublimation. There are three steps of freeze-drying, i.e., freezing, primary-drying, and secondary-drying (Martín et al., 2015; Zhu et al., 2021). The first step is to freeze the materials that need to be dried under lower temperatures (-80 °C to -40°C). Then, as the drying process starts, frozen water sublimates under vacuum conditions through primary drying. Finally, unfrozen water on the surface will be removed by desorption through secondary drying.

There are many advantages of this technique such as high viability, ease to store, and long shelf-life of the properties of probiotics. However, high viability does not mean that there is no viability loss of probiotics by applying freeze-drying, crystal formation, and high osmolarity. These may damage the cell membrane which can lower the viability of the probiotics (Martín et al., 2015), so plentiful protectants have already been adapted to the drying media before drying to protect the probiotics during the dehydration process. Some polysaccharides like maltodextrin, resistant starch, glucose, fructose, trehalose, etc., proteins like whey protein, denatured whey protein, skim milk, etc. are successfully used as protectants in the lyophilization process (Fu et al., 2021; Li et

al., 2016; Ma et al., 2021; Rajam & Subramanian, 2022). Besides the mentioned protectants, cryoprotectants behave the different mechanisms, as they can be added to the broth before fermentation to assist the probiotics to adapted to the environment and accumulate within the cells after fermentation to decrease the osmotic difference between the inside and outside of the cells (Martín et al., 2015). Apart from the advantages, the high production cost may be the most disadvantage of freeze-drying, especially regarding the cost of energy.

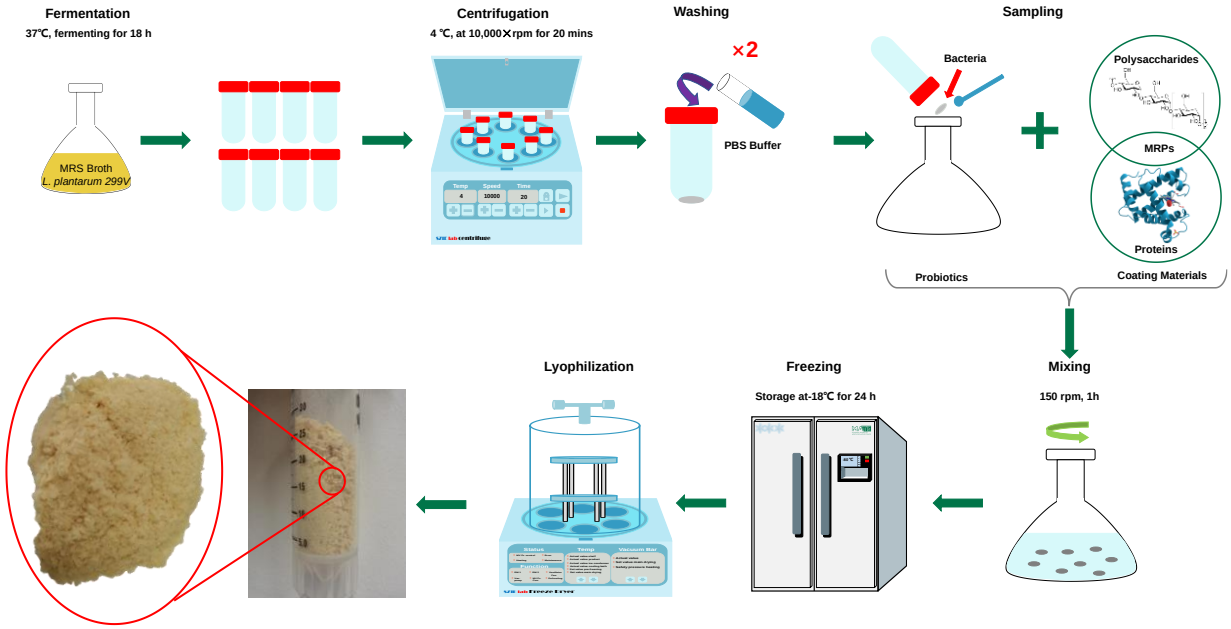

Figure 5. Scheme of freeze-drying (lyophilization) process

After the fermentation process, the probiotic cells were centrifugated and collected. Probiotics were mixed with coating materials and frozen for 24 hours. Samples were lyophilized and microcapsules were obtained.

2.2.3 Other types of microencapsulation techniques

2.2.3.1 Electrospinning

Electrospinning microencapsulation technology (**Figure 6**) bases on the electrostatic force to drive a polymer melt or solution to spray or eject to form ultrafine polymer fibers, which is generally between the range of 10 nm and several μm (Hirsch et al., 2021; Ma et al., 2021; Martín et al., 2015; Wei et al., 2021; Xu et al., 2022). When the high-voltage electrostatic field force is greater than the surface tension of the solution, it will eject from the needle or capillary and the jet will be split and stretched. With the evaporation of the jet, the extremely fine fibers are formed continuously and deposited on the ground or the oppositely charged electrode (Qiang et al., 2018). Generally, this kind of fiber has a large ratio surface and can be used to cover the surface of the

materials that need to be coated.

The advantages of this technology are attractive such as it does not produce any heat, does not need any organic solvents, and does not affect the properties of bioactive components (Ma et al., 2021; Xu et al., 2022). Besides, it can produce extremely thin fibers to form microcapsules on a nanometer scale with a high surface area (Wei et al., 2021). Furthermore, it is a technology that is ready to scale up to industrial production. Additionally, it also can be combined with other processes to be applied in different fields, e.g., the production of an expensive medicine targeted release at a specific point (Hirsch et al., 2021; Martín et al., 2015).

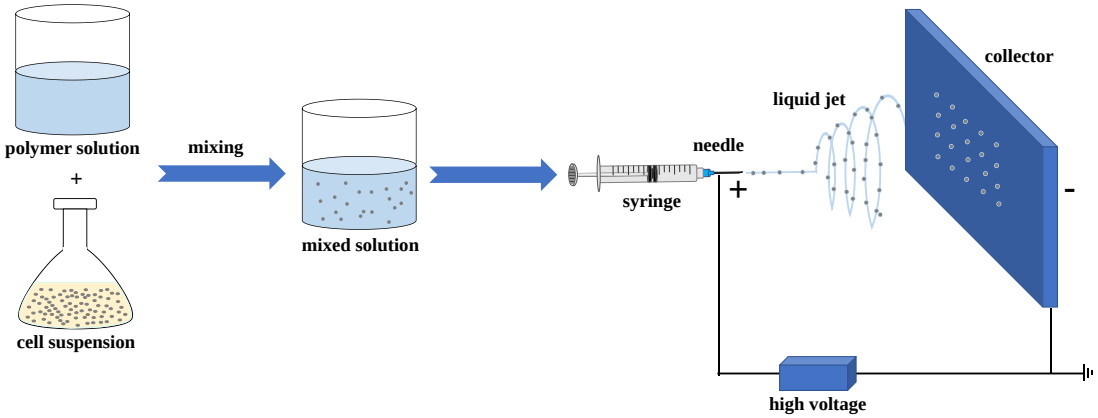

Figure 6. Scheme of electrospinning process (adapted from Martín et al., 2015)

The mixed solution of polymer and cell suspension was ejected to form ultrafine polymer fibers through a capillary by high-voltage electrostatic field force with the evaporation.

There are several materials that have been applied in the microencapsulation processes such as protein-based matrix (whey protein concentrate) and carbohydrate-based matrix (pullulan), gum Arabic, pectin, etc., and they have also been used in the process of spinning gradually. Ma and co-workers (2021) prepared and characterized gum Arabic (GA)-based nanofibers in combination with pullulan (PUL) by electrospinning, and used them as the wall material for *Lactobacillus* encapsulation. The results showed that the *Lactobacillus*-loaded GA/PUL 20:80 electrospin nanofibers showed better probiotic survivability (85.38–97.83%) compared to freeze-drying (80.92–89.84%) and retained viability during storage for 28 days at 4 °C. In addition, research focus on pectin-based nanofibers by electrospinning for encapsulation of probiotics was done by Xu and co-workers (2022). They used different proportions of electrospin nanofibers prepared by blending poly (vinyl alcohol) (PVA) with pectin (PEC), and the results showed that when the PVA:PEC proportion was 9:1, the viable count of *Lactobacillus rhamnosus* 1.0320 encapsulated by electrospinning technology was the largest, and the survival rate reached 89.26% titer. The

survival rate of PVA:PEC nanofiber-encapsulated *Lactobacillus rhamnosus* 1.0320 was 84.63% after storage at 4 °C for 21 days. These examples all show the ability of electrospinning microencapsulation technology in the production of probiotic microcapsules.

2.2.3.2 Spray chilling

Spray chilling (**Figure 7**) or spray cooling is a similar process to spray-drying for the production of encapsulated probiotic products (Favaro-Trindade et al., 2021; Figueiredo et al., 2022). On contrary to the hot air injected for spray-drying, cold air was applied in the spray chilling process. Due to the specific characteristics, lipids or fat are usually the carrier materials. The atomized active ingredients can be hydrophilic or hydrophobic, which results in dissolving or dispersing in the molten lipids and forming a solution or an emulsion based on the solubility of the substances. Then, the molten coating materials that contain probiotics or bioactive compounds are atomized and the formed droplets solidify when they encounter the cold air in the chamber. Finally, the spray-chilled microcapsules go through the cyclone and are collected at the collector of the system (Favaro-Trindade et al., 2021; Figueiredo et al., 2022; Martín et al., 2015).

There are a lot of factors that influence the result of spray chilling and there are some factors that can be optimized during the process, i.e., (i) the molten temperature of the lipid compounds; (ii) the atomization temperature; (iii) the atomization pressure; (iv) the temperature of the cooling chamber; (v) the speed of feeding coating materials, etc. (Favaro-Trindade et al., 2021; Figueiredo et al., 2022). Among these, the molten temperature of the lipid compounds is the most significant factor for the spray chilling process for probiotics, which is due to the high sensitivity of the probiotics to temperature. Generally, the molten temperature must be higher than 45°C to ensure the characteristics of the solid lipid microcapsules, for instance, low adhesion rate, good fluidity, high encapsulation efficiency, as well as good stability (Consoli et al., 2016) and this would be a problem for some probiotics that are not resistant to the heat. However, spray chilling does not need organic solvents and has a low cost compared with other microencapsulation technologies, which makes it considered the cheapest microencapsulation technology that has the possibility for large-scale production (Consoli et al., 2016; Martín et al., 2015). Hence, more and more probiotic strains that can be resistant to heat need to be selected and better characteristics of the low molten temperature lipid need to be found.

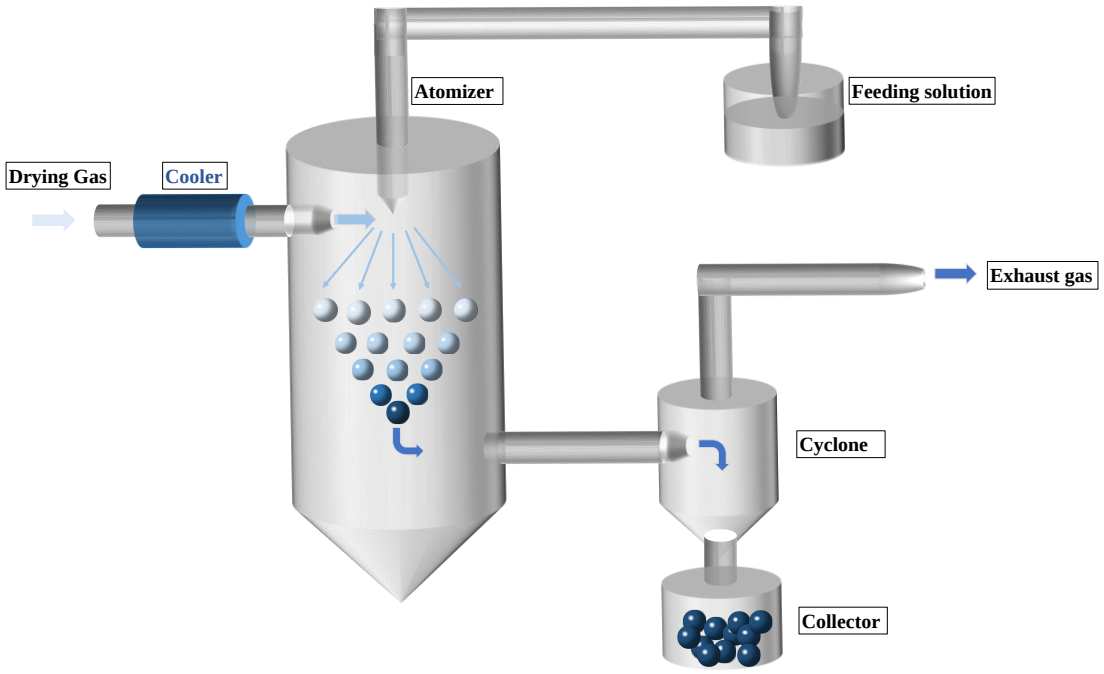

Figure 7. Scheme of spray chilling process
(adapted from Favaro-Trindade et al., 2021; Figueiredo et al., 2022; Martín et al., 2015)

The feeding solution of molten coating materials that contain probiotics was atomized and the formed droplets solidify when they encounter the cold air in the chamber. The spray-chilled microcapsules go through the cyclone and are collected at the collector of the system.

2.3 Coating materials

The selection of wall materials is crucial since it is related to the protective effect of the probiotics, especially during the digestion process. The wall materials used for the microencapsulation process are naturally large polymers and possess some characteristics such as follow: cheap, available, safe, hygienic, good biocompatibility with probiotics, good film-forming properties, good stability, and good enteric solubility (Cook et al., 2012; Figueiredo et al., 2022; Martín et al., 2015). The commonly used materials for microencapsulation of probiotics can be classified into three main groups (i) polysaccharides and their derivatives, including sodium alginate, maltodextrin, resistant starch, cellulose, inulin, etc.; (ii) proteins, including whey protein, casein, soy protein, gelatine, etc.; (iii) lipids, including waxes, fatty acids, triglycerides, etc.

2.3.1 Polysaccharides

2.3.1.1 Sodium alginate

Sodium alginate is the most commonly used material for the production of microcapsules, which

is a natural anionic polysaccharide, originating from the by-product of brown algae after the extraction of mannitol and iodine. L-guluronic acid (G unit) and D-mannuronic acid (M unit) are linked together by glycosidic bonds, and their spatial structure and biocompatibility are determined by the concentration ratio of G and M (Chen et al., 2016). When Na^+ ions in the G unit of sodium alginate are exchanged with divalent cations such as Ca^{2+} and Ba^{2+}, the cross-links within the molecule or inter molecules are formed creating network structure, and finally resulting the gel beads with both elasticity and strength properties (Chen et al., 2016; Cook et al., 2012; Martín et al., 2015; Zhang et al., 2016).

The stability of microcapsules is affected mainly by the concentration of coating materials, the concentration of probiotics, and the time for hardening. The concentration of sodium alginate directly affects the mechanical strength and morphological shapes of the microcapsules. In the case of low-concentration sodium alginate, the size of microcapsules is smaller, and the mechanical strength is weaker than in the case of high sodium-alginate. An increase in the concentration of sodium alginate will result in an increase in the mechanical strength of the microcapsules, while the increase in size is also markable. It means that the increase in the thickness of the wall and not good for the release of the probiotic in the microcapsules. Besides, the increase in viscosity also affects the uniform dispersion of probiotics. Meanwhile, the longer hardening time may assign the better mechanical strength of the microcapsules, however, long time exposure will affect the viability of the probiotics (Chen et al., 2016).

Due to the cheap, mild reaction conditions, non-toxic and good biocompatibility, sodium alginate is widely applied in the microencapsulation process, however, this coating material still faces some disadvantages. When placed in a high-affinity ion solution such as lactate, or citrate, the cross-linking of Ca^{2+} decomposes and the stability decreases. Besides, high concentrations of Na^+ or Mg^+, Ca^{2+} may be decomposed causing decreases in the stability, which may make the porous in the microcapsules and thus reduce the protective effect (Chen et al., 2016). Sodium alginate can be applied together with other traditional large molecular coating materials such as chitin, gelatine, whey protein or starch, etc., with single or multiple mixing.

2.3.1.2 Starch

Starch is the most significant storage carbohydrate in plants, which is made of α-D-glucose unit by the linkage of α-1→4 and α-1→6 glycosidic bonds (Kavitake et al., 2018). It is also the main ingredient of food for human consumption and rich in nutritional compounds. Besides, starch plays a crucial role in the prevention and treatment of junctional and rectal cancer (Chen et al., 2016). Generally, starches can be classified into amylose and amylopectin based on their chemical

structure characteristics. Besides, starch can be classified into porous starch and non-porous starch (Chao et al., 2012) based on the physical structure characteristics. Furthermore, according to the bioavailability of the starch in the small intestine, it can be divided into two groups: digestible starch and resistant starch (Chen et al., 2016). Based on the specific characteristics of starch, it can be used as a coating material for the microencapsulation process of probiotics to form the microcapsules controlling and targeted releasing at the colon, resistant to the digestion juice (Ahmad et al., 2019; Lin et al., 2022).

Resistant starch also known as anti-enzymatic starch or indigestible starch, which has the characteristics similar to cellulose that cannot be digested in the stomach and small intestine, but can be used by bacteria as a carbon source in the colon for human beings and animals (Chao et al., 2012; Chen et al., 2016; Tian et al., 2016). Moreover, resistant starch can reduce the pH, inhibit the growth of harmful bacteria, and give effective protection to the intestinal tract (Tian et al., 2016). Wang and co-workers (2022) reported that prebiotic activities of resistant starch nanoparticles (SNPs) on the probiotic *Lactiplantibacillus plantarum* subsp. *plantarum* was demonstrated. The results demonstrated that SNPs (0.5% w/v) could be continuously fermented by *Lp. plantarum* and that many viable cells was maintained at 9.5 log CFU/mL until the 70^{th} hour of cultivation. The viability of *Lp. plantarum* was merely 6.75 log CFU/mL when the glucose was used as a carbon source.

Porous starch is a new type of modified starch with a porous surface obtained by enzymatic hydrolysis of starch. It refers to the formulation of honeycomb porous modified starch after an enzyme with raw amylase activity is carried out on raw starch granules at a temperature lower than starch gelatinization temperature (Chen et al., 2016; Kavitake et al., 2018; Sen et al., 2015). The pores on the surface can be applied as a container to encapsulate the probiotics, which can reduce the impact of mechanical extrusion and gastrointestinal juice on probiotics, and make sure they can pass through the digestive tract to play their probiotic function. Porous starch has the characteristics of large pore volume, large surface area, low particle density, low bulk density, good absorption capacity, good mechanical strength, etc., which makes it a good coating material in the microencapsulation process of probiotics (Benavent-Gil et al., 2018; Chen et al., 2016; Sen et al., 2015). Benavent-Gil and co-workers (2018) developed a system to thermally stabilize probiotic bacteria based on porous starches and using biopolymers as coating materials (gelatinized starch, guar gum, and xanthan gum). The results revealed that the encapsulation yield with rice starch increased by around 10% due to the surface pores starch while this effect was not observed in porous corn starch. The highest encapsulation yield is between 92% and 100%, which was achieved by porous starches coated with gelatinized starch.

2.3.1.3 Maltodextrin

Maltodextrin is a hydrolysed starch that is used as a coating material in the microencapsulation process based on its relatively low price and good characteristics such as low viscosity, low bulk density under high concentration, neutral aroma, good solubility, exquisite mouthfeel, attractive surface sheen, effective binding and desirable filming properties (Parvez et al., 2022; Suryabhan et al., 2019). According to Suryabhan and co-workers (2019), two potential probiotic yeasts *Saccharomyces cerevisiae* KTP and *Issatchenkia occidentalis* ApC strains were microencapsulated by using maltodextrin and sucrose or sorbitol with an aim to improve its effectiveness by spray drying. The results revealed that the encapsulated yeast was remarkably improved. They grew in simulated gastrointestinal conditions (32–64% in gastric and 46–80% in bile juice) as compared to the non-encapsulated yeast.

2.3.2 Proteins

Compared to other types of coating materials, the film formed by the proteins has controllable permeability and high gelling strength and results in a good resistant ability against the harsh environment (Jiaojiao et al., 2016; Tao et al., 2019). Besides, proteins have a good buffing capacity and are a good source of nutrition, thus proteins are widely used as the coating materials for probiotics (Jiaojiao et al., 2016). Moreover, most of the protein solutions have low viscosity even under high concentration, which is beneficial for the forming of condensed gel network structure and furthermore has effective protective ability against the inner probiotics. Based on the original source of the proteins, they can be classified into animal proteins (milk, whey, casein, gelatine, etc.), plant proteins (soy, pea, rice, coconut, etc.), and other types of proteins.

2.3.2.1 Milk

Due to historical reason, milk is the largest consumed and well-studied protein product in the world. Whey protein is a well-researched coating material in probiotic microencapsulation. The cryoprotective potentials of 10% (m/v) skimmed milk, inulin, maltodextrin, and sucrose were investigated during freeze-drying by Oluwatosin and co-workers (2022). The results revealed that skimmed milk demonstrated the highest survival up to 91%. In addition, Würth and co-workers (2015) aimed to characterize the protective effects of milk-protein-based microcapsules both *in vitro* and in mice as a model consumers. Their results revealed that sodium caseinate (SC) and the newly developed, SGF-resistant fat SC (FSC) capsules significantly increased the survival of two *Lactobacillus* strains in SGF. These examples demonstrate the ability of milk as a coating material in the microencapsulation of probiotics.

2.3.2.2 Whey protein

Whey protein is a mixture of globular proteins that are isolated from whey. It is usually liquid waste after the production of cheese. Applying whey protein as a coating material in the microencapsulation process is not only increasing the added value of the products but also reduces environmental pollution. In addition, these proteins possess the ability to interact with a wide range of active molecules, which can protect the microencapsulated compounds and let them be released at the targeted position in the host. Moreover, the hydrolysis of the proteins by digestive enzymes can generate bioactive peptides and their physiological function in the human body (Martín et al., 2015). Etchepare and co-workers (2020) reported that the microencapsulated *L. acidophilus* was tested in simulated gastrointestinal conditions, and the treatment with a layer of whey protein provided greater protection for the probiotics in all the stages of the analysis, with a final count of 9.19 log (CFU/g). Currently, whey protein, whey protein concentrate, or whey protein isolate are often working as coating materials together with polysaccharides, which can overcome the disadvantages of easy hydrolyzed by pepsin, high hydrophobicity, and loss of structure of the microcapsules when the whey protein is applied alone as a coating material. Vanden Braber and co-workers (2020) evaluated the effect of the microencapsulation (by spray drying) of the whey-native probiotic yeast *Kluyveromyces marxianus* VM004 in matrices of whey protein concentrate (WPC) and water-soluble chitosan (WSCh) on the viability of the yeast during drying, storage and in simulated gastrointestinal conditions. The results revealed that the solids concentration in the suspension to be dried enhances the general probiotic count and sample with a suspension with 30% (w/v) solids (29:1 WPC:WSCh) showing 95% of viability after passing through gastrointestinal conditions. It can be concluded that matrices of WPC:WSCh constitute an excellent alternative to develop probiotic microcapsules as bioactive ingredients of functional foods.

2.3.2.3 Gelatine

Gelatine is obtained from the hydrolysis of collagen, which can be isolated from animal connective or epidermal tissue. Due to its good water solubility, exceptional emulsifying properties, great biocompatibility, superb biodegradability, and wonderful gel-forming property, gelatine is one of the earliest coating materials that have been applied in the microencapsulation process (Chen et al., 2016).

It is clear that gelatine, alginate, pectin, chitosan, gum Arabic, etc., are the most utilized biopolymers in the complex coacervation the microencapsulation process (Arslan-Tontul & Erbas, 2017; Brinques & Ayub, 2011; Darjani et al., 2016; Li et al., 2019; Liu et al., 2016; Olivares et al.,

2019; Tao et al., 2019). However, owing to the easily hydrolysed properties of gelation during the digestion process, it is commonly applied together with other types of coating materials to form a coating materials complex. This coagulation method is one of the most widely used methods for microencapsulation process with gelatine as coating material. Among those complexes, the gelatine-gum Arabic complex probably is the most frequently studied. It is based on the attractive interaction between two biopolymers with opposing electrical charges leading to the separation of two phases in the system and leading to the formation of coacervate complexes at a specific pH (Paula et al., 2019). When the pH is lower than the isoelectric point, the number of $-NH_3^+$ ions in the gelatine solution is more than -COO-, and the solution is positively charged; when the pH is higher than the isoelectric point, the molecular chain contains -COO- when gum Arabic in the aqueous solution, which is negatively charged. Adjusting the pH of the gelatine-gum Arabic aqueous solution can form and coagulate the complex from the system due to the opposite charges and desired microcapsules can be collected at the same time (Tian et al., 2016). According to a report from Paula and co-workers (2019), the probiotic cells of *Lactobacillus plantarum* were microencapsulated through a dual process consisting of emulsification followed by complex coacervation using gelatine and gum Arabic. The results illustrated that the optimized conditions for complex coacervation consisted of a 50:50 biopolymer ratio and pH 4.0. Meanwhile, the viability of the encapsulated cells was 80.4%, whereas it was only 25.0% for the free cells at 37 °C after *in-vitro* consecutive simulated gastric and small intestinal digestions. In addition, based on the study of Singh and co-workers (2018), gelatine and carboxymethyl cellulose emulsion were prepared as novel promising biobased matrices for the probiotic bacteria *Lactobacillus rhamnosus* GG (LGG). The results showed that the survival of the LGG cells, when exposed to the different model fluids namely Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF), and Simulated Intestinal Fluid (SIF), was improved after their entrapment in the w/w emulsions. It means the developed dispersions display high potential for probiotic encapsulation and eventual delivery into the intestinal tract with acceptable viability.

2.3.2.4 Soy protein

Soy protein is the most popular industrial plant protein source, which makes it a prevalent plant protein for the microencapsulation process (Can et al., 2015; Liu et al., 2014). Soy protein as a source of large-scale plant protein is cheaper than animal proteins and can reduce cholesterol, hyperlipidaemia, and other cardiovascular diseases (Fu et al., 2021). It has been used alone or combined with anionic polysaccharides by spray-drying, freeze-drying, and complex coacervation microencapsulation process. Fu and co-workers (2021) reported that soy protein isolate (SPI) and

α-lactose monohydrate were applied to form the Maillard-reaction-products (MRPs) as the coating materials to encapsulate *Limosilactobacillus reuteri*. The results clarified that the particle size of MRPs-microcapsules (68 μm) was smaller than that of SPI-microcapsules (91 μm). Besides, the viability of *Ll. reuteri* in simulated gastrointestinal digestion and under 30 days storage was improved. Additionally, the emulsification, foaming, and gel strength of SPI can be improved by the modifications with the Maillard reaction. The performance of Maillard reaction products (MRPs) prepared from soy protein isolate (SPI) and *I*-carrageenan (IC) by spray-drying and dry heat microencapsulation technology in the protection of *Bifidobacterium longum* was investigated by Mao and co-workers (2018). The results illustrated that among all the MRPs, the sample with a SPI:IC ratio of 1:3 was dry heated for 6 h after spray drying. It was the most effective process in the protection process. The results of the *in vitro* digestion test demonstrated that the viability of the bacteria was reduced by 2.38 and 0.74 log units after 120 min exposed to simulated gastric fluid and simulated intestinal fluid, respectively.

2.3.3 Lipids

Lipids, such as fatty acids, oils, triacylglycerols, and waxes can be used as the coating materials in the spray-chilling microencapsulation process (Consoli et al., 2016). Generally, the active materials or probiotics are mixed with the molten coating materials to form an emulsion or suspension, then the mixture was automized into the chamber of the spray-chilling machine with an air temperature lower than the molten temperature of the lipids, hence, the microcapsules or droplets were formed with solidification process (Consoli et al., 2016; Figueiredo et al., 2022; Pedroso et al., 2012; Silva et al., 2018). Due to the crystalline structure and polymorphic arrangement properties during the lipid solidification and crystallization process, the microcapsules may have some weaknesses, such as low microencapsulation rate and capacity, exposure of embedded materials, etc., (Pedroso et al., 2012). However, spray-chilling is the most economical microencapsulation technology and has the potential to be applied in industrial-scale production. Moreover, this method can generate smaller capsules, which is preferable for further food processing (Champagne & Fustier, 2007). According to the study of Pedroso and co-workers (2012), solid lipid microparticles are made by palm oil and palm kernel through a spray-chilling microencapsulation technique that contains *Bifidobacterium lactis* and *Lactobacillus acidophilus*. The resistances of the probiotics to the spray-chilling process, to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), and their stability during storage for 90 days were tested. The results demonstrated that the free and encapsulated cells of *B. lactis* were resistant to SGF and SIF and the viability of the cells was not affected by microencapsulation, but microencapsulation by

lipids provided protection for *L. acidophilus* against SGF and SIF. Besides, during the storage of the samples at 37 °C, the free and encapsulated microorganisms lost their viability. However, when the samples were stored under refrigerated and frozen conditions, promising results were obtained. Based on the research of Silva and co-workers (2018), *Lactobacillus acidophilus* LA3 and *Bifidobacterium animalis* subsp. *lactis* BLC1 were microencapsulated by vegetable fat together with gelatine and gum Arabic through spray-chilling and freeze-drying. The results revealed that lipids were effective in protecting the probiotics subjected to environmental stress conditions and SGF and SIF. It is demonstrated that the viability of probiotics encapsulated by spray-chilling was about 7.2 log (CFU/g), while the viability of probiotics was approximately 6.7 log (CFU/g) in the freeze-dried lipid particles covered by gelatine and gum Arabic. Both encapsulation methods were successful in protecting the probiotics in environmental stress conditions and simulated gastrointestinal conditions.

2.4 Application of probiotic microcapsules in food

Traditionally, milk matrix was used as a probiotic carrier, which includes the fermented dairy products, such as yoghurt, cheese, etc., and non-fermented dairy product such as frozen desserts, etc. Recently, overwhelming number of non-dairy probiotic products are considered an attractive choice for those consumers who are allergic to milk protein or have lactose intolerance (Frakolaki et al., 2021).

2.4.1 Dairy food

2.4.1.1 Yoghurt

Microencapsulated probiotics have already been applied in many yoghurt products, as the first choice of dairy products to carry probiotics (Frakolaki et al., 2021). Most of the research mainly focuses on the protecting ability of probiotics during the storage and digestion process (Capela et al., 2006; Pavunc et al., 2011; Shoji et al., 2013). Probiotics also perform low viability during the storage process of yoghurt due to some hurdles such as low pH value (pH 4.2-4.6), high water activity, high oxygen rate, organic acid and hydrogen peroxide content, etc. (De Prisco & Mauriello, 2016; Frakolaki et al., 2021). Pavunc and co-workers (2011) reported that the microencapsulated probiotic *Lactobacillus helveticus* M92 has better survival ability than free cells in the yoghurt during storage. A similar result was revealed by Shoji and co-workers (2013), when they applied the microencapsulated *Lactobacillus acidophilus* by complex coacervation onto buffalo milk yoghurt. They found that lower values for post-acidification and greater stability were obtained in the yoghurts fortified with microencapsulated probiotics compared to those with free cells.

Furthermore, it has been reported by many authors that the incorporation of probiotic microcapsules does not influence any changes in the colour, appearance, flavour, etc., of the yoghurt, while it can facilitate the texture properties, for example, increasing the smoothness (De Prisco & Mauriello, 2016; Frakolaki et al., 2021; Wenjing et al., 2019). This may be due to the production of exopolysaccharides by microcapsules, which increases the viscosity of the yoghurt, prevents the precipitation of the whey protein, and result in the enhancement of the texture of the yoghurt (Wenjing et al., 2019).

However, the addition of microencapsulated probiotics may result in an undesirable mouth feeling in the yoghurt, but it depends on microencapsulation technology and coating materials. On the one hand, it is well-known that the thicker cover has a better protecting ability meaning the better resistance of the microcapsules. On the other hand, the increase in the size of the microcapsules or beads can increase the grainy feeling of the texture of the food (De Prisco & Mauriello, 2016), which is an extreme disadvantage for those soft and liquid types of food, like yoghurt, beverage, soft cheese, etc.

In summary, the survival ability and survival period of the probiotics can be greatly improved by microencapsulation technology when they are applied to soft or liquid products, especially yoghurt products. However, the addition of probiotic microcapsules may also reduce the sensory properties of the yoghurt since it is directly related to the size of the microcapsules, and the compatibility of the coating materials with yoghurt. Consequently, further research for the reducing of the particle size and analyzing the compatibility of the coating materials with yoghurt is needed to be performed to realize the application of probiotic microcapsules in yoghurt.

2.4.1.2 Ice-cream

Ice cream is a kind of dairy product in the summertime, and it could be a potential carrier or food matrix for the application of probiotics (Farias et al., 2019; Homayouni et al., 2008; Mohammadi et al., 2011). Since the increase in the claim of consumers to healthy nutrition and the spreading of the concept of functional foods around the world, the application of probiotics in ice cream is an attractive strategy (Mohammadi et al., 2011). Ice cream has very high acceptance from different groups due to its sweet taste, soft texture, high nutrition, and easy digestion properties (Farias et al., 2019). In addition, compared with the protecting ability of fermented dairy products, ice cream has significantly higher support due to relatively lower temperature during the production and storage process (Mohammadi et al., 2011). However, the viability of the probiotics will lose somehow be unavoidable during the formulation, production, storage, and melting process. During these periods, the factors such as pH value, acidity level, osmotic level, sugar content, shearing

stress, freezing time, formulation, microencapsulation or not, etc., will all influence the viability of the probiotic in the final product before consumption. Farias and co-workers (2019) microencapsulated *Lactobacillus rhamnosus* and *Lactobacillus casei* with alginate-chitosan by an extrusion method and tested the viability of free and microencapsulated probiotics in the ice cream samples. The results illustrated that microcapsules reduce the viability loss of probiotics in the frozen process and in the simulated digestion process. They also concluded that *L. rhamnosus* ASCC 290 is more beneficial to apply in the ice cream. Homayouni and co-workers (2008) however, found that the survival ability of microencapsulated *L. casei* Lc-01 and *B. lactis* Bb-12 was significantly higher than the free cells in the ice cream sample storage for 180 days at -20 °C. The sensory properties of the ice cream with the addition of resistant starch as prebiotics were not significantly affected by the addition of encapsulated probiotics.

2.4.2 Non-dairy food

2.4.2.1 Plant-based products

Dairy products like milk, yoghurt, cheese, etc., are the ideal matrices for carrying probiotic bacteria. However, during the past years, the increase in vegetarianism involves increased the demand for plant-based probiotic products correspondingly (Gandomi et al., 2016). Additionally, plant-based products have some advantages over dairy probiotic products such as lactose-free (for those who are lactose intolerant), not contain milk protein (milk protein allergy), and not contain cholesterol (De Bellis et al., 2021). However, some texture and sensory issues may limit the consumption of plant-based products by consumers, thus intensive development of technology is needed to solve the problems of sensory, acceptability of plant-based probiotic foods (De Bellis et al., 2021). Apart from the vegetable-based matrix, fruit is another option for application as raw material for probiotic products. Fruit juices are rich in many nutrients such as antioxidants, minerals, vitamins, etc., and they have a fresh taste and pleasant aroma which is acceptable for all ages (Gandomi et al., 2016). Moreover, the sensory properties of the fruit-based matrix will not be influenced by probiotic microcapsules during the whole manufacturing process. However, in order to exhibit the health benefits of the probiotics, they must be alive through the production, storage, the digestion process, and start the fermentation process at a specific place. Besides, probiotics should not negatively affect the sensory properties like taste, aroma, and flavour of the fruit juice. Therefore, the application of microencapsulation technology to embed the probiotics and applied the microcapsules in the fruit juice is the possible method to realize this target. Gandomi and co-workers (2016) investigated the viability and sensory properties of microencapsulated *Lactobacillus rhamnosus* GG that was coated by alginate and chitosan, with or without inulin

during storage for 90 days at 4°C and 25°C in apple juice. The results demonstrated that after storage for 90 days, the viability of the microencapsulated *L. rhamnosus* was 4.5 times higher than those that are free. Besides, microencapsulation technology of the samples increases the sensory scores in all aspects. This result revealed that microencapsulation technology can enhance the probiotic viability during storage in apple juice and strengthen the sensory characteristics of the apple juice.

2.4.2.2 Bakery products

Bakery products like biscuits, bread, etc., can also be an ideal food matrix for probiotics because of their large consumption especially the European countries (De Prisco & Mauriello, 2016). However, there are still some obstacles for the application of probiotics in bakery products. Apart from the chemical reactions of this specific food matrix, such as the change of pH, the production of ethanol, the reaction of Maillard reaction, etc., the biggest problem is the high heating temperature during the baking process and long storage time for some specific products like biscuits (Semwal et al., 2022; Soukoulis et al., 2014). Consequently, edible film or filling of the probiotics in the cream layer of the cake is the main methods that can assure the viability of the probiotics before consumption (De Prisco & Mauriello, 2016). The edible film refers to a kind of layered structure that was made by the biopolymers and can be applied onto the surface of the products to control the shelf-life or as a carrier for bioactive compounds through dipping or spraying methods (Semwal et al., 2022; Soukoulis et al., 2014; Zoghi et al., 2020). Soukoulis and co-workers (2014) developed a new strategy for the inclusion of probiotics in bakery products by applying probiotic *Lactobacillus rhamnosus* GG hydrogel solution made by sodium alginate and whey protein concentrate on pan bread followed by air drying at 60 °C for 10 min. The results revealed that the viability of *L. rhamnosus* GG increased significantly during the air drying and storage at room temperature due to the presence of WPC. Moreover, the consumption of 30-40 g of this pan bread will meet the requirement of WHO recommended living cell number for probiotics to be delivered to the human gut. Semwal and co-workers (2022) found that the viability of the probiotic was improved in the presence of edible film which was produced by chia mucilage and sodium caseinate. Besides, the following application of this new edible film was assessed on the wheat buns, and the results highlighted the viability of the probiotic maintained for 3 weeks at 4 °C and 2 weeks at 25 °C. They also concluded that chia mucilage and sodium caseinate-based edible film can perform a potential carrier for the delivery of probiotics to the human gut safely.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Microorganisms

A probiotic strain *Lactiplantibacillus plantarum* 299v strain (Probi Corp., Lund, Sweden) was from the Collection of the Department of Bioengineering and Alcoholic Drink Technology, Institute of Food Science and Technology, Hungarian University of Agriculture and Life Sciences, Budapest, Hungary.

3.1.2 Coating materials

Two types of coating materials were applied in the microencapsulation process. Polysaccharides maltodextrin and resistant starch were from Ingredion (Germany), while whey protein was from Nutriversum® Ltd. (Hungary). The denatured whey protein was prepared in-house by heating 20% (w/w) whey protein solution at 90 °C for 20 min. MRPs were prepared by maltodextrin and whey proteins are dissolved in saline solution and the concentration was modified to 20% (w/w). The pH was adjusted to 8.0 with 4 N NaOH. The solutions were heated in the water bath at 90 °C for 4 h to form the Maillard reaction products (MRPs) as the coating materials. The MRPs solutions were cooled down and stored at 4 °C for further practice (Fu et al., 2021).

3.1.3 Chemicals and solutions

3.1.3.1 MRS medium

MRS (Man, Rogosa, and Sharpe) medium was usually used for maintaining *Lp. plantarum* 299v strain. The composition of the growth medium is listed in **Table 2.** The final pH of the medium was adjusted to pH 6.8 to pH 7.0 and sterilized in the autoclave at 121°C for 15 min.

Table 2. Composition of MRS medium

Ingredients	Amount	Unit
Proteose peptone	10.0	g
Meat (beef) extract	8.0	g
Yeast extract	4.0	g
D (+)-glucose	20.0	g
Dipotassium hydrogen phosphate	2.0	g
Sodium acetate	5.0	g
Tri-ammonium citrate	2.0	g
Magnesium sulphate heptahydrate	0.2	g
Manganous sulphate tetrahydrate	0.05	g
Tween 80	1.0	mL
Distilled/deionized water	1000.0	mL

3.1.3.2 PBS solution

Phosphate buffer saline (PBS) (0.1 M and pH 7.4) was used in the sampling process, and prepared by dissolving 8 g NaCl, 0.2 g KCl, 1.15 g $Na_2HPO_4.2H_2O$ and 0.2 g KH_2PO_4 in 1L distilled/deionized water. This solution was sterilized in the autoclave at 121°C for 15 min.

3.1.3.3 Saline solution

Saline solution (0.85%, w/v) was prepared by dissolving 8.5g NaCl in 1L distilled/deionized water. The solution then was dispensed into test tubes with a volume of 4.5 mL/each and sterilized in the autoclave at 121°C for 15 min.

3.1.3.4 Simulated gastric fluid

Simulated gastrointestinal fluid (SGF) was made by dissolving the pepsin (727 U/mg) (Sigma, Germany) in the sterilized 5 g/L saline solution (pH 2). The pH value of the saline solution was previously adjusted to pH 2 by 6N HCl before sterilization (Nguyen et al., 2019).

3.1.3.5 Simulated intestinal fluid

Simulated intestinal fluid (SIF) was made by dissolving the bile salt (Sigma, Germany) in the sterilized K_2HPO_4 and KH_2PO_4 solution (pH 7.4). The K_2HPO_4 and KH_2PO_4 solution was made by dissolving 5.43 g K_2HPO_4 and 2.56 g KH_2PO_4 in 1L distilled/deionized water and sterilized in the autoclave at 121°C for 15 min (Nguyen et al., 2019).

3.1.4 Apple juice

Unfiltered high-quality HAZÁNK Kincsei apple juice (Lidl, Hungary) was purchased from a local supermarket. The pH of apple juice was adjusted to pH 6 by 4 N NaOH solution prior to use.

3.2 Methods

3.2.1 Microencapsulation process

The *Lp. plantarum* 299v strain was activated twice in MRS medium at 37°C for 18 h when the cell count reached around 10^9 CFU/mL at the end of the incubation. Then the cells were collected by centrifugation at 10.000×rpm at 4 °C for 20 min. After that, they were washed twice with phosphate-buffered saline solution (0.1 M, pH 7.4). Then the wet pellet of the cells was mixed with wall materials based on the ratios of core-to-wall, and the ratios between wall materials **(Table 3)**. In the cases of a combination of maltodextrin and whey protein, the Maillard reaction was priorly carried out by heating the solutions at 90°C for 3 h. After the mixing procedure, samples

were gently shaken at 150×rpm and 4°C for 1 h.

Table 3. Experimental design for coating probiotic bacterium *Lp. plantarum* 299v strain

Coating materials	Ratios of core-to-wall	Wall materials	Ratios of wall materials	MD (g)	RS (g)	*Lp. plantarum* 299v (g)
Polysaccharides	1:1	MD	-	10.00	0.00	10
		MD:RS	3:1	7.50	2.50	10
		MD:RS	1:1	5.00	5.00	10
		MD:RS	1:3	2.50	7.50	10
		RS	-	0.00	10.00	10
	1:1.5	MD	-	15.00	0.00	10
		MD:RS	3:1	11.25	3.75	10
		MD:RS	1:1	7.50	7.50	10
		MD:RS	1:3	3.75	11.25	10
		RS	-	0.00	15.00	10
Proteins	1:1	WP	-	10.00	0.00	10
		WP:DWP	3:1	7.50	2.50	10
		WP:DWP	1:1	5.00	5.00	10
		WP:DWP	1:3	2.50	7.50	10
		DWP	-	0.00	10.00	10
	1:1.5	WP	-	15.00	0.00	10
		WP:DWP	3:1	11.25	3.75	10
		WP:DWP	1:1	7.50	7.50	10
		WP:DWP	1:3	3.75	11.25	10
		DWP	-	0.00	15.00	10
Polysaccharides + Proteins	1:1	MD:WP	3:1	7.50	2.50	10
		MD:WP	1:1	5.00	5.00	10
		MD:WP	1:3	2.50	7.50	10
	1:1.5	MD:WP	3:1	11.25	3.75	10
		MD:WP	1:1	7.50	7.50	10
		MD:WP	1:3	3.75	11.25	10

MD-Maltodextrin; RS-Resistant Starch; WP-Whey protein; DWP-Denatured whey protein

The suspensions were dispensed into sterilized and clean-drying glass bottles (**Figure 8**). Then, the samples were placed in the freezer (-18°C) for 24 h. The lyophilization was carried out by laboratory-scale Christ Alpha 2-4 freeze dryer (Martin Christ, Germany). The dried pressure and temperature were 0.250 mbar and 17°C, respectively. The drying process lasted for 3 days. The dried microcapsules were grounded manually under aseptic conditions, then transferred into sterilized vials, and stored at 4°C for future analysis. All preparations were conducted in duplicate.

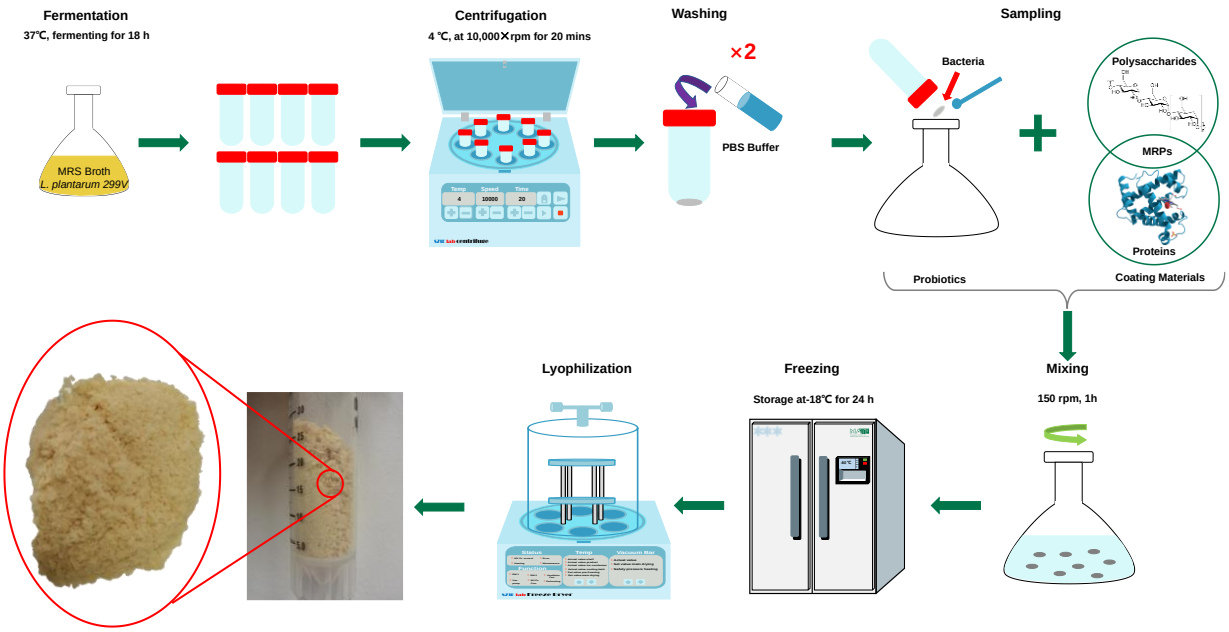

Figure 8. Scheme of microencapsulation process of *Lp. plantarum* 299v by lyophilization

3.2.3 Determination of viable cell numbers

The viable bacterial cells were enumerated by pour plating method using MRS agar and the serial dilutions were made with sterile 0.85% w/v sodium chloride solution. Generally, 0.5 mL or 0.01 g sample was mixed with 4.5 mL sterilized saline solution (0.85% w/v NaCl) and diluted to 10^{-7} in 1 mL with 10-fold stepwise. After that, 0.1mL samples were taken and poured on the MRS agar plate and incubated at 37 °C for 48-72 h (Nguyen et al., 2019) for the development of colonies. After incubation, the plates containing around 30-300 colonies were counted and expressed as CFU/g of dried samples or CFU/mL of solution. All enumerations were achieved in duplicate.

3.2.4 Determination of encapsulation yield

Encapsulation yield was determined by weighting the total amount of solid materials before and after lyophilization. The percentage of the ratio of total solid weight after and before lyophilization was depicted as yield (Eq. 1).

$$Y = \frac{m_t}{m_0} \times 100\% \qquad \text{(Eq. 1)}$$

Where Y refers to the yield (%), m_0 and m_t means the total solid weight (g) before and after lyophilization, respectively (Paula et al., 2019; Rajam & et al., 2015)..

3.2.5 Determination of bulk density

Bulk density was typified by measuring the volume of 1 g microcapsule sample in a 5 mL cylinder

after being tapped on a vortex for 2 min (Eq. 2).

$$\rho = \frac{m}{v} \quad \text{(Eq. 2)}$$

Where ρ is the bulk density (kg/m^3), m is the mass (kg) of the sample, and v is the volume (m^3) occupied in the cylinder (Rajam & Anandharamakrishnan, 2015; Sun et al., 2023).

3.2.6 Scanning electron microscopy

Scanning electron microscopy (SEM) was used to observe the morphological structure of *Lactiplantibacillus plantarum* 299v microcapsules with different core-to-wall ratios and wall material formulations. The samples were transferred and stuck on a plate in the vacuum chamber and gradually decreased to 200 Pa. Observation of the samples was carried out in Thermo Scientific^{TM} Prisma^{TM} E (Waltham, Massachusetts, USA) SEM under an accelerating voltage of 15 kV. The samples were examined under 1000x and 14000x magnifications (Sun et al., 2023).

3.2.7 Effect of storage

The viability of the microencapsulated probiotics during storage at 4 °C and at 25 °C was determined by enumeration on MRS agar. It was carried out every 2 weeks and the process lasted for 10 weeks. Moreover, both fermented and fortified apple juices were stored at 4°C and 25°C for 8 weeks. The samples were taken two weekly and viable cells were determined by the plate-counting method. All experimental runs were prepared in duplicate. Different kinetic models were regressed based on the experimental data obtained for the estimation of changes in viable cells during storage.

3.2.8 Tolerance study

Tolerance of probiotics in microcapsules during the digestion process was carried out at *in vitro* conditions (Dimitrellou et al., 2016; Nguyen et al., 2019; Rajam & Anandharamakrishnan, 2015). Briefly, 0.1g microcapsules of samples were added into 9.9 mL simulated gastric fluid (SGF) or simulated intestinal fluid (SIF). Samples were taken at the incubation time 0 h, 0.5 h, 1 h, 2 h, 3 h, and 0 h, 3 h, 6 h in the cases of SGF or SIF, respectively. Viable cell numbers were enumerated by the plate-counting method (Ashwar et al., 2018; Nguyen et al., 2019).

3.2.9 Application potential of microencapsulated probiotic bacteria

The probiotic capsules were applied for fermentation and fortification of apple juice. In the case of fermentation of apple juice, 0.2 g microcapsules were added into 90 mL apple juice and then

incubated at 37°C for certain hours. The fermentation process was monitored by the changing of pH, and it was completed when the pH value dropped to below pH 5.0. In the case of fortification of apple juice, 0.2 g microcapsules were directly added to 90 mL apple juice.

3.3 Statistical methods

All experiments were performed in duplicates and the results were presented as means ± standard deviation. ANOVA (analysis of variance), unpaired and paired Student's t-tests with a significance level of $\alpha = 0.05$ was used to determine statistical differences among the independent variables by using SPSS AU (www.spssau.com/en).

4. RESULTS AND DISCUSSION

4.1 Encapsulation of *Lp. plantarum* 299v strain with polysaccharides

Polysaccharides such as maltodextrin and starch are good polymers for the formation of networks for the encapsulation of bacterial cells. Maltodextrin has good solubility and low bulk density (Parvez et al., 2022), while resistant starch has a relatively weaker solubility and higher density. However, RS can reduce the pH value in the colon thus inhibiting the growth of harmful bacteria (Tian et al., 2016), and can be used by probiotics as a carbon source in the colon for human beings and animals (Chao et al., 2012; Chen et al., 2016; Tian et al., 2016). In my research, two polysaccharides maltodextrin and resistant starch were selected and encapsulations with different ratios of core-to-wall as well as the ratios of maltodextrin to resistant starch were designed and applied for lyophilization of probiotics.

4.1.1 Yield of encapsulation process

Yield is an essential parameter during the manufacturing, packaging, and storage process of probiotic microcapsules. The yields of the microencapsulation process were determined and summarized in **Figure 9**.

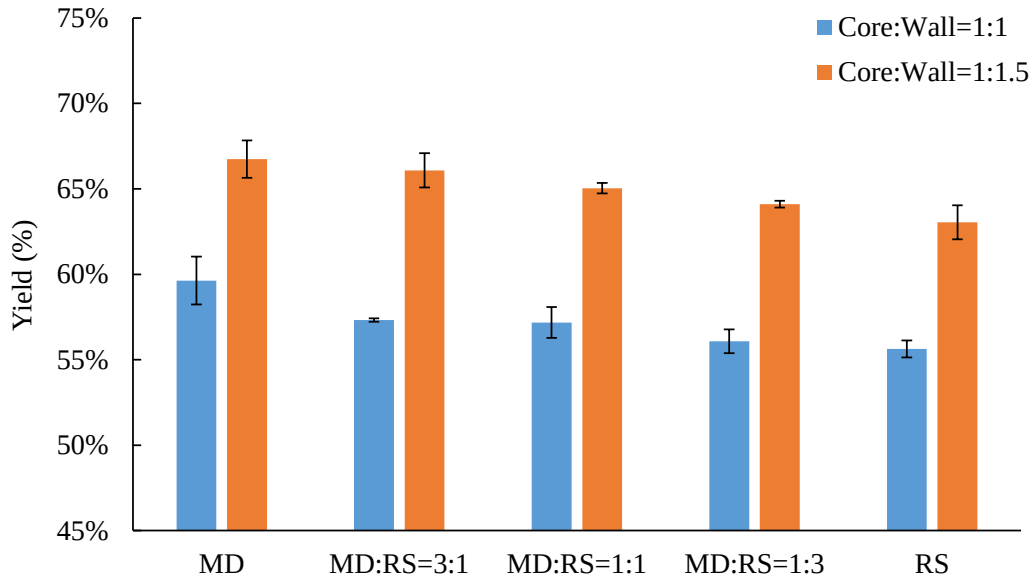

Figure 9. Yield of microencapsulated *Lp. plantarum* 299v with different ratios of core-to-wall and different ratios of wall materials MD: maltodextrin, RS: resistant starch

The yields varied from 55.63% to 59.63% with a ratio of core-to-wall 1:1 and 63.04% to 66.74% with a ratio of core-to-wall 1:1.5. The yields of samples with the ratios of core-to-wall 1:1.5 were significantly higher than those with ratios of core-to-wall 1:1. A possible explanation is that the

total water content in the probiotic samples before lyophilization is quite different. Under the same scale of probiotics, more dry coating materials will result in a higher total solid content after the lyophilization, which finally caused the significant difference in yields between the two groups. Meanwhile, the yield of microcapsules decreased synchronously with the decrease of MD content in the microcapsule complex. It is likely that MD has high solubility and more easily combines water molecules than RS (Parvez et al., 2022; Suryabhan et al., 2019), thus causing less water content loss during the lyophilization process.

4.1.2 Cell number and bulk density of microcapsules

The cell number of living microorganisms is an indicator for all the probiotic products, it is recommended by FAO/WHO with a minimal number of 6 log (CFU/g). Some researchers suggest that this number should be increased to 7 log (CFU/g) (Alfaro-Galarza et al., 2020). Nevertheless, most of the researchers have already used the microencapsulation method to achieve the minimum requirements with 6 log (CFU/g) or 7 log (CFU/g). Different types of porous maize starches (Li et al., 2016) and skim milk (Otero et al., (2007) were applied as a coating material to microencapsulate *Lp. plantarum* 299v and *L. gasseri* CRL1421 by the freeze-drying method and achieved the microcapsules with a cell count of 9.21 log (CFU/g) and 10.89 log (CFU/g), respectively.

In our case, the cell number varied from 10.01 CFU/g to 11.93 log (CFU/g) (**Figure 10**), the highest cell number of our microcapsules was quite larger than the research above mentioned. Therefore, in the real industrial production of probiotic products, more excipients are able to add, or fewer microcapsules are needed in order to save the production cost, while still achieving a relatively high probiotic cell number. In addition, probiotics coated with a ratio of core-to-wall 1:1.5 had a significantly higher viability than those of probiotics coated with a ratio of core-to-wall 1:1. One possible implication is that relatively more coating materials have a better-protecting ability during lyophilization. Similar results were obtained by Rajam and Anandharamakrishnan (2015), who microencapsulated *Lactiplantibacillus plantarum* (MTCC 5422) with fructooligosaccharide by spray-drying.

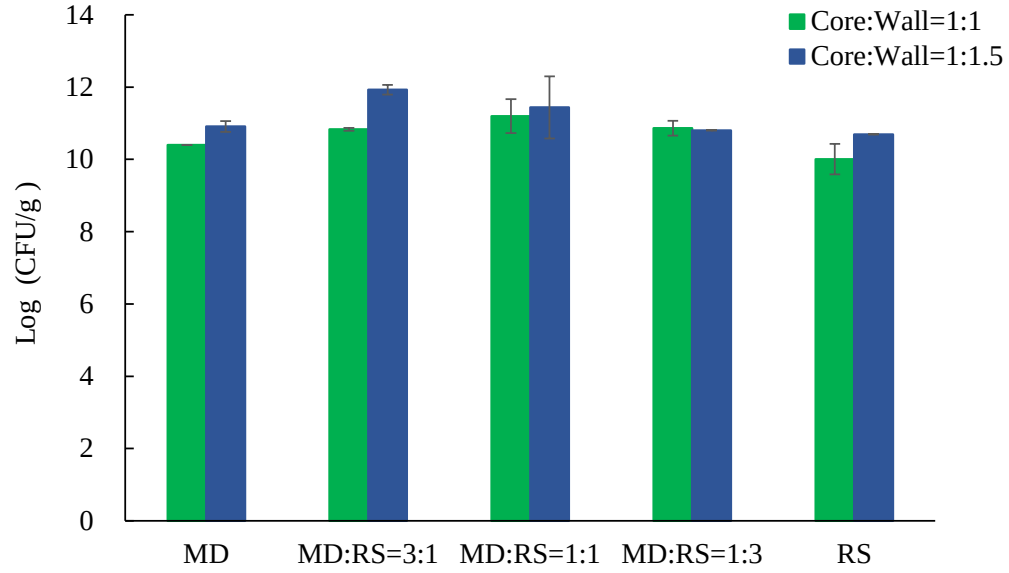

Figure 10. Cell number of microencapsulated *Lp. plantarum* 299v formed with different ratios of core-to-wall and of wall materials MD: maltodextrin, RS: resistant starch

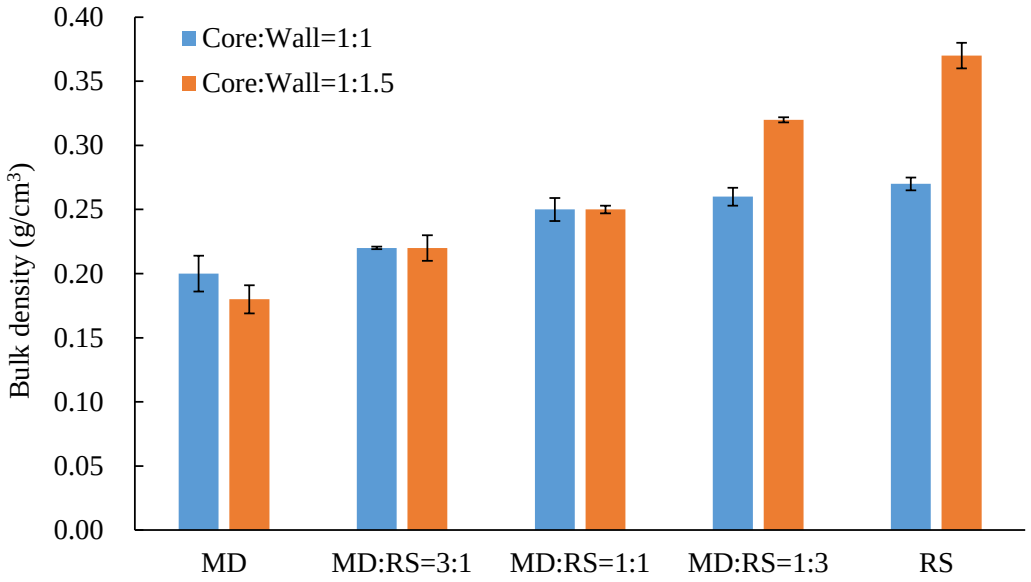

Figure 11. Bulk density of microencapsulated *Lp. plantarum* 299v formed with different ratios of core-to-wall and of wall materials MD- maltodextrin, RS- resistant starch

The bulk density ranged from 0.20 kg/m^3 to 0.27 kg/m^3 with ratios of core-to-wall 1:1 and 0.18 kg/m^3 to 0.37 kg/m^3 with ratios of core-to-wall 1:1.5 (**Figure 11**). It was obvious that with the decrease of ratios of MD in the microcapsules, the bulk density decreased in both groups of ratios of core-to-wall 1:1 and 1:1.5. It is probable that the natural characteristics of maltodextrin with spongy microcapsules and lower bulk density results in this phenomenon (Goyal et al., 2015; Karrar et al., 2021). Fuchs and co-workers (2006) reported a close relationship between bulk

density and the composition of microcapsules, i.e., a high bulk density value indicates high weight, which means small particle size. This characteristic also has a connection with the easy solubility of the powders. Hence, the bulk density of the microcapsules has some reference value for the application in the functional beverage.

4.1.3 Morphology

Scanning electron microscope (SEM) is an electron microscope that produces images of a sample by scanning the surface with a focused beam of electrons that can reflect the size and shape of microcapsule unit structure (Wang et al., 2022). Images of the microstructure of microencapsulated *Lp. plantarum* 299v with different ratios of core-to-wall and ratios of wall materials were shown in **Figure 12C&V**.

The rod-shaped free *Lp. plantarum* 299v cells were observed clustered together clearly under a magnification of 14000x **(Figure 12B)**. Besides, all other 14000x magnification microscopes demonstrate that the rod-shaped *Lp. plantarum* 299v cells were homogenously microencapsulated and covered in the coating materials. Moreover, the results of microencapsulated samples were in accordance with the conventional observation of matrix-type microcapsules that the cells were dispersed in the coating materials while there may present on the surface of the materials (Ahmad et al., 2019; Halim et al., 2017). This means that the probiotics are randomly distributed over the surface and within the microcapsules. Moreover, it was amazing that the surface of microcapsules with a ratio of core-to-wall 1:1.5 was smoother, the structure was more uniform, and the spherical shape was more abundant than in microcapsules with a ratio of core-to-wall 1:1. Initial observations suggest that there may be a link between this special surface structure and the increasing content of coating materials, especially the resistant starch content, which exposes more starch granules on the surface (Ahmad et al., 2019). Hence, the thicker coating materials may have not only a better protection ability, against harsh environmental conditions, but also serve as a protective barrier for cells with the function of preventing water uptake (Savedboworn et al., 2020). However, several tiny cracks and holes on the microcapsules were visible at higher magnification of samples with a ratio of core-to-wall 1:1. It is almost certain that during the microencapsulation process, the microcapsules are first subjected to low temperature and lead to the formation of the ice crystals from the residual water present. Hence, the sublimation of the ice crystals under vacuum conditions results in the formation of porous structures on the dried microcapsules (Ashwar et al., 2018).

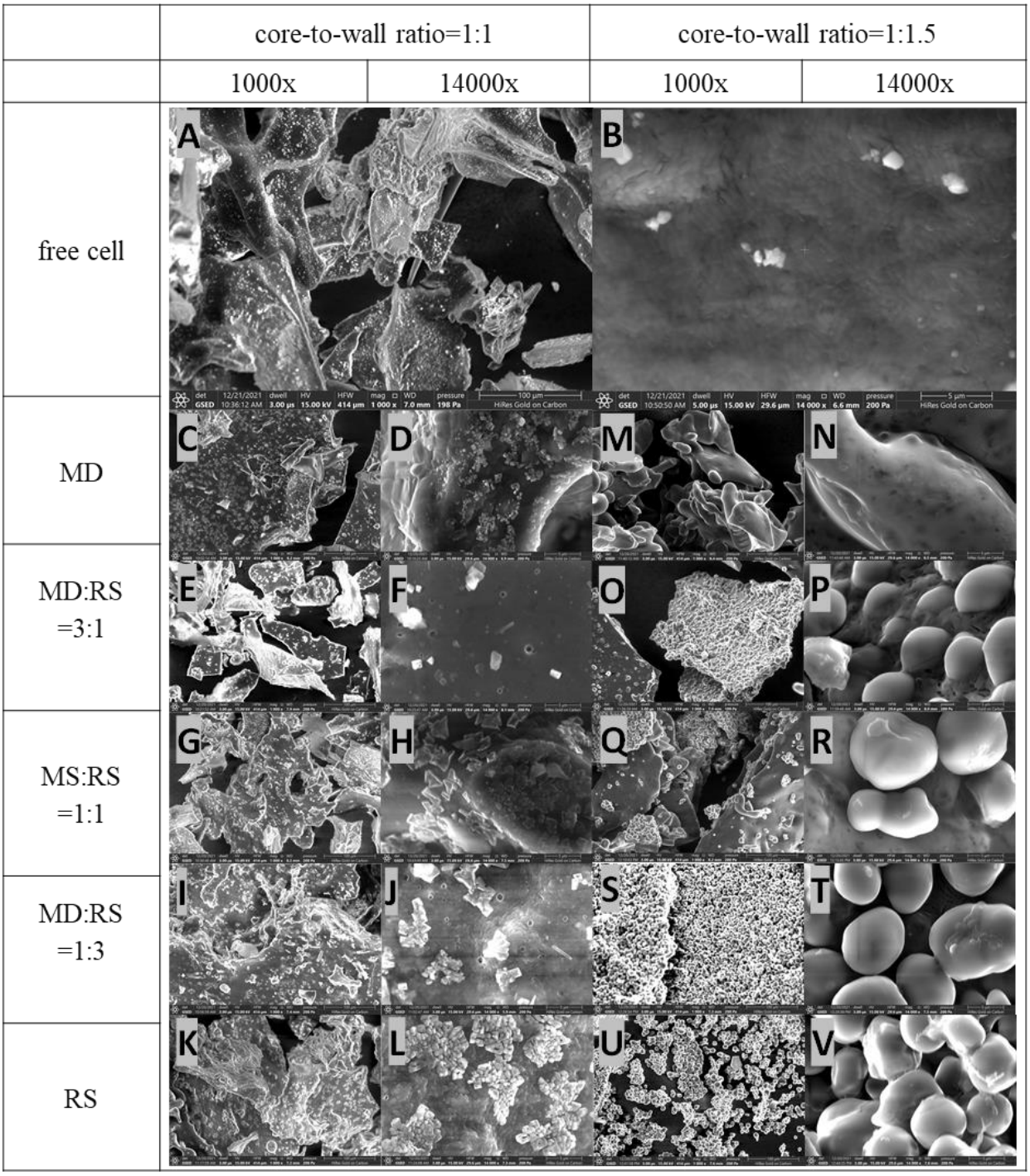

Figure 12. Scanning electron microscope (SEM) images of *L. plantarum* 299v cells under 1000x and 14,000x magnification

A, B are free cells; C, D are ratios of core-to-wall 1:1 and MD; E, F are ratios of core-to-wall 1:1 and MD:RS 3:1; G, H are ratios of core-to-wall 1:1 and MD:RS 1:1; I, J are ratios of core-to-wall 1:1 and MD:RS 1:3; K, L are ratios of core-to-wall 1:1 and RS; M, N are ratios of core-to-wall 1:1.5 and MD; O, P are ratios of core-to-wall 1:1.5 and MD:RS 3:1; Q, R are ratios of core-to-wall 1:1.5 and MD:RS 1:1; S, T are ratios of core-to-wall 1:1.5 and MD:RS 1:3; U, V are ratios of core-to-wall 1:1.5 and RS; MD: maltodextrin; RS: resistant starch

4.1.4 Effect of storage on viability

The viability of the probiotics is the most important indicator to measure whether the probiotic products meet the standard of WHO. However, membrane lipid oxidation during the storage process may cause the viability loss of the microencapsulated probiotics (Rajam & Anandharamakrishnan, 2015). Hence, analysing the viability change of the microencapsulated probiotics focuses on protecting ability of coating materials, temperature, etc., during storage process can not only check the quality of the microcapsules but also predict the shelf-life of the microcapsules.

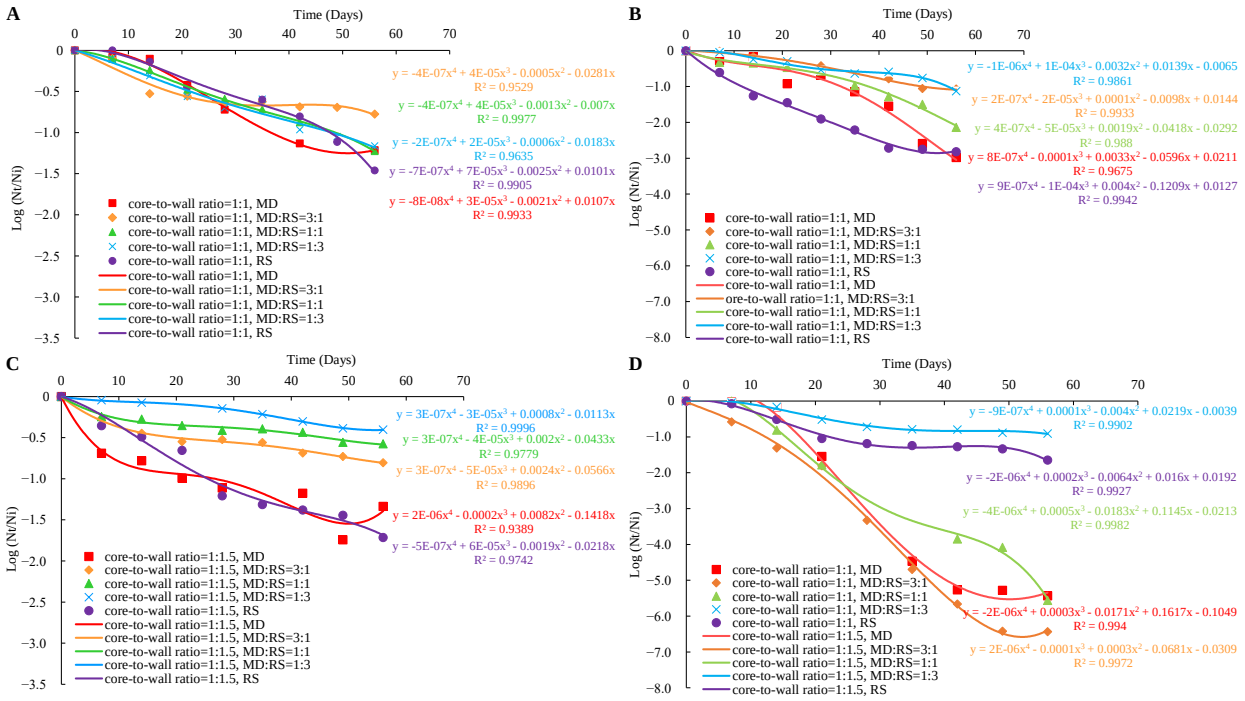

Figure 13. Viability loss of lyophilized *Lp. plantarum* 299v with different wall ratios of core-to-wall and different ratios of wall materials at 4°C and 25°C

A: 4°C, ratios of core-to-wall 1:1; B: 25°C, ratios of core-to-wall 1:1; C: 4°C, ratios of core-to-wall 1:1.5; D: 25°C, ratios of core-to-wall 1:1.5; MD- maltodextrin, RS- resistant starch

The effectiveness of different ratios of core-to-wall and ratios of wall materials on the storage stability of microencapsulated *Lp. plantarum* 299v stored at 4°C and 25°C for 8 weeks were demonstrated in **Figure 13**. In the case of samples stored at 4°C with a ratio of core-to-wall 1:1 (**Figure 13A**), probiotics coated with MD:RS 1:1, MD:RS 1:3, and RS almost had the same constant viability loss rate, the only difference was that the viability decreased sharply in case of probiotics coated with RS from the end of the 6th weeks. As for probiotics coated with MD started the viability loss from the 1st week till the 6th week, and from then on, the viability seemed no change. Moreover, it was a similar trend for probiotics coated with MD:RS 3:1. However, the

unchanged state started from the 4th week. It seems possible that these results are due to probiotics coated with MD and MD:RS 3:1 was protected well by the coating materials with a dense structure and only the probiotics exposed outside were oxidized during the first 6 and 4 weeks of storage. In the case of samples stored at 4℃ with a ratio of core-to-wall 1:1.5 (**Figure 13C**), the viability loss of probiotics coated with a mixture of MD and RS is significantly lower than probiotics coated with a single material.

In the case of samples stored at 25℃ with a ratio of core-to-wall 1:1 (**Figure 13B**), probiotics coated with MD:RS 3:1 had a constant viability loss rate for 8 weeks of the experiment. Similarly, probiotics coated with MD:RS 1:3, MD:RS 1:1, and MD had a constant viability loss rate in the previous weeks. However, from the 4th week and the 7th week, the viability of probiotics coated with MD:RS 1:1, MD, and probiotics coated with MD:RS 1:3 decreased sharply, respectively. As for probiotics coated with RS, the viability losing rate was almost constant until the 7th week, from the 7th to the 8th week, this viability remained unchanged. The same explanations can be addressed here that the formation of a dense structure after oxidation and absorption of water molecules for several weeks changed the structure of the microcapsules. In the case of storage at 25℃ with a ratio of core-to-wall 1:1.5 (**Figure 13D**), probiotics coated with MD, MD:RS 3:1, and MD:RS 1:1 decreased the viability sharply in the first 7 weeks. From the 7th to the 8th week, the viability of probiotics coated with MD:RS 1:1 decreased even more seriously than in the previous 7 weeks, while the viability of probiotics coated with MD and MD:RS 3:1 remain unchanged. Moreover, the viability loss rate of probiotics coated with MD:RS 1:3 and RS almost remains constant and is significantly lower than that of the three samples mentioned above. It seems possible that these results are due to RS content has a positive effect on the protecting ability of the probiotics under the circumstance of a ratio of core-to-wall 1:1.5 during the long-term storage at 25℃.

In summary, all the microencapsulated probiotics lost viability during the storage. However, the viability changing patterns of *Lp. plantarum* 299v were different when it comes to microcapsules coated with MD and RS with different ratios of core-to-wall and ratios of wall materials. It indicated that the compactness and oxidation resistance of each microcapsule was quite different, even during the different storage stages, because some microcapsules can form a dense structure even after several weeks of storage. Lower storage temperature can decrease the oxidation rate of the cell membrane, thus decreasing the viability loss rate.

4.1.5 Tolerance study

The stomach is the main digestion organ of human beings with low pH, which is extremely harmful to probiotics. Moreover, the high bile salt in the gastrointestinal region is also a stress factor that

has harmful damage to the probiotics. Therefore, the resistance of microencapsulated probiotics to harsh environments is a crucial property of microcapsules (Tao et al., 2019). Hence, the investigation of those factors mentioned above may have some guidance on the production of microcapsules to achieve the goal of high survival ability during the simulated gastric and intestinal digestion process. The viability of lyophilized *Lp. plantarum* 299v with different ratios of core-to-wall and different ratios of wall materials after being exposed to the simulated gastric fluid containing 0.3% pepsin for 3 h at 37°C were shown in **Figure 14**.

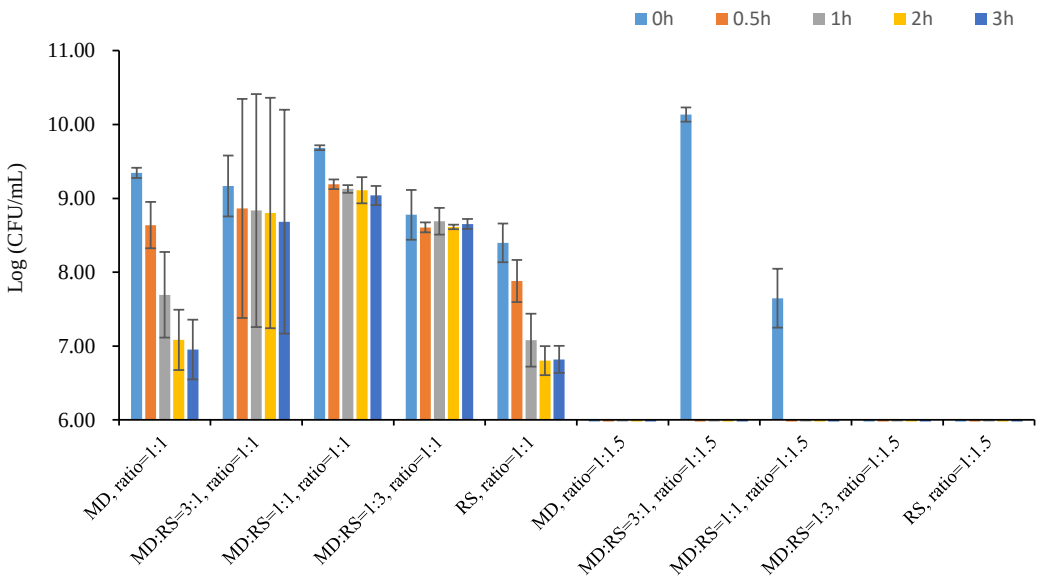

Figure 14. Viability of lyophilized *Lp. plantarum* 299v with different ratios of core-to-wall and of wall materials after exposed to SGF for 3 h at 37°C
MD: maltodextrin, RS: resistant starch

As can be seen in **Figure 14**, almost all the microencapsulated probiotics were shown a loss of viability during the SGF digestion process. This proved that low pH or high acidity as a harsh factor for the probiotics is almost certain (Liao et al., 2017; Rajam & Anandharamakrishnan, 2015; Tao et al., 2019). In addition, as for the samples with a ratio of core-to-wall 1:1, the average reduction of each sample after 3 h SGF digestion is 2.39 log (CFU/g), 0.48 log (CFU/g), 0.65 log (CFU/g), 0.12 log (CFU/g), and 1.58 log (CFU/g), respectively. It demonstrated that samples added resistant starch enhanced the resistance of probiotics to low pH values. Similar results were found by Li and co-workers (2016). The final cell number after 3 h SGF digestion of each sample is 6.95 log (CFU/g), 8.68 log (CFU/g), 9.04 log (CFU/g), 8.65 log (CFU/g), and 6.82 log (CFU/g), respectively. Samples coated with a mixture of MD and RS had a higher survival ability than samples coated with a single material, which might be that the combined wall material has synergistic resistance to simulated gastric fluid compared to the single coating material. Moreover,

many researchers (Chao et al., 2012; Chen et al., 2016; Tian et al., 2016) mentioned that RS can reduce the pH, inhibit the growth of harmful bacteria, and can be utilized by probiotic bacteria as a carbon source in the colon, in our case, RS coated microcapsules did not have high cell number after SGF test. Therefore, the target should still focus on the high viability of the probiotics after passing through the digestion process against low pH. Furthermore, samples with ratios of core-to-wall 1:1 and MD:RS 1:1 had the best protecting ability in SGF, probably not only due to the protecting ability of the combined wall material but also because it has a relatively high initial cell number of the microcapsules.

Bile salt is the compound that involves the digestion of fat content, and high bile salt concentration is the main factor that can influence the survival of the probiotics apart from low pH. Hence, the survival ability of the probiotics against bile salt is a significant property (Liao et al., 2017; Tao Tao et al., 2019). Therefore, the tolerance of lyophilized *Lp. plantarum* 299v with different ratios of core-to-wall and different ratios of wall materials after being exposed to SIF for 6 h at 37°C were shown in **Figure 15**.

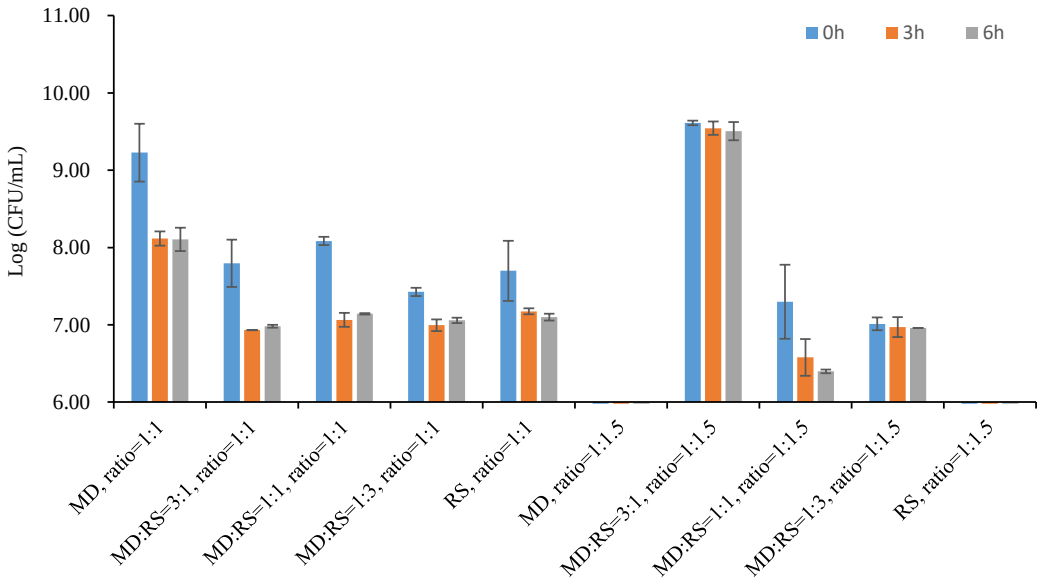

Figure 15. Viability of lyophilized *Lp. plantarum* 299v with different ratios of core-to-wall and of wall materials after exposed to SIF for 6 h at 37°C
MD: maltodextrin, RS: resistant starch

As can be seen from **Figure 15**, after 6 h SIF digestion, the final cell number of each sample was 8.11 log (CFU/g), 6.98 log (CFU/g), 7.14 log (CFU/g), 7.06 log (CFU/g) and 7.10 log (CFU/g) in the group with a ratio of core-to-wall 1:1 and <6.0 log (CFU/g), 9.51 log (CFU/g), 6.40 log (CFU/g), 6.96 log (CFU/g) and <6.0 log (CFU/g) in the group with ratios of core-to-wall 1:1.5. Among these two groups, the sample with a ratio of core-to-wall 1:1.5 and MD:RS 3:1 had the

highest 9.51 log (CFU/g) with only 0.11 log (CFU/g) reduction after 6 h SIF digestion. This discrepancy could be attributed to the formation of a dense structure during the storage or SIF digestion process. Besides, although the cell counts of microcapsules in the group with a ratio of core-to-wall 1:1 did not reach the maximum value, it was still promising that the viability of all microencapsulated probiotic samples is higher than 6 log (CFU/g).

4.1.6 Application of microcapsules in fermented and fortified apple juice

The stability of microencapsulated cells is quite different from diverse food matrices, even for the same kind of food matrices, the properties will change after the fermentation process. Hence, the stability of the microencapsulated cells in food matrices is related not only to the characteristics of the food matrices but also has a linkage with the coating materials (Saarela et al., 2006). Ying and co-workers (2013) observed that the whey protein isolate (WPI) or WPI combined with resistant starch (RS) had better-protecting ability than singular RS on *Lactobacillus rhamnosus* GG under all storage conditions. Zhang and co-workers (2020) suggested using a mixture of 5.0% (w/v) lactose, mannitol, trehalose, ascorbic acid, gelatine, and 10.0% (w/v) skim milk to access the optimal protecting composition by freeze-drying for *Pseudoalteromonas nigrifaciens*. In our research, the viability of microencapsulated *Lp. plantarum* 299v in fermented and fortified apple juice at 4℃ for 11 weeks and 25℃ for 8 weeks are shown in **Figure 16**.

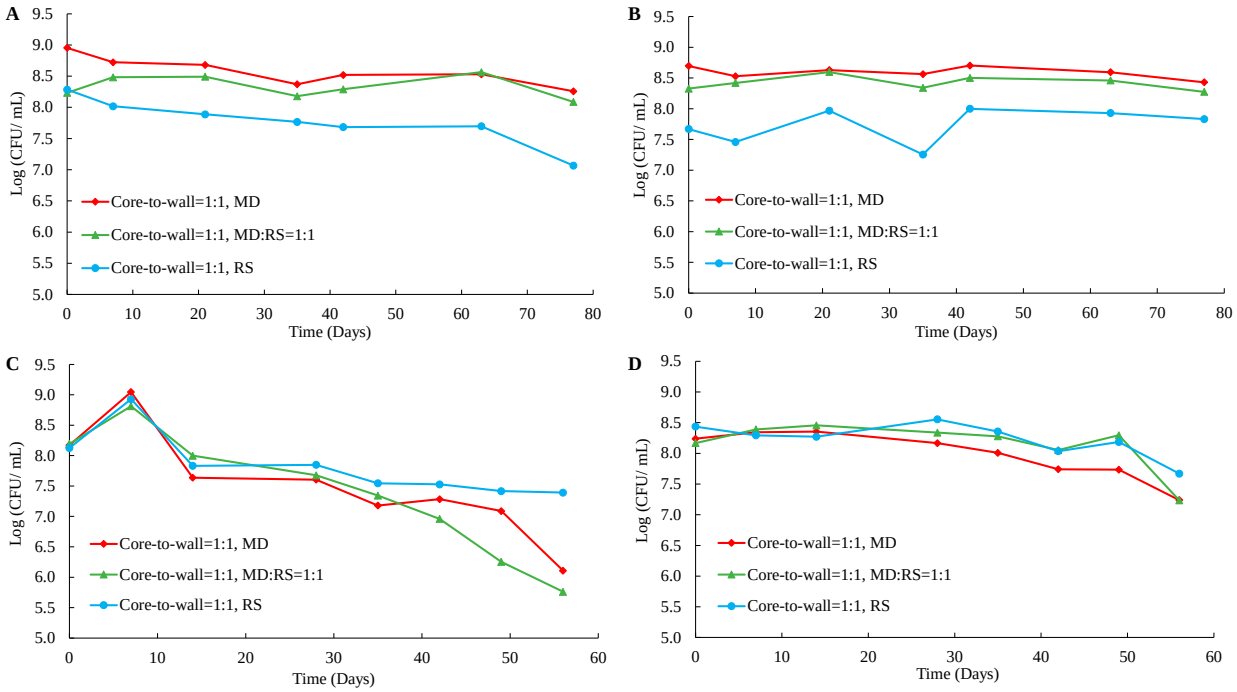

Figure 16. Viability of microencapsulated *Lp. plantarum* 299v in fermented and fortified apple juice at 4℃ for 11 weeks and 25℃ for 8 weeks

A: 4℃, Fermented; B: 4℃, Fortified; C: 25℃, Fermented; D: 25℃, Fortified;
MD: maltodextrin, RS: resistant starch

Based on **Figure 16**, after 8 weeks of storage, only the sample coated with ratios of core-to-wall 1:1 and MD:RS 1:1 that storage at 25℃ with fermented process did not meet the recommended level announced by WHO and FAO (Brinques & Ayub, 2011; Cheow et al., 2016; Nguyen et al., 2019). Moreover, samples stored under 4℃ for 11 weeks had significantly higher viability than samples stored under 25℃ for 8 weeks. The result suggested that lower temperature is more suitable for maintaining the viability of the microencapsulated probiotics (Li et al., 2019). In the case of **Figure 16C**, the initial cell number showed an increase after a weak and then it rapidly decreases. It is possible that the cells multiplied in the capsule did not get enough nutrients later and died faster or probably water activity inside the capsule was insufficient for the bacterial enzymes to function. In the meantime, there was a significantly higher viability in the apple juice fortified by probiotics than those fermented under the same storage temperature, which means the fortified methods were more suitable for maintaining the viability of probiotics in the apple juice. Furthermore, the viability of the probiotics coated with ratios of core-to-wall and MD as well as ratios of core-to-wall and MD:RS 1:1 in the fortified juices at 4°C, was higher than those coated with RS. The reduced viability of the sample with MD:RS 1:1 was lower than probiotics coated with MD. Hence, the more suitable condition for the application of *L. plantarum* 299v in apple juice to achieve the goal of high survival ability in this functional beverage is listed as follows: ratios of core-to-wall 1:1 and MD:RS 1:1 for the coating material, with a fortified process, and storage at 4℃.

4.2 Encapsulation of *Lp. plantarum* 299v strain with proteins

In my research, two proteins, whey proteins (WP) and denatured whey protein (DWP) were selected and encapsulations with different ratios of core-to-wall as well as the ratios of the two coating materials were designed and applied for lyophilization of *Lp. plantarum* 299v strain.

Whey proteins (WP) are the by-product of the dairy industry, and are rich sources of valuable biological proteins as well as riboflavin and minerals (Eckert et al., 2017). WP is considered an exceptional coating material due to their specific physical and chemical properties, such as excellent emulsification, superb gelation, and exquisite fill-forming properties. Denatured whey proteins (DWP) are originated from WP by treatment with acid or heat, which can contribute to some specific properties including high tensile property and low oxygen permeability (Goyal et al., 2015; Moayyedi et al., 2018; Rajam & Anandharamakrishnan, 2015; Ying et al., 2013). Based on the concept of development of new value-added applications of dairy wastes to enlarge the application area and avoid discarding the whey protein, it is appropriate to adopt the practice of using whey proteins as coating materials for the microencapsulation process (Rama et al., 2019).

4.2.1 Yield of encapsulation process

Yield is a key parameter during the manufacturing, packaging, and storage process of probiotic microcapsules. The yields of encapsulation process of *Lp. plantarum* 299v strain with two ratios of core-to-wall and five ratios of wall materials after lyophilization were shown in **Figure 17**. These values varied from 49.35% to 68.02%. Additionally, the samples with a ratio of core-to-wall 1:1.5 had a significantly higher yield than samples with a ratio of core-to-wall 1:1. It is likely caused by the wetness of the probiotics core. With the same scale of wet probiotics, the higher amount of dry powder will result in a higher total solid amount after the drying process. Meanwhile in the case of samples with a ratio of core-to-wall 1:1, the yield of the sample with only DWP as coating material was significantly lower than the others, whereas, in the case of samples with a ratio of core-to-wall 1:1.5, the yield of the sample with only WP was the lowest. It is possible that ratios of core-to-wall 1:1 and WP, as well as ratios of core-to-wall 1:1.5 and DWP have a poor combination of water molecules together with probiotic bacteria, hence causing more water loss during the lyophilization process.

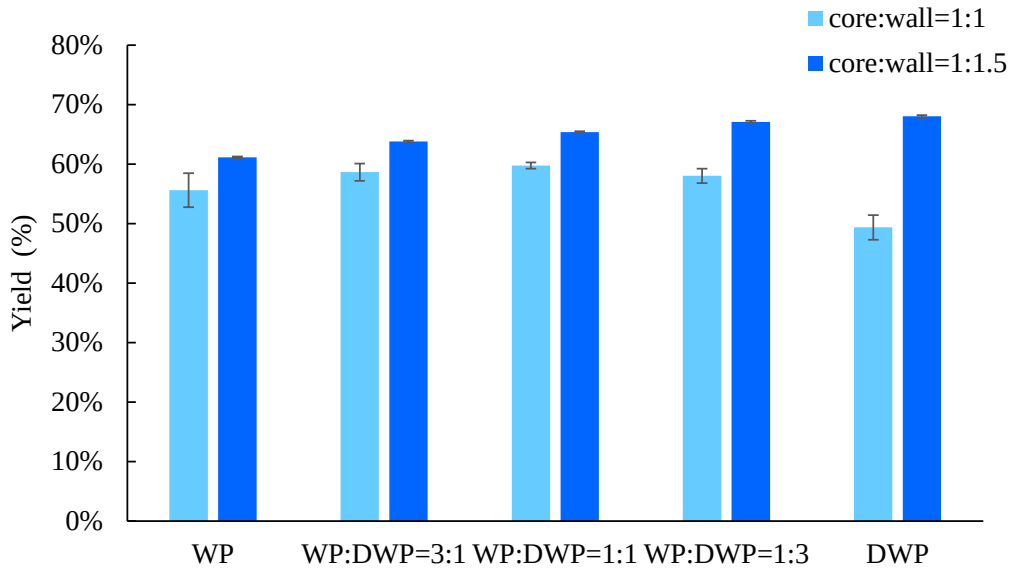

Figure 17. Yield of microencapsulated *Lp. plantarum* 299v strain with different ratios of core-to-wall and ratios of wall materials WP: whey protein, DWP: denatured whey protein

4.2.2 Cell number and bulk density of microcapsules

The viable cell number or cell number of the microcapsules is a noteworthy indicator for checking the quality of probiotic products (Kavitake et al., 2018). The cell number of microencapsulated *Lp. plantarum* 299v samples varies from 7.66 to 11.34 log (CFU/g) (**Figure 18**). All samples showed numeration above 6 log (CFU/g) values, the minimal requirement of probiotics as a functional food (FAO/WHO, 2001). Additionally, the cell number was approximately 10 log (CFU/g) of most

samples except the DWP sample. These results are similar to the values published in the studies of *Kluyveromyces marxianus* VM004 (Vanden Braber et al., 2020) and *Bifidobacterium animalis* subsp. *lactis* INL1 (Loyeau et al., 2018) when they microencapsulated microorganisms with whey protein and spray-drying technology. Besides, the cell number of samples with a ratio of core-to-wall 1:1 was significantly higher than that of those samples with a ratio of core-to-wall 1:1.5. It is possible that the lower ratios of wall materials leading to a higher number of probiotic bacteria in a certain microcapsules complex, which can result in a higher cell number in truth. In addition, the cell number of the samples with WP and WP:DWP 3:1 was significantly higher than samples with WP:DWP 1:1, WP:DWP 1:3, and DWP; the samples with WP and WP:DWP 1:1 was significantly higher than samples with DWP; the samples with WP:DWP 1:1 and WP:DWP 3:1 was significantly higher than samples with DWP. There is a possibility that the stable membrane stabilization properties of whey proteins, which can avoid cell damage, and with a decrease in ratios of core-to-wall of whey protein in the microcapsules, more probiotic bacteria lost their viability during the lyophilization process.

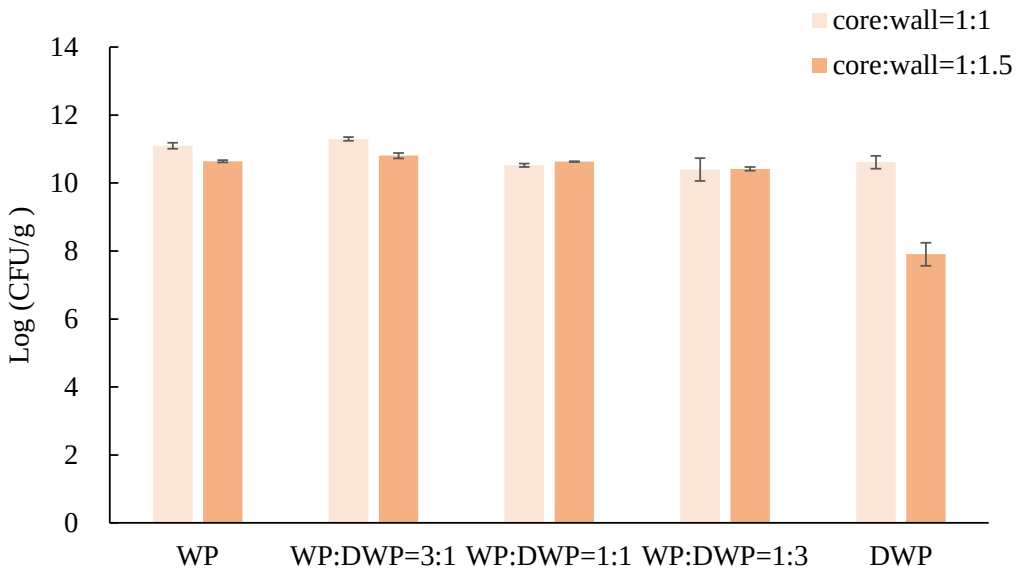

Figure 18. Cell number of microcapsules with different ratios of core-to-wall and ratios of wall materials WP: whey protein, DWP: denatured whey protein

The bulk densities were between 0.23 g/cm^3 to 0.27 g/cm^3 (**Figure 19**), which were lower than the results (approximately 0.5 g/cm^3) reported by Rajam and Anandharamakrishnan (2015). The difference may be due to the use of different coating materials and drying technology, the combined whey proteins, and fructooligosaccharide as coating materials and applied spray-drying technology. There is a possibility the high-water evaporation ability of the spray-drying method, resulted in less water content remaining in the microcapsules while using lyophilization in our case. Furthermore, there was no significant difference in bulk density between different ratios of

wall materials. However, the bulk density of samples with a ratio of core-to-wall 1:1 was significantly higher than that of samples with a ratio of core-to-wall 1:1.5. The different ratios of coating materials resulted in a fluffier structure and in a significantly lower bulk density of samples with a ratio of core-to-wall 1:1.5 than 1:1.

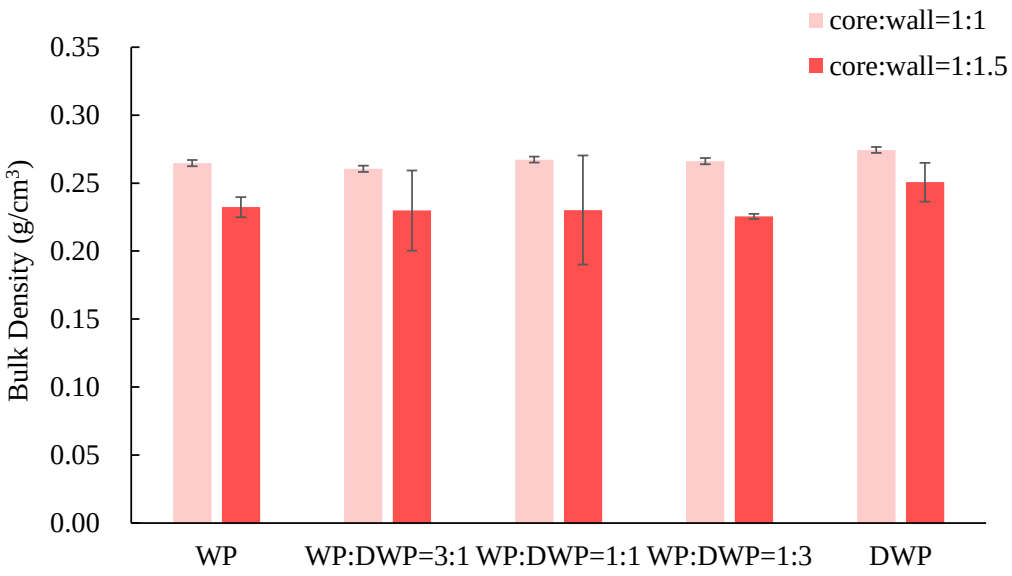

Figure 19. Bulk density of microcapsules with different ratios of core-to-wall and ratios of wall materials WP: whey protein, DWP: denatured whey protein

4.2.3 Morphology

A scanning electron microscope (SEM) can generate pictures of a sample by scanning the surface with a focused beam of electrons that can reflect the size and shape of the structure of the microcapsules (Wang et al., 2022). The micrographic images of free and microencapsulated *Lp. plantarum* 299v strain with whey protein and denatured whey protein were shown in **Figure 20**. The structure of the microcapsules under 1,000x magnification was irregular and rod-shaped cells are clustered together and closely aligned under 14,000x magnification. It is almost certain that the evaporation of water during the lyophilization results in a condensed structure and can be seen clearly with large magnification (Savedboworn et al., 2020). Furthermore, the cells of *Lp. plantarum* 299v can be found in microcapsules with different coating materials (**Figure 20 C–V**), which are similar to the results reported by Savedboworn and co-workers (2020). The thick materials and tight arrangement of the probiotic might work as protective walls and prevent water uptake. However, there were still some free cells that can be found on the surface of the coating materials.

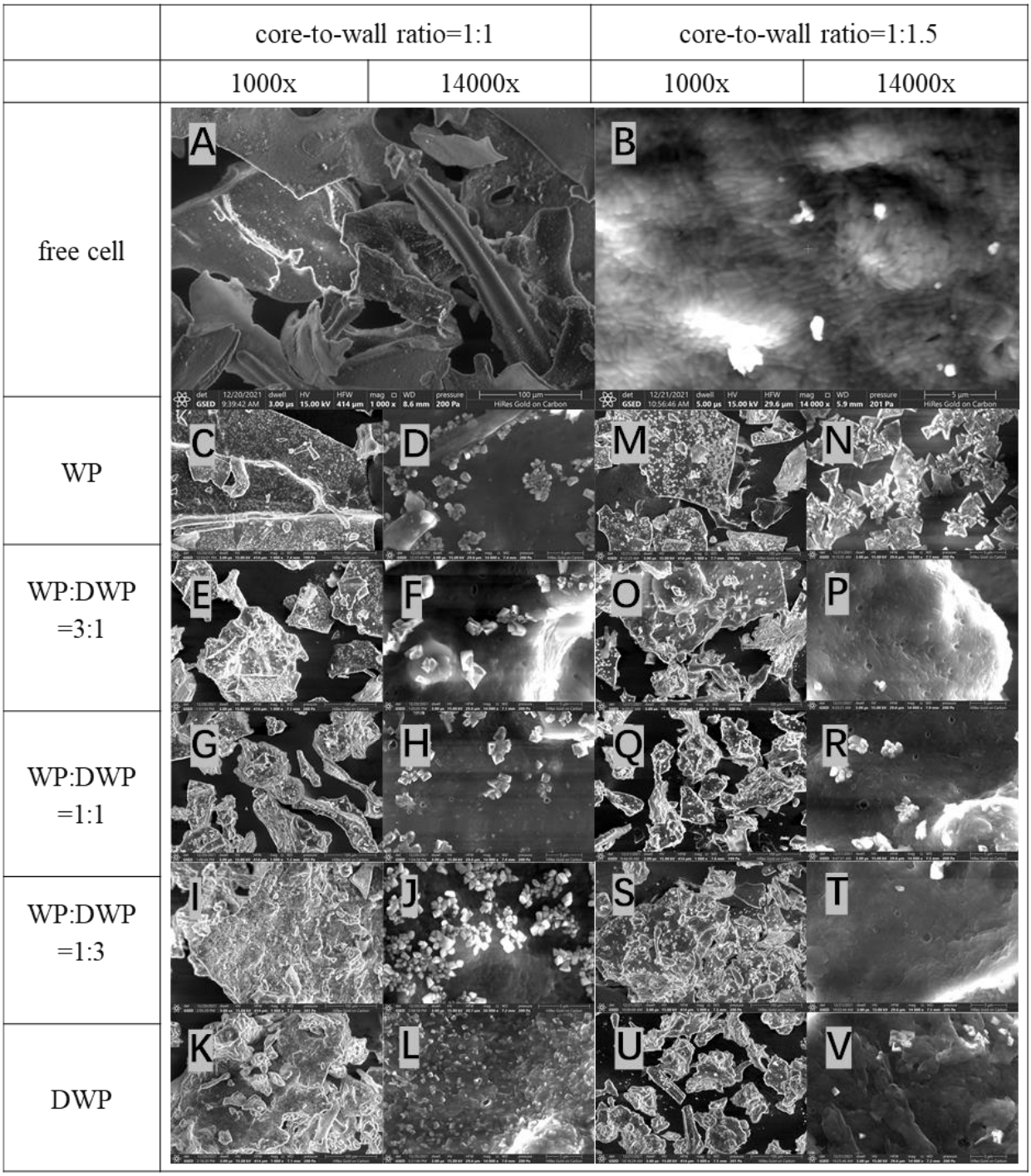

Figure 20. Scanning electron microscope (SEM) images of *Lp. plantarum* 299v cells under 1,000x and 14,000x magnification

A, B are free cells; C, D are ratios of core-to-wall 1:1 and WP; E, F are ratios of core-to-wall 1:1 and WP:DWP 3:1; G, H are ratios of core-to-wall 1:1 and WP:DWP 1:1; I, J are ratio 1:1 and WP:DWP 1:3; K, L are ratios of core-to-wall 1:1 and DWP; M, N are ratios of core-to-wall 1:1.5 and WP; O, P are ratios of core-to-wall 1:1.5 and WP:DWP 3:1; Q, R are ratio core-to-wall 1:1.5 and WP:DWP 1:1; S, T are ratio core-to-wall 1:1.5 and WP:DWP 1:3; U, V are ratios of core-to-wall 1:1.5 and DWP; WP: whey protein and DWP: denatured whey protein

This inspection is in accordance with traditional features for matrix-type microcapsules that core molecules are commonly distributed in the coating materials, while some core materials may also

present on the surface (Ahmad et al., 2019; Halim et al., 2017). In addition, several tiny cracks and holes on the microcapsules were visible at higher magnification on the microcapsules on samples with a ratio of core-to-wall 1:1 and a ratio of core-to-wall 1:1.5. Halim and co-workers (2018) revealed that the cracking-like structure was found on the surface of chitosan, while it was not observed on the samples with alginate beads and free cells. It is likely that the specific structure and unique characteristics of whey protein and denatured whey proteins. Furthermore, there was no significant morphology difference as well as in the bulk density between the samples in the group of a ratio of core-to-wall 1:1 and a ratio of core-to-wall 1:1.5. Morphology of particles plays important roles in the formation of some physical properties, such as directly influences the bulk density, flowability and rehydration characteristics of the powders (Rajam & Anandharamakrishnan, 2015). However, in the group of samples with a ratio of core-to-wall 1:1.5 under 14,000x magnification, the sample coated with DWP with a ratio of core-to-wall 1:1.5 seemed to have more folds and bulges than other samples with a ratio of core-to-wall 1:1.5, which may be linked to the effect of atomization mechanism and film properties of WP and DWP for the microencapsulation process.

4.2.4 Effect of storage on viability

Viability during manufacturing or storage is also a fundamental standard for the use of probiotic products. Many researchers have demonstrated that microencapsulated probiotics samples have significantly higher cell density than those samples without microencapsulation (Guerin et al., 2017; K. Li et al., 2019; Liao et al., 2017; Muhammad et al., 2017). Specifically, the membrane lipid oxidation is the main reason that caused the viability loss (Rajam & Anandharamakrishnan, 2015). Thus, protection ability or forming ability of coating materials, temperature, water activity, oxygen content, etc. are the main factors during storage. The effectiveness of ratios of core-to-wall and ratios of wall materials on the storage stability of microencapsulated *Lp. plantarum* 299v were investigated. The storage was done at 4 °C and 25 °C for 70 days and the results were demonstrated in **Figure 21**.

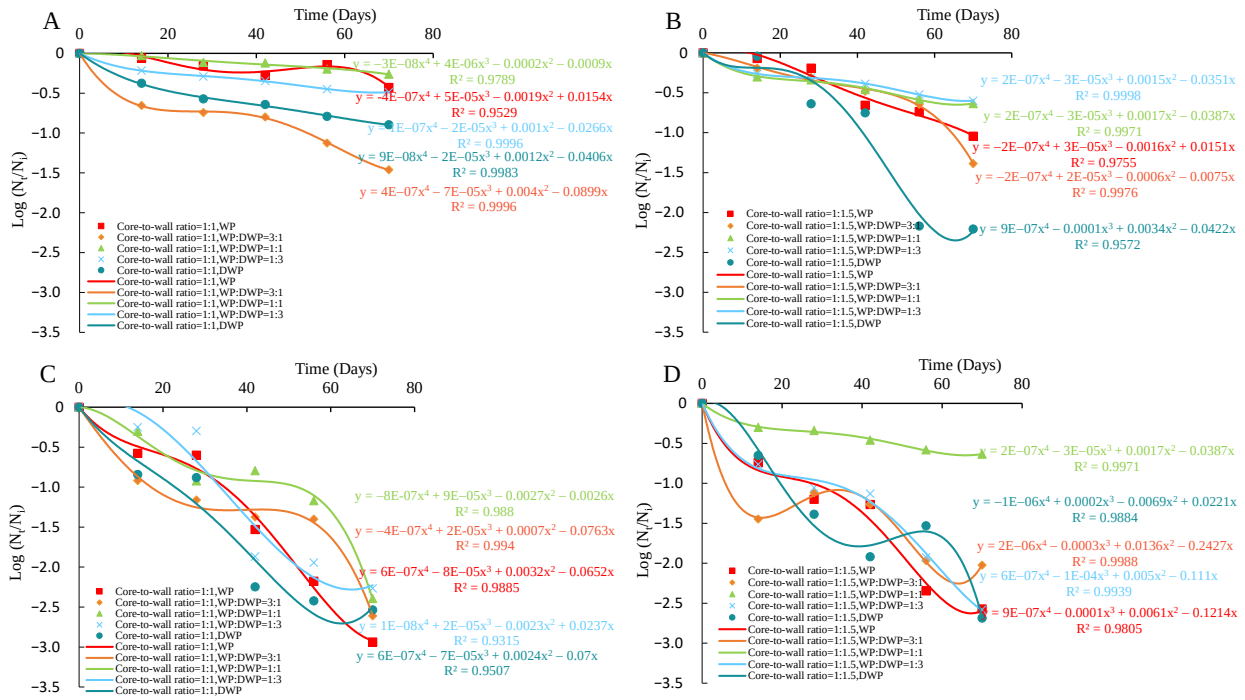

Figure 21. Viability loss of lyophilized *Lp. plantarum* 299v strain with different ratios of core-to-wall and ratios of wall materials at 4 °C and 25 °C A: 4 °C, ratios of core-to-wall 1:1; B: 4 °C, ratios of core-to-wall 1:1.5; C: 25 °C, ratios of core-to-wall 1:1; D: 25 °C, ratios of core-to-wall 1:1.5; WP: whey protein, DWP: denatured whey protein

In the case of samples stored at 4 °C with a ratio of core-to-wall 1:1, the cell number decreased gradually in the first two weeks, then it remained constant till the 6th week. After that the cell number started decreasing again. A similar cell-losing pattern was found on samples coated with WP. In this case, the cell number started decreasing sharply after the 8th week. This may be due to the stability of the coating materials with a ratio of core-to-wall 1:1 and WP:DWP 3:1 as well as a ratio of core-to-wall 1:1 and WP. However, the losing dynamic was quite different for samples coated with WP:DWP 1:1, WP:DWP 1:3, and DWP. The cell number of those three samples started decreasing from the beginning of the storage process and after 4 weeks with almost a constant loss rate. In the case of storage at 4 °C with a ratio of core-to-wall 1:1 and 1:1.5, samples coated with DWP exhibited a significantly higher loss rate in cell number than the other samples. The cell number decreased slowly in the first 4 weeks and then it started decreasing faster. Samples coated with WP:DWP 3:1 had the same losing pattern as samples coated with DWP after storage for 6 weeks. This may be due to the stability of the coating materials DWP and DWP 3:1 with ratios of core-to-wall 1:1.5. The cell numbers decreased sharply after storage for 4 and 6 weeks under 4 °C, respectively. Additionally, samples coated with WP, WP:DWP 1:1, and WP:DWP 1:3 shared the same losing dynamic.

In the cases of samples stored at 25 °C with a ratio of core-to-wall 1:1 of bacteria to coating material, the cell numbers of samples coated with WP:DWP 3:1 and WP:DWP 1:1 exhibited the same trends, i.e., decreased in the first 4 weeks, then kept constant till the 8th week, and at the end phase, they dropped drastically. Generally, the cell numbers of samples with a ratio of core-to-wall 1:1 stored at 25 °C had a higher loss rate than those of samples stored at 4 °C. There is a possibility that the change of moisture of the microcapsules and thus caused the disruption and deactivation of the cell membrane. The increase in moisture content during the storage period may be linked to the absorption of water from the environment (Minj & Anand, 2022). In the case of samples stored at 25 °C with a ratio of core-to-wall 1:1.5, the cell number of samples coated with WP:DWP 1:1 had a significantly lower loss rate than by other ratios of wall materials. It is possible due to the relatively condense structure. The cell number of samples coated with WP and WP:DWP 1:3 started to decrease in the first two weeks and almost remained constant till the 4th week of storage. After that, it decreased again until the end of the experiment. Moreover, the cell number of samples coated with WP:DWP 3:1 and DWP showed quite a different pattern compared with those cell number losing styles. After decreasing for 2 weeks and 6 weeks, they increased until the 6th and 8th week, respectively, and then decreased again until the end. These results indicated that the ratios of core-to-wall, ratios of wall materials, and storage temperature play an important role in the cell viability of the microcapsules during storage. In conclusion, the samples with a ratio of core to wall 1:1 and WP:DWP 1:1 and the samples with a ratio of core-to-wall 1:1.5 and WP:DWP 1:1 were the best ones in the storage period at both temperatures 4 °C and 25 °C, respectively.

In summary, all the microencapsulated probiotics lose viability during storage. However, the viability-losing pattern of *L. plantarum* 299v is different when it comes to microcapsules coated with WP and DWP with different ratios of core-to-wall and ratios of wall materials. It indicates that the compactness and oxidation resistance of each microcapsule is quite different, even during the different storage stages since some microcapsules can form a dense structure even after several weeks of storage. Specifically, microcapsules with a ratio of core-to-wall 1:1 stored under 4°C have relatively lower viability losing rate. Lower storage temperature can decrease the oxidation rate of the cell membrane, thus decreasing the viability loss rate. However, due to the strength or weakness of the structure, there are always some special cases. For instance, microcapsules coated by DWP with a ratio of core-to-wall 1:1.5 have a relatively bad storage ability under 4°C, while microcapsules coated by WP:DWP=1:1 with a ratio of core-to-wall 1:1.5 and have a relatively good storage ability under 25°C.

4.2.5 Tolerance study

Low pH and high bile salt content indicate the harsh environment that probiotics may suffer during the digestion process. Consequently, the sustainability of the living characteristics during the digestion process is the other paramount property of probiotics incorporated with coating materials, which can effectively consolidate them into functional foods (Muhammad et al., 2017). Microencapsulating effect on probiotic survival ability manifests a conspicuous increase in viable cell number (Dimitrellou et al., 2016; Eckert et al., 2017; Li et al., 2019; Loyeau et al., 2018), ensures more viable cells pass through the digestion process with low pH and high bile salt conditions safely, minimizes the viability loss of probiotic product, together with safeguarding complete releasing microencapsulated probiotic bacteria into the intestine in quantities large enough for further colonization (Liu et al., 2016; Tao et al., 2019). Ratios of core-to-wall and of wall materials have an influence on the survival ability during the digestion process. Thus, investigating those factors on the viability change of microencapsulated probiotic bacteria during the SGF and SIF digestion process has some guidance in improving viable cell numbers in the production of a probiotic product.

The stomach is the main digestion process that can cause the loss of viability of probiotics due to the low pH and pepsin enzyme. The acidity of the stomach is usually approximately pH 2.0, which is affected by eating time, quality, and quantity of diet (Arslan-Tontul & Erbas, 2017; Liao et al., 2017).

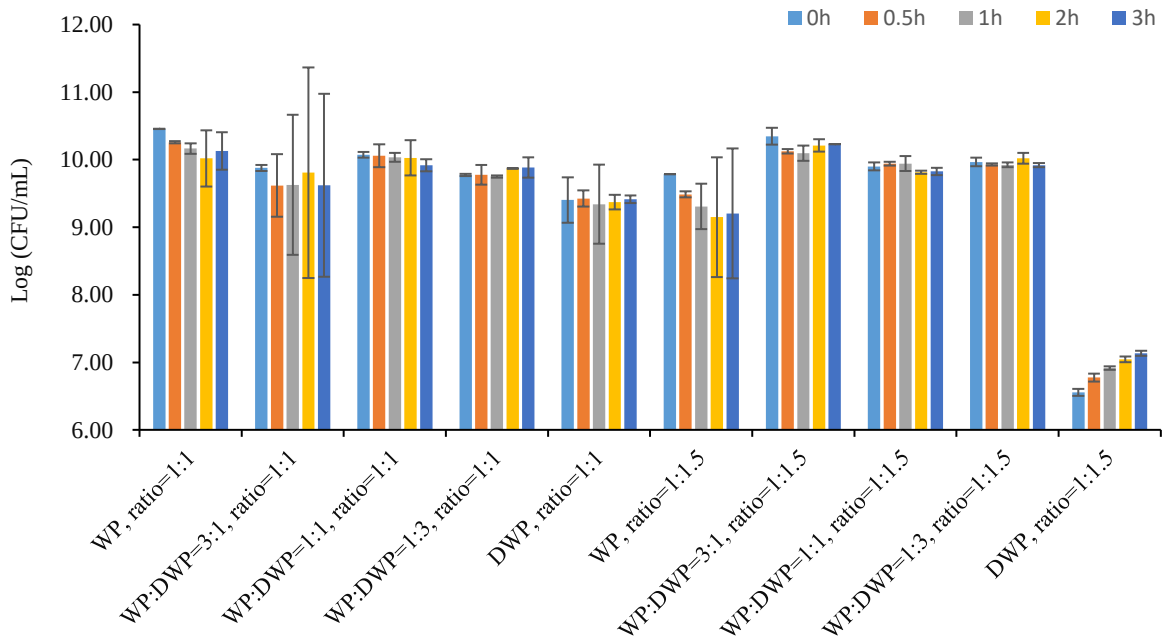

Figure 22. Viability of lyophilized *Lp. plantarum* 299v with different ratios of core-to-wall and of wall materials after exposed to SGF at 37 °C for 3 h

WP: whey protein, DWP: denatured whey protein

As can be seen from **Figure 22**, all samples except samples with a ratio of core-to-wall 1:1.5 coated by DWP did not show any significant differences in viable cells during the SGF experiment. It is likely due to the collective effect of the acidity stress (Dimitrellou et al., 2016; Hernández-López et al., 2018; Rajam & Anandharamakrishnan, 2015) and pepsin at low pH partially hydrolyses whey protein that caused the death of probiotics as well as may release the probiotics from the microcapsules. Denatured proteins are usually broken down more efficiently, for example, the samples with a ratio of core-to-wall 1:1 and WP had a significantly lower in cell number in SGF. Besides, samples with a ratio of core-to-wall 1:1.5 and coated with DWP gradually increased the release of probiotics from microcapsules during the 3 h of incubation may be due to the deep hydrolysed whey protein in the microcapsules and the activity of the pepsin lost gradually, thus caused the gradual releasing of probiotics from the capsules. In addition, the samples with a ratio of core-to-wall 1:1 had a significantly higher cell number than those with a ratio of core-to-wall 1:1.5. It is possible owing to the physical properties of the microcapsules, which means under the same circumstance, the higher the viable cells of the probiotics together with properties of the coating materials can exhibit higher protecting ability against pepsin and low pH digestion environment. Based on our overall results of the lyophilization, storage, and digestion process, it can be stated that probably probiotics coated by DWP with a ratio of core-to-wall 1:1.5 may not good choice. In addition, the samples with a ratio of core-to-wall 1:1 coated with WP, WP:DWP 1:1, and WP:DWP 1:3 as well as the samples with ratios of core-to-wall 1:1.5 coated with WP:DWP 3:1, WP:DWP 1:1, and WP:DWP 1:3 had relatively higher cell numbers than others. Therefore, the physicochemical composition, ratios of wall materials, and concentration of coating materials need to be taken into consideration when planning to produce probiotic microcapsules with high gastrointestinal juice resistance.

Bile salt is the main component of bile juice that is involved in the digestion and absorption process of fat content. In addition, it can dissolve bacterial cell membranes and result in viability loss (Arslan-Tontul & Erbas, 2017); thus, the resistance of probiotic bacteria to bile salt environment is an imperative property (Dimitrellou et al., 2016; Hernández-López et al., 2018; Liao et al., 2017). Therefore, the viability of lyophilized *Lp. plantarum* 299v strain with different ratios of core-to-wall and ratios of wall materials was investigated. Time, ratios of core-to-wall, ratios of wall materials, and the combination of ratios of core-to-wall and ratios of wall materials had a significant effect on the viability during the *in vitro* SIF digestion process (**Figure 23**).

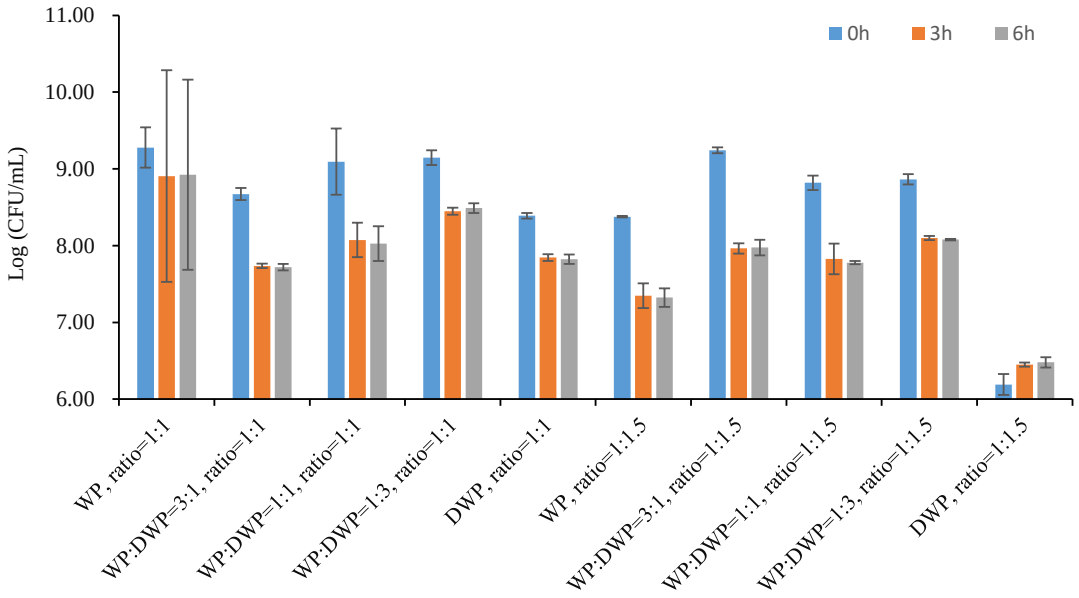

Figure 23. Viability of lyophilized *Lp. plantarum* 299v strain with different ratios of core-to-wall and of wall materials after exposure to SIF for 6 h at 37 °C
WP: whey protein, DWP: denatured whey protein

As can be analysed from **Figure 23**, the cell number of each sample significantly decreased in the first 3 h of incubation, and then did not change. Similar results were reported by Arslan-Tontul & Erbas (2017) of *Saccharomyces boulardii* after 180 min incubation. A possible explanation for this phenomenon may be explained by the adaptation properties of the cells to the bile salt or by synthesizing bile salt hydrolase (Kumar et al., 2014; Liao et al., 2017).

The ratios of core-to-wall is another factor that influences cell viability in the SIF experiment. In the case of samples with a ratio of core-to-wall 1:1 of the cell to coating material, the cell numbers were significantly higher than those with a ratio of core-to-wall 1:1.5. It is likely due to the increase the amount of wall material did not provide any higher protecting effect than ratios of core-to-wall 1:1.5. Rajam & Anandharamakrishnan, (2015) found that single or double coating materials can take advantage in the elimination of the influence of bile salt on the probiotic bacteria (Rajam & Anandharamakrishnan, 2015). In addition, capsules coated with WP, WP:DWP 1:3, WP:DWP 1:1 and WP:DWP 3:1 contained a significantly higher cell number than ones coated with DWP. Although the differences of cell numbers of those four ratios of wall materials were not significant. Meanwhile, the capsules coated with WP with a ratio of core-to-wall 1:1 exhibited the lowest cell number reduction at approximately 0.35 log (CFU/g), whereas the highest reduction of cell number (approximately 1.27 log (CFU/g)) was obtained in the case of capsules coated with a ratio of core-to-wall 1:1.5 and WP:DWP 3:1.

4.2.6 Application of microcapsules in fermented and fortified apple juice

The perception of applying probiotic bacteria into fruit juice can compensate for the nutrition loss due to the manufacturing process. Additionally, the probiotic bacteria also increase the functionality via viable cells that have already been accepted by customers and experts. The cell numbers of fermented and fortified apple juices with different ratios of core-to-wall and ratios of wall materials stored at 4 °C and 25 °C for 10 weeks were shown in **Figure 24**.

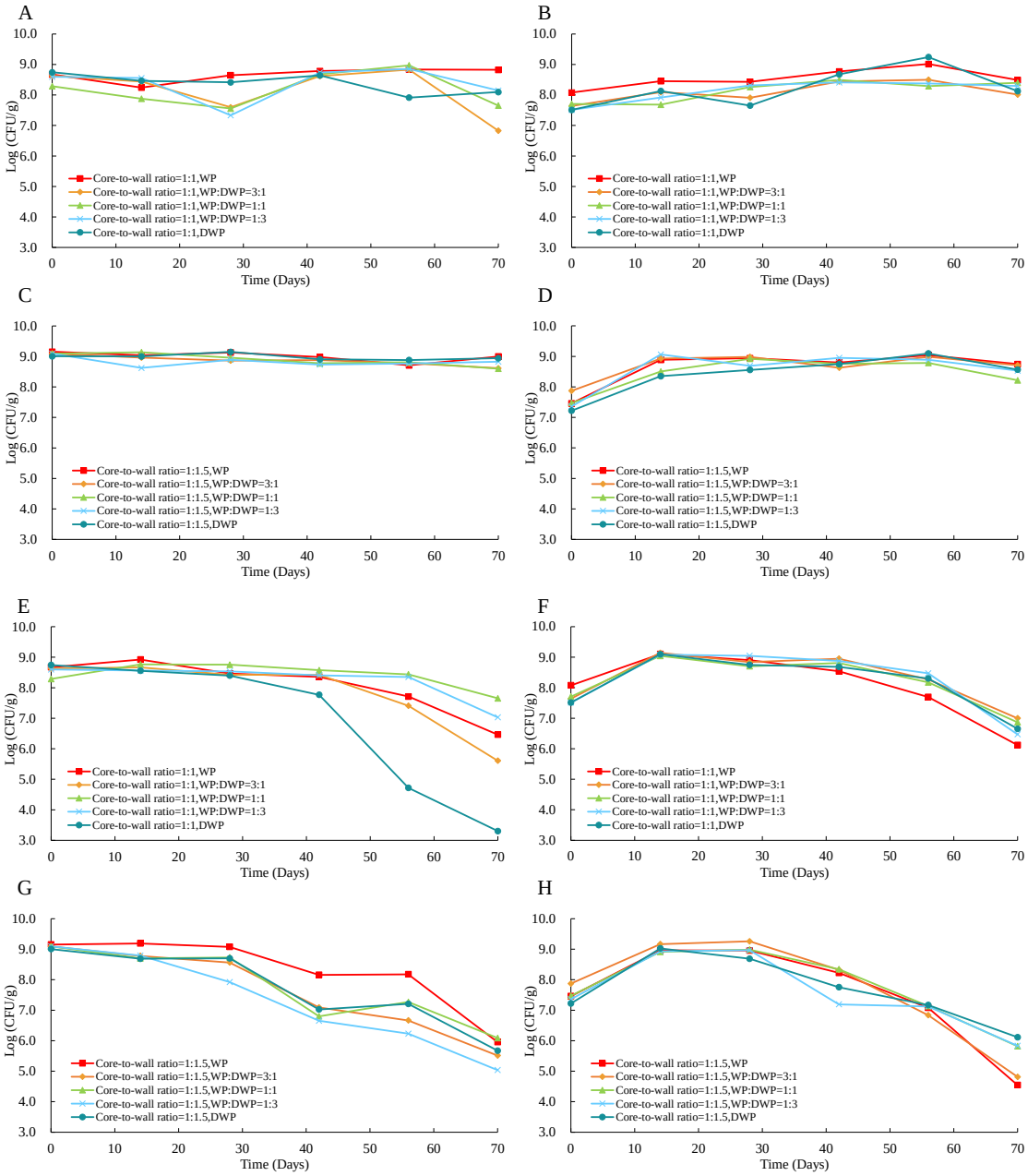

Figure 24. Cell number of fermented and fortified apple juices

A: 4 °C, fermentation, ratios of core-to-wall 1:1; B: 4 °C, fortification, ratios of core-to-wall 1:1; C: 4 °C, fermentation, ratios of core-to-wall 1:1.5; D: 4 °C, fortification, ratios of core-to-wall 1:1.5; E: 25 °C, fermentation, ratios of core-to-wall 1:1; F: 25 °C, fortification, ratios of core-to-wall 1:1; G: 25 °C, fermentation, ratios of core-to-wall 1:1.5; H: 25 °C, fortification, ratios of core-to-wall 1:1.5; WP: whey protein, DWP: denatured whey protein

Storage time and storage temperature had a significant effect on the changes in cell numbers of probiotic apple juices during storage. However, ratios of core-to-wall, ratios of wall materials, and fortification or fermentation methods did not significantly affect the viability of the probiotics. Storage temperature is a factor that influences the viability of probiotics in apple juice. Meanwhile, storage at a high temperature, such as a room or higher temperature, may initiate some metabolisms by other microbes especially some pathogens that thus will suppress the probiotics, whereas storage at low temperatures, such as at 4 °C can keep the bacteria alive (**Figure 24A–C**). Viable cells in all capsules were maintained at the initial levels (approximately 9 log (CFU/g)) for a whole storage period (two months). The effect of temperature on the viability of cells can also be observed when doing a comparison of data in **Figure 24C**&**G)**. Meanwhile, the cell number of those samples fermented by microcapsules with a ratio of core-to-wall 1:1.5 (**Figure 24C**) was kept unchanged (9 log (CFU/g)), whereas it started to decrease from the 4th week and reached 5.04 log (CFU/g) at the end of storage. It was worth noting that this value was much lower than the viable cell criterion of probiotic products (7 log (CFU/g)).

Storage time is also a crucial factor that influences the viability of cells. There was no significant difference in the cell numbers during the storage of apple juices for 0, 2, 4, and 6 weeks. However, the cell number started to significantly decrease from the 8th week. This trend was accelerated after week 10 (**Figure 24E–H**). The cell number apple juice fermented with microcapsules coated with DWP with a ratio of core-to-wall 1:1 reached even 3.3 log (CFU/g) at the end of the storage period.

Different ratios of core-to-wall, as well as WP to DWP, did not affect the trend of change of viable cells of both fermented and fortified apple juices stored at 4 °C. The cell numbers were kept constant for the whole storage period (**Figure 24A–D**). In the case of storage at 25 °C, the cell numbers of fermented samples with microcapsules coated with pure WP or DWP (**Figure 24E&G**) as coating materials. Instead, they may influence the dynamics of changes of cell numbers. In addition, these changes were also not affected by fortification and fermentation methods, but generally, the viable cell numbers of fermented samples were significantly higher than fortified samples. Meanwhile, in the fermentation process, the bacteria grew, thus resulted in an increase in cell number, whereas it was missed in the case of fortification. The same result was also observed by Ying and co-workers (2013). However, at the end of the storage process for a long time (some months), this is no significant difference in the cell number between the two methods (fermentation and fortification).

In summary, storage time and temperature are the two main factors that influence the viability of the probiotic cells in apple juice, while the ratios of core-to-wall, ratios of wall materials,

fermentation and fortification technologies did not affect it. Storage at 4 °C is suggested for probiotic apple juice. At this temperature, both fermented and fortified probiotic apple juices can maintain their viable cells more than 6 weeks.

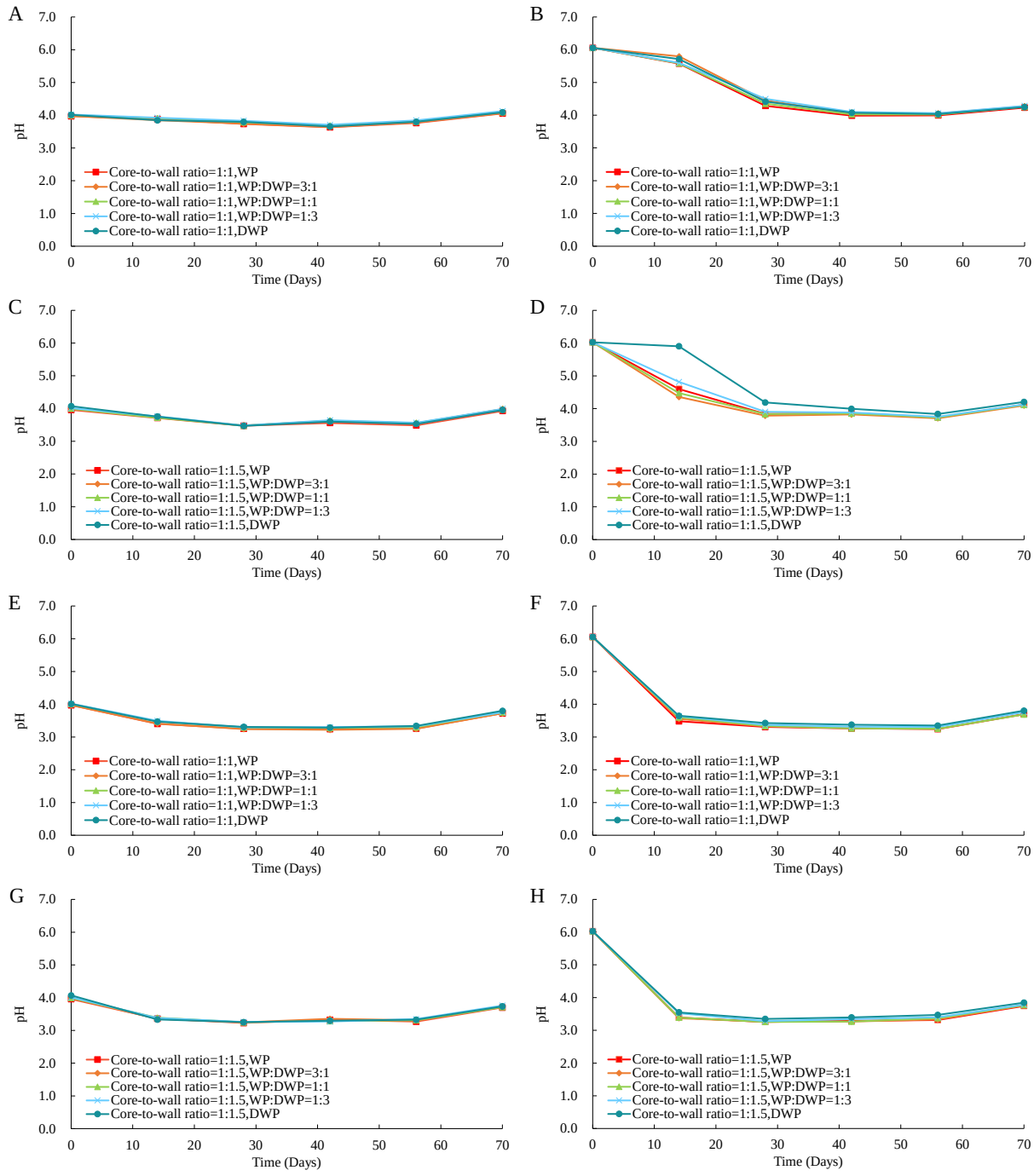

Figure 25. pH of fermented and fortified apple juices

A: 4 °C, fermentation, ratios of core-to-wall 1:1; B: 4 °C, fortification, ratios of core-to-wall 1:1; C: 4 °C, fermentation, ratios of core-to-wall 1:1.5; D: 4 °C, fortification, ratios of core-to-wall 1:1.5. E: 25 °C, fermentation, ratios of core-to-wall 1:1; F: 25 °C, fortification, ratios of core-to-wall 1:1; G: 25 °C, fermentation, ratios of core-to-wall 1:1.5; H: 25 °C, fortification, ratios of core-to-wall 1:1.5. WP: whey protein, DWP: denatured whey protein

The change in pH can be used as an indicator to monitor both fermentation and storage processes quickly. The pH changes of fermented and fortified apple juices with different ratios of core-to-wall and ratios of wall materials of storage at 4 °C and 25 °C for 10 weeks were illustrated in **Figure 25**. Meanwhile, the pHs of all fermented samples were approximately pH 4.0 and did not change significantly during storage (**Figure 25A, C, E, G**), whereas the storage time had a significant effect on the changes in pHs of the probiotic apple juice. In the case of fortification, the pH values dropped to approximately pH 4.0 when stored at 4 °C and 25 °C for 28 days and 14 days, respectively (**Figure 25B, D, F, H**). During the period of the starting point to the pH stable stage, the probiotics in the microcapsules gradually released and caused the fermentation process to start. Similar results were reported by Sohail and co-workers in 2012 as well as Gandomi and co-workers in 2016. They reported that the orange juices fermented with *Lactobacillus rhamnosus* GG stored at 4 °C for 30 days exhibited lower acidification compared to one stored at 25 °C for 12 days and it may result in better sensory properties.

In summary, while the ratios of core-to-wall, storage time, and temperature as well as fermentation or fortification methods significantly affected the changes of pH of probiotic apple juices during storage, the ratios of wall materials of the microcapsules do not have any.

4.3 Encapsulation of *Lp. plantarum* 299v strain with Maillard reaction products

The polysaccharide-protein binary complexes can be made through the Maillard reaction and get Maillard reaction products (MRPs) (Fu et al., 2021; Liu et al., 2016, 2017; Mao et al., 2018). There are several advantages of MRPs that have been reported. They have excellent antioxidant and emulsifying characteristics. In addition, they also perform prebiotic functionality because they are resistant to digestion compared with the non-glycated proteins, which means more dietary glycoconjugates are available for endogenous microbiota utilization in the distal colon. Therefore, MRPs microencapsulated probiotics can pass through the digestion tract and colonize in the intestine successfully (Mao et al., 2018). Hence, the combination of both carbohydrates and proteins, i.e., polysaccharide-protein binary complexes show a great potentiality as the delivery carrier for bioactive substances. In my research, maltodextrin and whey protein were used as the base materials to produce the MRPs as the coating materials for the probiotic *L. plantarum* 299v.

4.3.1 Yield of encapsulation process

Yield is a key indicator of microcapsules during manufacturing. The yields of *L. plantarum* 299v formed with different ratios of core-to-wall and ratios of wall materials MRPs after lyophilization were shown in **Figure 26**.

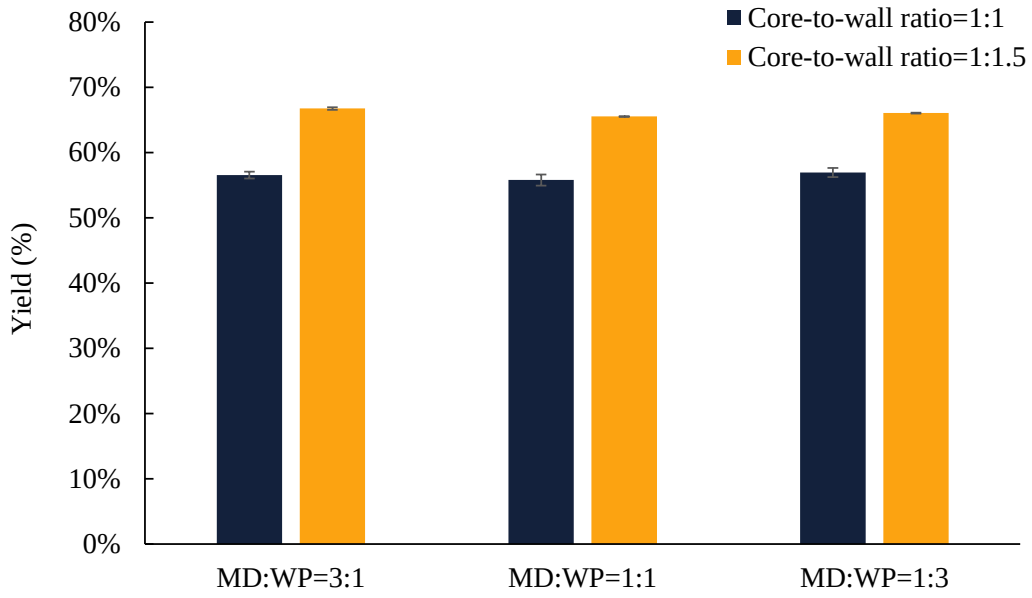

Figure 26. Yield of microencapsulated *Lp. plantarum* 299v formed with different ratios of core-to-wall and ratios of wall materials MRPs MD: maltodextrin, WP: whey protein

The yield of the microcapsules varied from 55.18% to 66.89% (**Figure 26**). The samples with a ratio of core-to-wall 1:1.5 had a significantly higher yield than samples with a ratio of core-to-wall 1:1. It is possible that there was less water content in the coating materials than the probiotics, which resulted in a higher total solid content of samples with a ratio of core-to-wall 1:1.5 after the lyophilization. However, there was no significant difference between the samples with ratios of wall materials. It is likely that there was no significant molecular weight change during the Maillard reaction.

4.3.2 Cell number and bulk density of microcapsules

Cell number has a fundamental indicator function on the microcapsules. The cell number of microencapsulated *Lp. plantarum* 299v varies from 11.25 to 13.76 log (CFU/g) (**Figure 27**), which was quite higher than the recommended dose by the WHO and FAO. Moreover, ratios of core-to-wall did not affect the cell number of samples formed with the same ratios of wall materials. It is more likely that MRPs with ratios of core-to-wall 1:1 and 1:1.5 had the same degrees of crosslinking network structure. However, MRPs formed with different ratios of wall materials had an effect on cell numbers. Samples coated by MD:WP 1:1 had a significantly higher cell number than those samples coated by MD:WP 3:1 and MD:WP 1:3. It is almost certain that the degree of crosslinking of the samples coated by MD:WP 1:1 was higher than the others since the cell number increased with the increase in the degree of the crosslinking of the polymer network structure of

the polymer (Liu et al., 2016). Many researchers reported that the polymers concentration, temperature, etc., can affect the glycation extent of proteins during the Maillard reaction, which can further influence the degree of crosslinking network of the polymers and finally affect the viability of the microcapsules (Fu et al., 2021; Liu et al., 2016, 2017).

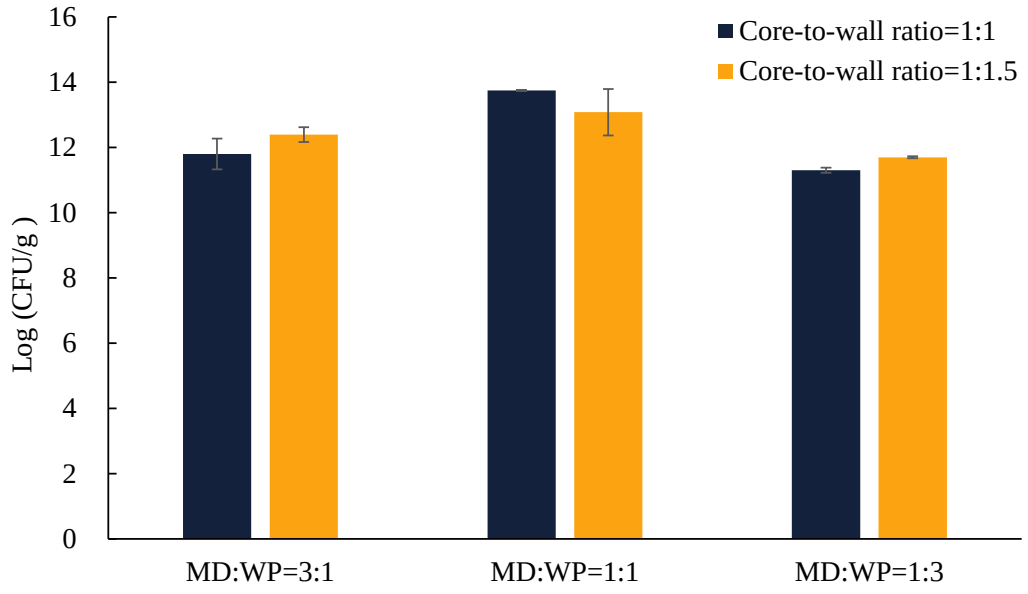

Figure 27. Cell number of microencapsulated *L. plantarum* 299v formed with different ratios of core-to-wall and ratios of wall materials MRPs
MD: maltodextrin, WP: whey protein

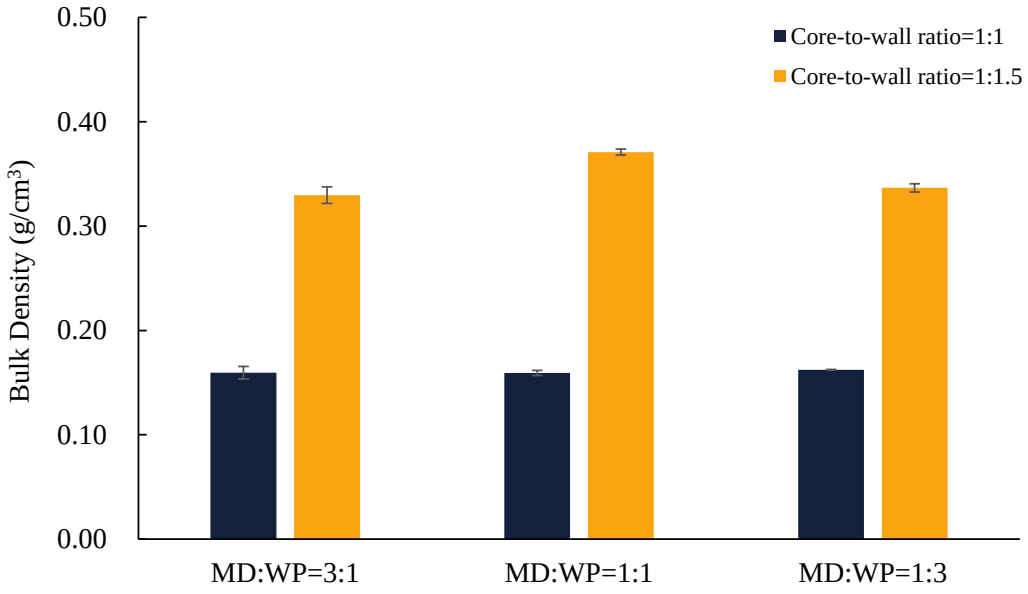

Figure 28. Bulk density of microencapsulated *Lp. plantarum* 299v formed with different ratios of core-to-wall and ratios of wall materials MRPs
MD: maltodextrin, WP: whey protein

The bulk density of microencapsulated *L. plantarum* 299v strain with three wall materials ratio and two core-to-wall ratios after lyophilization were shown in **Figure 28**. The bulk density of the microcapsules varied from 0.16 g/cm^3 to 0.37 g/cm^3. The samples with a ratio of core-to-wall 1:1.5 had a significantly higher bulk density than samples with a ratio of core-to-wall 1:1. Similar explanation can be applied here to the yield. Specifically, the samples with a ratio of core-to-wall 1:1.5 with MD:WP 1:1 had the highest bulk density. It is almost certain that a more condensed polysaccharides-proteins complex was formed which leads to a change in the structure of the two molecules (Goyal et al., 2015; Karrar et al., 2021).

4.3.3 Morphology

Scanning electron microscopy (SEM) can be used to observe the morphology of the microcapsules, from which the functional properties of the protein can be reflected (Fu et al., 2021). In order to check whether the ratios of core-to-wall and ratios of core-to-wall and the occurrence of Maillard reaction products formed from different ratios of wall materials could modify the particle state and the surface morphology of probiotic microcapsules, SEM observation was carried out and the results were shown in **Figure 29**.

The micrographs observed by SEM demonstrated a quite compact structure of microcapsules after lyophilization although there were still some small cracks and poles presented under 1000× magnification. This would be helpful for the resistance to the mechanical force during the production, the struggle against gastrointestinal juice during digestion process, etc. (Rajam & Anandharamakrishnan, 2015). Moreover, **Figure 29C&I** represented a rigid sheet-like and flake-like structure with little aggregations for microcapsules coated with MD:WP 3:1 in both ratios. **Figure 29E&K** exhibited a granule-flake-like structure with some aggregations occurring, and **Figure 29G&M** illustrated a rougher granule-like structure with many aggregations occurring. It is certain that the rough surface was owing to the Maillard reaction at high temperatures and the sublimation of water during the lyophilization. It can be explained by the changes in protein structure through glycosylation and lead to the construction of a more compact and uneven structure. This indicated that the compact penetration-resistant surface can be served as a strong physical barrier that protects probiotic cells during the passage through the gastrointestinal tract (Fu et al., 2021). Furthermore, as can be discovered from the figures under 14,000× magnification, the surface of microcapsules coated by MD:WP 1:3 has more condensed and fewer cracks and exposed probiotic cells than with MD:WP 1:1 and MD:WP 3:1 with both ratios of core-to-wall 1:1 and 1:1.5. Moreover, the size of free *Lp. plantarum* 299v cells are smaller than the encapsulated ones. It explained the protecting ability of the wall materials to prevent free bacteria from losing

too much water. In the contrast, long-time dehydration led to the shrinking of cytoplasm and resulted in a smaller size of the free cells (Mao et al., 2018), while the microencapsulated cells were more possible to maintain their original cytomorphology.

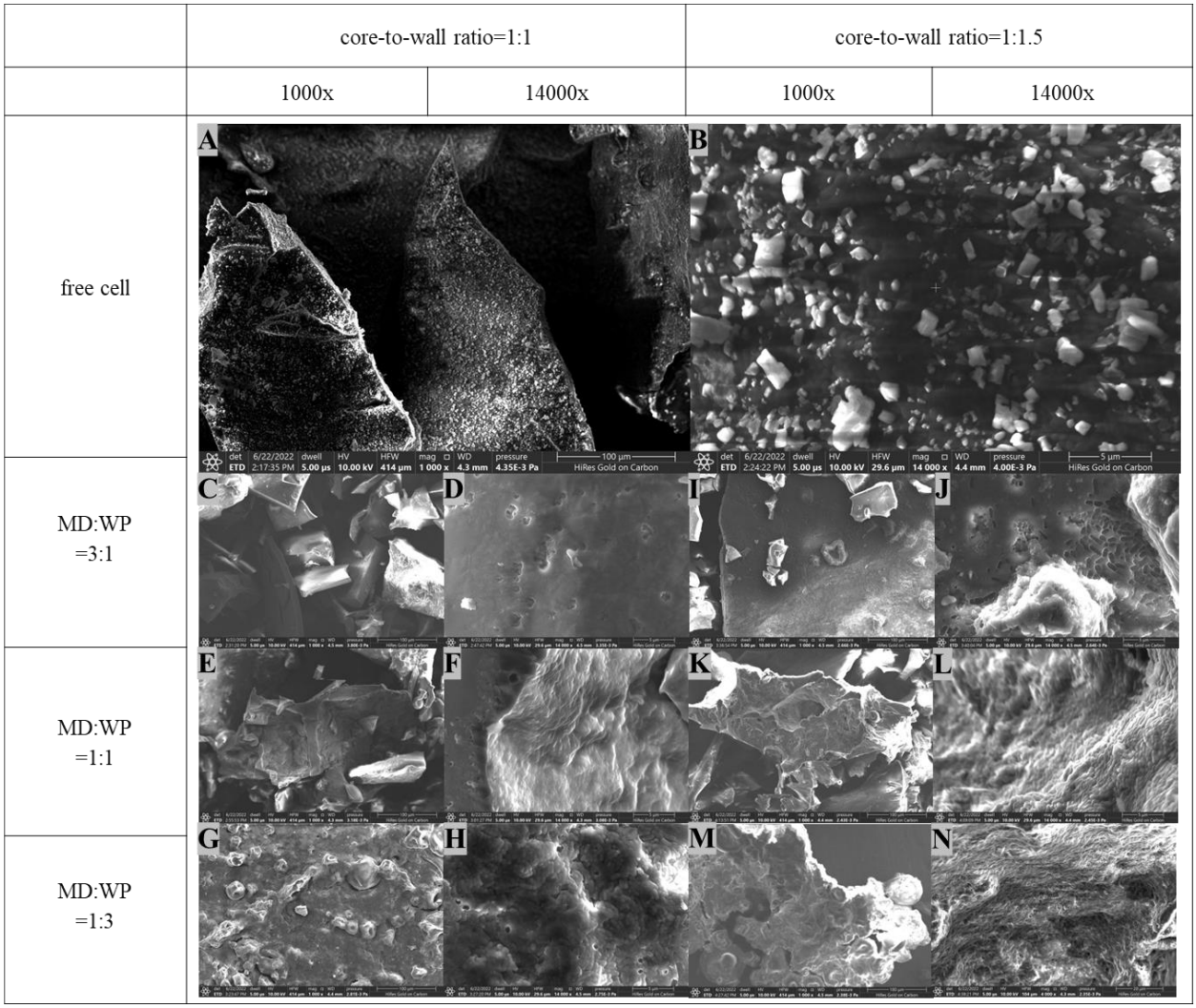

Figure 29. Scanning electron microscope (SEM) images of *Lp. plantarum* 299v cells under 1000x and 14,000x magnification

A, B are free cells; C, D are ratios of core-to-wall 1:1 and MD:WP 3:1; E, F are ratios of core-to-wall 1:1, MD:WP 1:1; G, H are ratios of core-to-wall 1:1 and MD:WP 1:3; I, J are ratios of core-to-wall 1:1.5 and MD:WP 3:1; K, L are ratios of core-to-wall 1:1.5 and MD:WP 1:1; M, N are ratios of core-to-wall 1:1.5 and MD:WP 1:3; MD: maltodextrin, WP: whey protein

4.3.4 Effect of storage on viability

The viability loss of the probiotics during storage is mainly owing to the membrane lipid oxidation (Rajam & Anandharamakrishnan, 2015), thus oxygen content and temperature are the crucial factors that affect probiotic viability. The effectiveness of different ratios of core-to-wall and ratios of wall materials on the storage stability of microencapsulated *Lp. plantarum* 299v were investigated. The storage was done at 4℃ and 25℃ for 12 weeks and the results were

demonstrated in **Figure 30**.

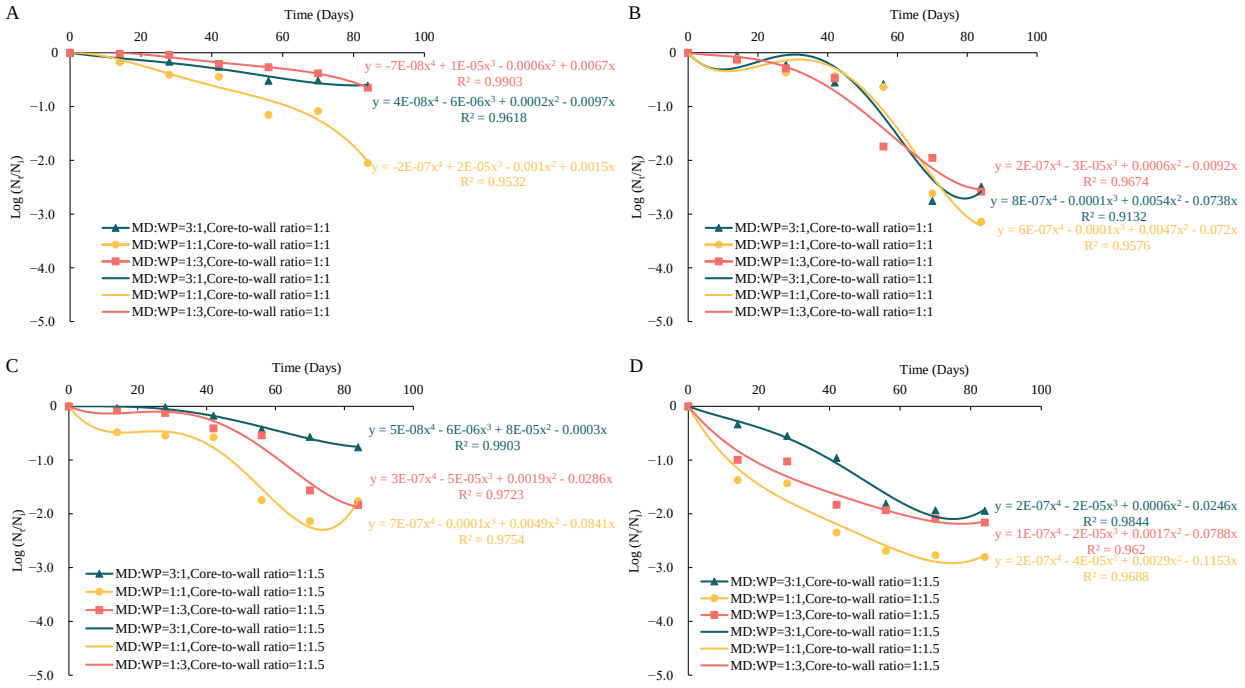

Figure 30. Viability loss of microencapsulated *Lp. plantarum* 299v formed with different ratios of core-to-wall and ratios of wall materials MRPs at 4°C and 25°C

A: 4°C, ratios of core-to-wall 1:1; B: 25°C, ratios of core-to-wall 1:1; C: 4°C, ratios of core-to-wall 1:1.5; D: 25°C, ratios of core-to-wall 1:1.5; MD: maltodextrin, WP: whey protein

In the case of samples stored at 4°C with a ratio of core-to-wall 1:1 (**Figure 30A**), the 10th week was the critical point for the viability of probiotics coated with MD:WP 1:1 and the viability decreased continuously, but after the critical point, the viability decreased sharply. Probiotics coated with MD:WP 3:1 and with MD:WP 1:3 lost viability much slower and did not even reach the decrease of 1 log (CFU/g) for 12 weeks of cold storage. It is likely that probiotics coated with MD:WP 3:1 and MD:WP 1:3 had a denser structure compared to probiotics coated with MD:WP 1:1 under the circumstance of a ratio of core-to-wall 1:1 at 4°C. In the case of samples stored at 4°C with a ratio of core-to-wall 1:1.5 (**Figure 30C**), the viability loss degree of probiotics coated with these three ratios of wall materials was similar until 6 weeks. However, the trend of the viability loss was quite different after 6 weeks of storage. As for probiotics coated with MD:WP 3:1, there was almost no viability loss during the first four weeks, and it started to lose viability continuously from the four weeks. As for probiotics coated with MD:WP 1:3, the viability of the probiotics decreased during the first weeks and then kept unchanging till the 4th week, later showed a fastened viability loss rate for the end of the storage process. The final decrease in viability was 2 log (CFU/g) at the end of the 12-week storage. Samples coated by MD:WP=1:1 had the same

losing pattern as samples coated by MD:WP 1:3 after storage for 10 weeks. In the last two weeks, increased viability can be seen. This strange phenomenon may be due to the non-uniform of the probiotics in microcapsules.

In the case of samples stored at 25℃ with a ratio of core-to-wall 1:1 (**Figure 30B**), probiotics coated with MD:WP 1:3 gradually decreased the viability till the 10^{th} week and slow down the loss rate from the 10^{th} to the 12^{th} week. Probiotics coated with MD:WP 3:1 and MD:WP 1:1 almost had the same trend; the viability decreased slowly in the 8 weeks and sharply till the 10^{th} week. From this time, for probiotics coated with MD:WP 3:1 the viability did not decrease further, while for probiotics coated with MD:WP 1:1 keep losing the cell count till the end of the storage. In the case of samples stored at 25℃ with a ratio of core-to-wall 1:1.5 (**Figure 30D**), the viability loss trend of probiotics coated with these three ratios of wall materials was almost the same from the beginning to the end. From the beginning to the 10^{th} week, a gradual viability loss can be observed, and from that time the viable cell count did not show a further decrease.

Temperature significantly affected the viability of encapsulated probiotics with MRPs during storage. In summary, all the microencapsulated probiotics lose viability during storage. Generally, the cell numbers of samples stored at 25 °C have a higher loss rate than those of samples stored at 4 °C. The viability loss reached 3 log (CFU/g) in case of storage at room temperature. However, the viability-losing pattern of *L. plantarum* 299v is different when it comes to microcapsules coated with MRPs formed with different ratios of core-to-wall and ratios of wall materials. Every single case should be analysed based on the storage conditions and protecting ability of the coating materials.

4.3.5 Tolerance study

During the digestion process, low pH and high bile salt concentration combined with digestion enzymes are harsh factors that may be harmful to probiotics and may cause the loss of viability or even the death of the probiotic strains (Rajam & Anandharamakrishnan, 2015; Tao et al., 2019). Hence, the protective effect of different ratios of core-to-wall and ratios of wall materials on the viability of probiotics after being exposed to SGF for 3 h at 37**℃** were investigated (**Figure 31**).

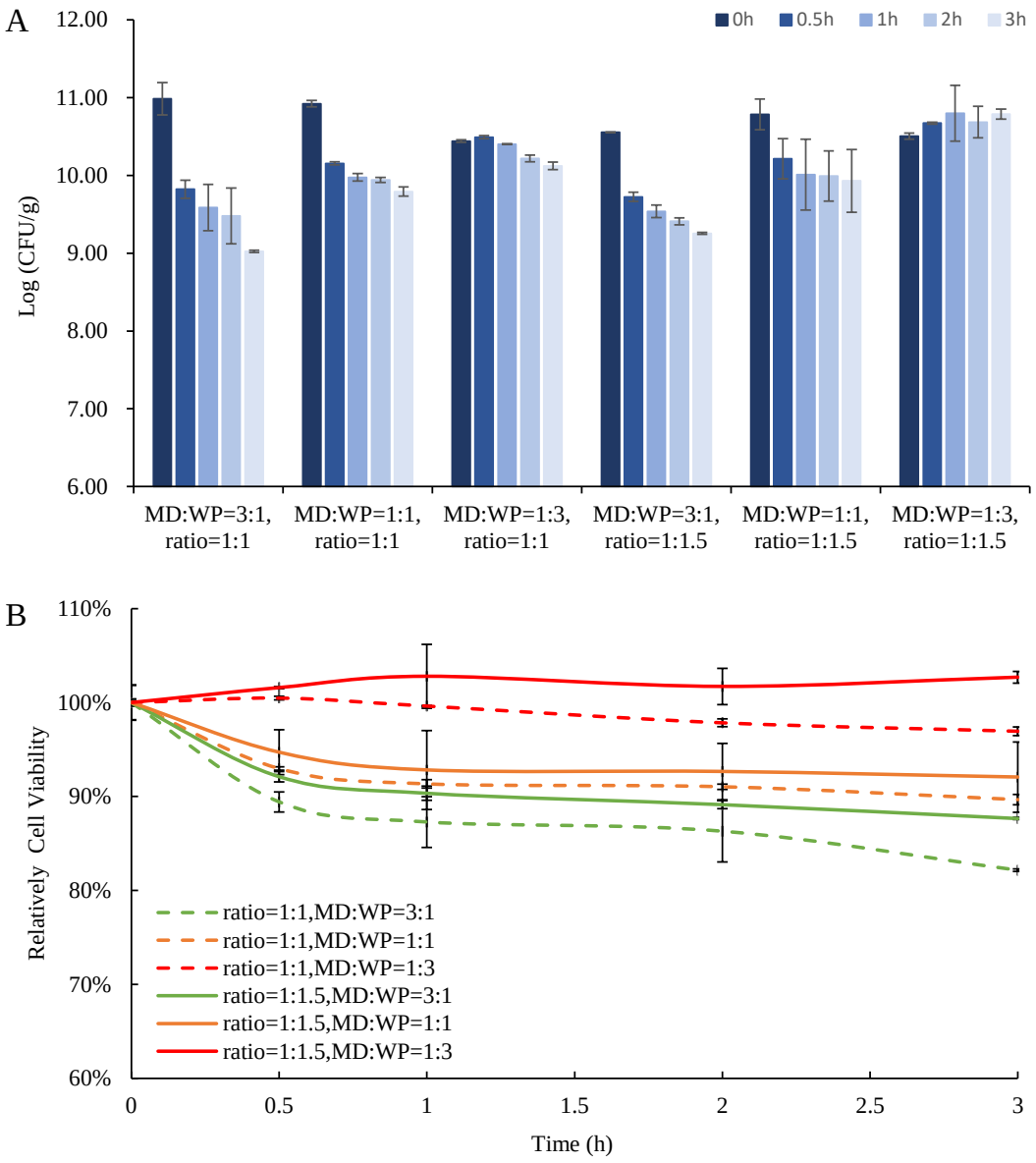

Figure 31. Cell number (A) and relative cell number (B) of lyophilized *Lp. plantarum* 299v formed with different ratios of core-to-wall and ratios of wall materials MRPs after being exposed to SGF for 3 h at 37°C

The cell number of the microencapsulated probiotics varied from 9.02 to 10.98 log (CFU/g), which was considerably higher than the recommended limitation viability, i.e., 6 log (CFU/g) after 3 h SGF conditions. In addition, the viability loss rate of the microencapsulated probiotics was decreased in the following order MD:WP 3:1, MD:WP 1:1, and MD:WP 1:3. It is possible that dense hydrogel network formed by MRPs in the order above reduced the diffusion rate of the acid and enzymes into the microcapsules (Fu et al., 2021; Liu et al., 2016). The pH in the inner part of the microcapsules was higher than outside, and thus provide protection to the probiotics. Moreover, the buffering capacity of the WP which can protect the probiotic microorganism from digestive stress (Vanden et al., 2020) increased, and the increasing WP content in the formulation was

probably another piece of evidence that can explain these results. Likewise, microencapsulated probiotics with a ratio of core-to-wall 1:1.5 had significantly higher viability than those with a ratio of core-to-wall 1:1. It is likely the thicker of the coating materials, the better protecting ability to the probiotics (Arslan-Tontul & Erbas, 2017; Rajam & Anandharamakrishnan, 2015). In summary, these results highlighted those microcapsules coated with a ratio of core-to-wall 1:1.5 and MD:WP 1:3 present the best protection ability of *Lp. plantarum* 299v in SGF conditions. The results of cell number and relative cell number of lyophilized *Lp. plantarum* 299v formed with different ratios of core-to-wall and ratios of wall materials MRPs after being exposed to SIF for 6 h at 37°C were shown in **Figure 32**.

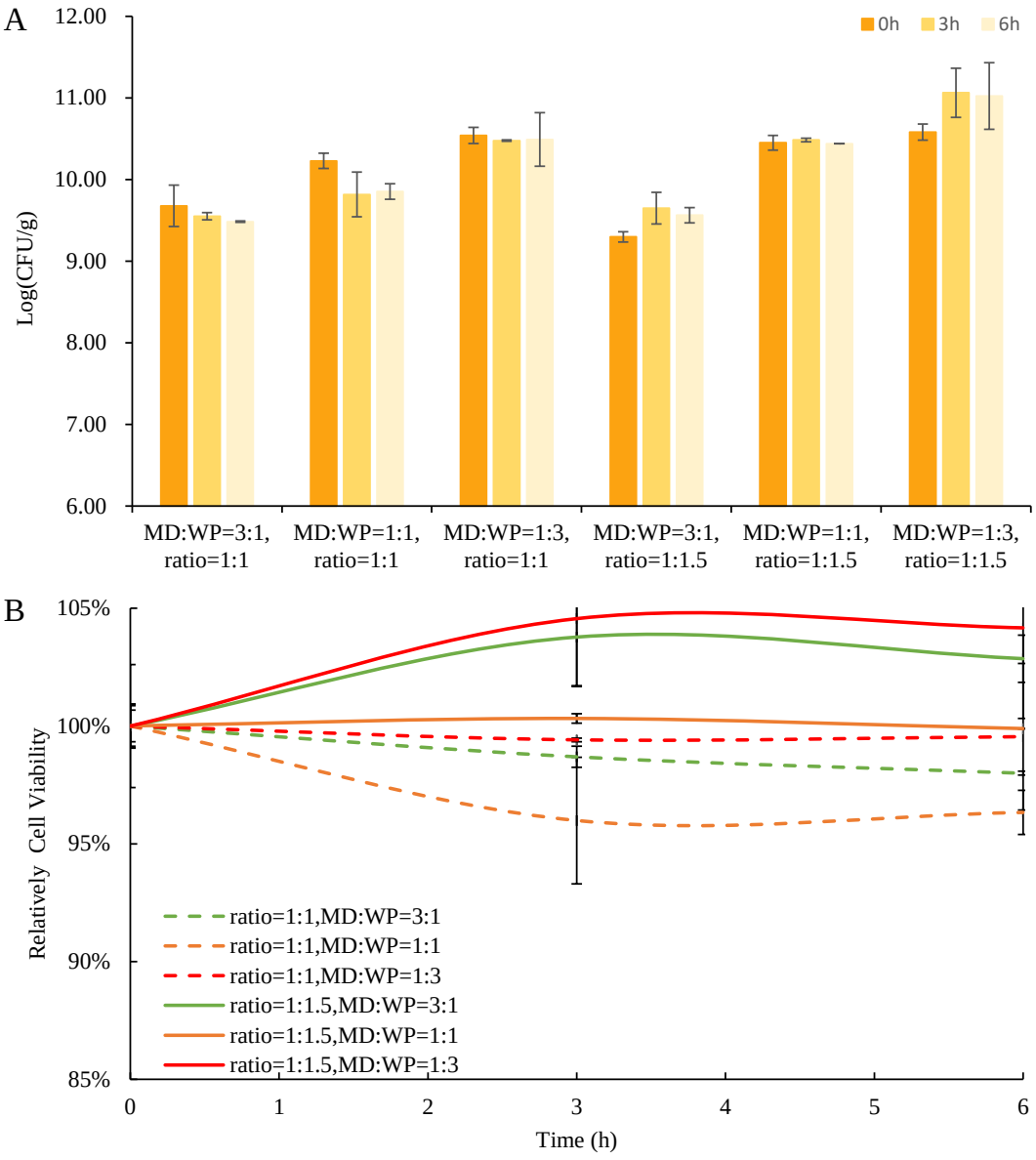

Figure 32. Cell number (A) and relative cell number (B) of lyophilized *Lp. plantarum* 299v formed with different ratios of core-to-wall and ratios of wall materials MRPs after being exposed to SIF for 6 h at 37°C MD: maltodextrin; WP: whey protein

The cell number of the microencapsulated probiotics varied from 9.48 to 11.06 log (CFU/g). Likewise, microencapsulated probiotics with a ratio of core-to-wall 1:1.5 had significantly higher viability than those with a ratio of core-to-wall 1:1 after 6 h of exposure to SIF. It has similar results and explanations to the SGF experiment. Moreover, similar results can be found comparing to the experiment in SIF to the above-mentioned SGF digestion experiment where probiotics were coated with MD:WP 1:3 and MD:WP 3:1 with a ratio of core-to-wall 1:1.5 during 6 h. During the SIF digestion process, due to the controlling release characteristics of the specific structure, the cell count did not decrease. These results highlight that wall materials with a ratio of core-to-wall of 1:1.5 and MD:WP 1:3 present better protection ability of *Lp. plantarum* 299v in SIF conditions. In summary, samples coated by MD:WP 1:3 with the ratio of core-to-wall 1:1.5 has the best results after the SGF and SIF test.

4.3.6 Application of microcapsules in fermented and fortified apple juice

The low pH can influence the viability of the probiotics even though they are microencapsulated. However, it depends on the type and content of the fruit juice. Moreover, fruit juices contain compounds that can be used as substrates for *Lactobacillus* and other compounds may have an antimicrobial function (Olivares et al., 2019). Hence, microcapsules formed with different ratios of core-to-wall and ratios of wall materials MRPs were applied to produce fermented and fortified apple juice. The viability of microencapsulated probiotics and pH of the apple juices during storage at 4℃ and 25℃ were analysed every 2 weeks. The change in the viability of microencapsulated probiotics and the pH of fermented and fortified apple juice stored under 4℃ were shown in **Figure 33**.

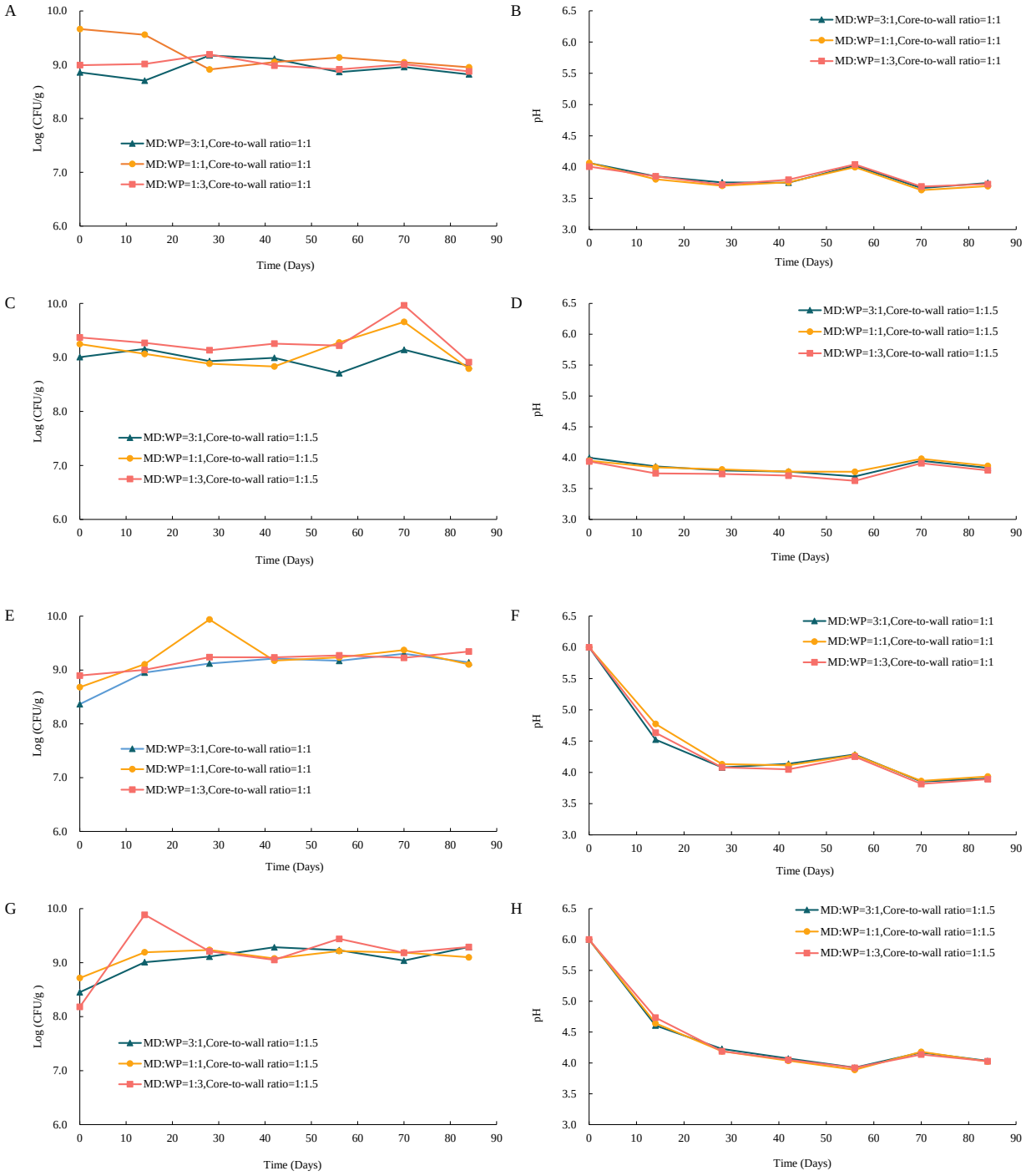

Figure 33. The change of the viability of microencapsulated probiotics and pH of fermented and fortified apple juice stored under 4℃ A, B: fermented, ratios of core-to-wall 1:1; C, D: fermented, ratios of core-to-wall 1:1.5; E, F: fortified, ratios of core-to-wall 1:1; G, H: fortified, ratios of core-to-wall 1:1.5; MD: maltodextrin, WP: whey protein

For the fermented samples, the viable cell number of the microencapsulated probiotics after fermentation was within 9 to 10 log (CFU/g). These values can be considered as the initial cell count for storage. The initial cell count of microencapsulated probiotics in fortified samples was

within 8 to 9 log (CFU/g). During the later storage period, microencapsulated probiotics in fermented samples still kept high viability and constant pH value.

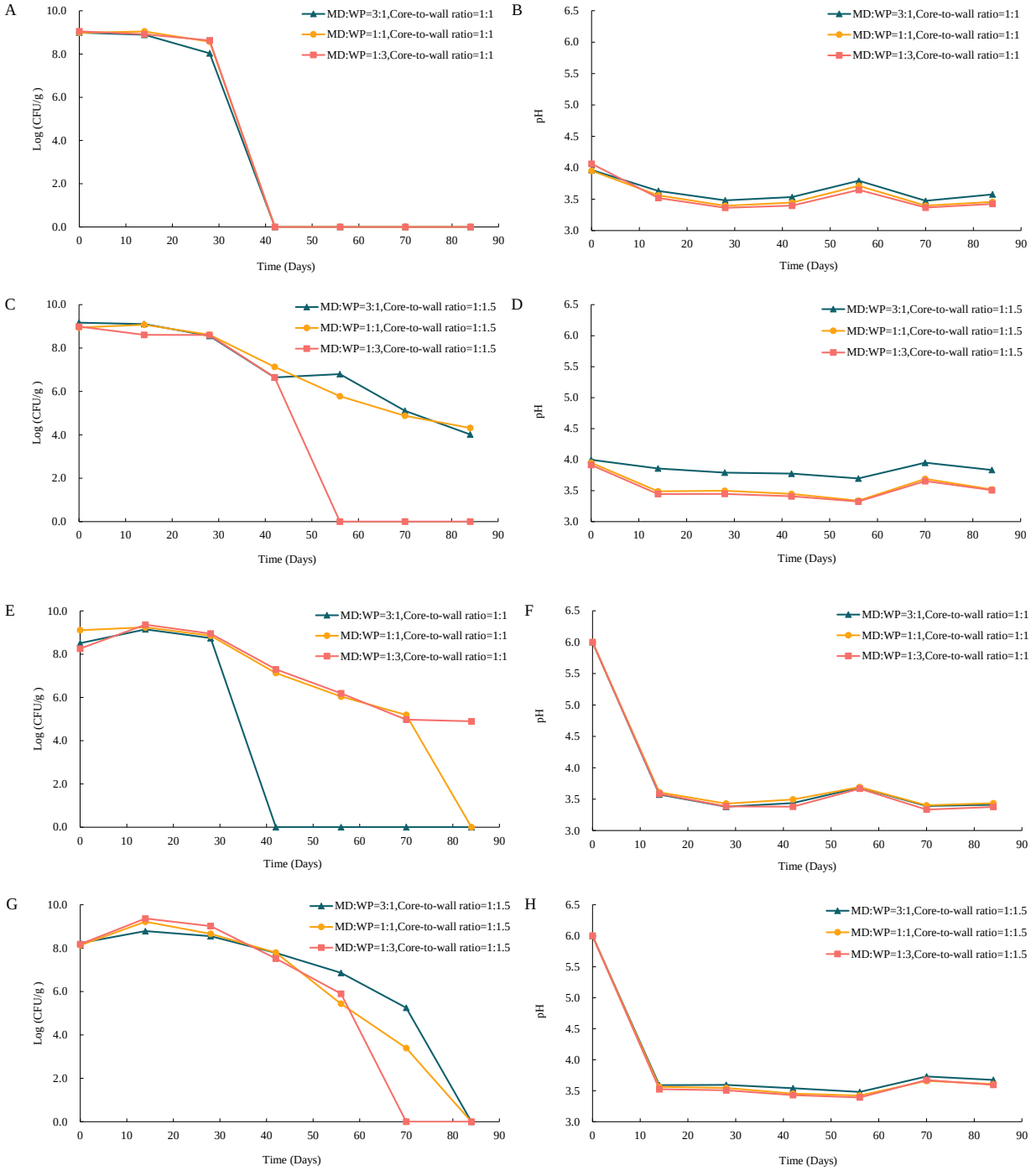

Figure 34. The change of the viability of microencapsulated probiotics and pH of fermented and fortified apple juice stored under 25°C A, B: fermented, ratios of core-to-wall 1:1; C, D: fermented, ratios of core-to-wall 1:1.5; E, F: fortified, ratios of core-to-wall 1:1; G, H: fortified, ratios of core-to-wall 1:1.5; MD: maltodextrin, WP: whey protein

While in the fortified samples, a violent change in the viability and the pH of the microencapsulated probiotics can occur in the first 6 weeks. During this period, the apple juice began to ferment slowly increasing the cell number of probiotics and decreasing the pH together with the apoptosis of old cells. After this process was completed, the cell number of the probiotics in the apple juice was maintained at around 9 log (CFU/g) and the pH at 3.5 to 4.0.

The change in the viability of probiotics and pH of fermented and fortified apple juice stored at 25℃ are shown in **Figure 34**. For the fermented samples, after the fermentation process, the viability of the probiotics of each sample was within 9 to 10 log (CFU/g), as the starting point of the storage, while the cell count of the probiotics in fortified samples was within 8 to 9 log (CFU/g). At the end of the fermentation process, the viability of probiotics in fermented samples began to decline. As it was shown in **Figure 34A&C**, probiotics coated with a ratio of core-to-wall 1:1 in fermented apple juice decreased their viability lower than the limitation, i.e., 10^6 (CFU/g) after the 4^{th} week. While probiotics coated with a ratio of core-to-wall 1:1.5 in fermented apple juice lost their viability lower than the limitation after the 6^{th}, 8^{th}, and 10^{th} week for samples coated by MD:WP 3:1, MD:WP 1:1, and MD:WP 3:1, respectively. While the fortified samples increased the viability of probiotics for the first two weeks and then started the viability-decreasing process. It is likely that there were many nutrition compounds in the fortified apple juice for the fermentation process. As it was shown in **Figure 34E&G**, probiotics coated with ratios of core-to-wall 1.1 and 1:1.5 in fortified apple juice decreased their viability below the limitation, i.e., 10^6 (CFU/g) after the 6^{th} week. Likewise, the viability loss of probiotics in both fermented and fortified groups stored at 25℃ was extremely higher than that at 4℃. It is almost certain that temperature significantly affected the viability of probiotics. Thus, the storage at 4℃ was a better option for the probiotic apple juice to maintain the cell number at an appropriate level.

Furthermore, when considering the overall viability of the microencapsulated probiotics for 12 weeks of storage, microencapsulated probiotics stored at 4℃ had a significantly higher viability than those stored at 25℃. Besides, storage time also had significant effect on the viability of the probiotics in apple juice, with the best consumption time being before the fourth week. However, ratios of core-to-wall, ratios of wall materials, and fermented or fortified technology did not have any significant effect on the viability of the microencapsulated probiotics. There is a possibility that the microcapsules may have been penetrated and disintegrated in the fruit juice during long-time soaking during storage and may only have a short-term effect on viability. For example, after a certain time of storage, the microcapsules may swell and rupture, and the effect of fermentation technology that increased the viability of probiotics may gradually develop.

5. SUMMARY

Probiotics are living microorganisms that have health benefits on the host when they are administrated to a sufficient amount (FAO/WHO, 2001) that are generally recommended as more than 6 log (CFU/g). Most probiotic food products are dairy-based, but plant-based matrices such as fruit juice can also serve as very good carriers for the delivery of probiotic cells. Fruits are considered as fresh, nutritious, and disease-avoiding foods due to their nutritional and functional properties, thus fruit juices are the popular and a good preference of consumers worldwide. In the production, storage, and digestion processes, many factors such as oxygen content, high temperature, low pH value, high bile salt content, etc., may affect the viability and functionality of probiotics. Microencapsulation technology offers a promising solution for the protection of probiotics. The effectiveness of protection is influenced by many factors including the types and properties of the coating materials, the ratios of core-to-wall or the ratios of wall materials, etc. My Ph.D. work focused on the encapsulation of the probiotic *Lp. plantarum* 299v strain. Investigation of the influence of different types and ratios of different coating materials such as polysaccharides (maltodextrin and resistant starch), proteins (whey protein and denatured whey protein), Maillard reaction products as well as ratios of core-to-wall on the protection of probiotics was aimed. Additionally, the application potentials of probiotic microcapsules were also aimed to explore.

Probiotic microcapsules were successfully produced by encapsulation with different coating materials, ratios of core-to-wall, and ratios of wall materials. The highest cell numbers were observed in the microcapsules coated with polysaccharides (ratio of core-to-wall 1:1.5 and MD:RS 3:1), proteins (ratio of core-to-wall 1:1 and WP:DWP 3:1), and MRPs (ratio of core-to-wall 1:1 and MD:WP 1:1) were 11.93 log (CFU/g), 11.29 log (CFU/g) and 13.75 log (CFU/g), respectively. In terms of yield, the best results were 66.74%, 68.02%, and 66.76%, respectively corresponding to polysaccharides (ratio of core-to-wall 1:1.5 and MD), proteins (ratio of core-to-wall 1:1.5 and DWP), and MRPs (ratio of core-to-wall 1:1.5 and MD:WP 3:1).

Scanning electron microscopy was performed to study the morphological properties of the microcapsules. The rod-shaped *Lp. plantarum* 299v cells were all homogenously microencapsulated and coated. Additionally, in the case of polysaccharides with a ratio of core-to-wall 1:1.5, the surface of the microcapsules was smoother, and had a more uniform structure compared to the samples with a ratio of core-to-wall 1:1. This observation was not noted in the microcapsules coated with proteins nor MRPs.

Table 4. Summary table of the highest viability, viability reduction and final cell number of probiotics coated by three kind of coating materials (storage for 8 weeks) at 4°C and 25°C

	parameters	polysaccharides	proteins	MRPs
4°C	ratio of core-to-wall	1:1.5	1:1	1:1
	ratio of wall materials	MD:RS 3:1	WP	MD:WP 1:1
	cell number changes	-0.81 log (CFU/g)	-0.14 log (CFU/g)	-1.16 log (CFU/g)
	highest number	**11.12 log (CFU/g)**	**10.96 log (CFU/g)**	**12.59 log (CFU/g)**
25°C	ratio of core-to-wall	1:1	1:1	1:1
	ratio of wall materials	MD:RS 1:3	WP:DWP 3:1	MD:WP 1:1
	cell number changes	-0.91 log (CFU/g)	-1.40 log (CFU/g)	-0.64 log (CFU/g)
	highest number	**9.90 log (CFU/g)**	**9.90 log (CFU/g)**	**13.11 log (CFU/g)**

MD: maltodextrin; RS: resistant starch; WP: whey protein; DWP: denatured whey protein

Different models obtained by regression analysis of experimental data were applied for monitoring the viability change of the probiotics. The viability of bacterial cells in microcapsules stored at 4°C had significantly lower loss compared to those stored at 25°C. However, no significant difference in viability loss was observed between the samples with a ratio of core-to-wall 1:1 and 1:1.5 even stored at 4°C or at 25°C. The comparison of the highest viability after 8 weeks of storage of three types of coating materials is listed in **Table 4**. The highest viability of probiotics coated by polysaccharides, proteins, and MRPs are 11.12 log (CFU/g), 10.96 log (CFU/g), and 12.59 log (CFU/g) when stored at 4°C. The highest viability of probiotics coated by polysaccharides, proteins, and MRPs are 9.90 log (CFU/g), 9.90 log (CFU/g), and 13.11 log (CFU/g) when stored at 25°C. In the case of storage during storage at 4°C, the best combinations of coating materials for the protection of probiotics were polysaccharides with a ratio of core-to-wall 1:1.5 and MD:RS 3:1, proteins with a ratio of core-to-wall 1:1 and WP, and MRPs with a ratio of core-to-wall 1:1 and MD:WP 1:1. These samples had viability reduction of 0.81 log (CFU/g), 0.14 log (CFU/g), and 1.16 log (CFU/g), respectively. In the case of storage during storage at 25°C, the best combinations of coating materials for the protection of probiotics were polysaccharides with a ratio of core-to-wall 1:1 and MD:RS 1:3, proteins with a ratio of core-to-wall 1:1 and WP:DWP 3:1, and MRPs with a ratio of core-to-wall 1:1 and MD:WP 1:1. These samples had viability loss of 0.91 log (CFU/g), 1.40 log (CFU/g), and 0.64 log (CFU/g), respectively, during storage at 25°C for 8 weeks.

The tolerance of different probiotic microcapsules to SGF and SIF was evaluated using an *in vitro* simulated gastrointestinal testing system. The comparison of the highest viability of probiotics coated by three kinds of coating materials after the SGF and SIF test is listed in **Table 5**.

Table 5. Summary table of the highest viability, viability reduction and final cell number of probiotics coated by three kind of coating materials after SGF and SIF test

	parameters	polysaccharides	proteins	MRPs
SGF	ratio of core-to-wall	1:1	1:1.5	1:1.5
	ratio of wall materials	MD:RS 1:1	WP:DWP 3:1	MD:WP 1:3
	cell number changes	-0.65 log (CFU/g)	-0.12 log (CFU/g)	-0.28 log (CFU/g)
	cell number after SGF	**9.04 log (CFU/g)**	**10.23 log (CFU/g)**	**10.79 log (CFU/g)**
SIF	ratio of core-to-wall	1:1.5	1:1	1:1.5
	ratio of wall materials	MD:RS 3:1	WP	MD:WP 1:3
	cell number changes	-0.11 log (CFU/g)	-0.35 log (CFU/g)	-0.52 log (CFU/g)
	cell number after SIF	**9.51 log (CFU/g)**	**8.92 log (CFU/g)**	**11.02 log (CFU/g)**

MD: maltodextrin; RS: resistant starch; WP: whey protein; DWP: denatured whey protein

The highest viability of probiotics after the SGF test coated by polysaccharides, proteins, and MRPs are 9.04 log (CFU/g), 10.23 log (CFU/g), and 10.79 log (CFU/g), respectively. The highest viability of probiotics after the SIF test coated by polysaccharides, proteins, and MRPs are 9.51 log (CFU/g), 8.92 log (CFU/g), and 11.02 log (CFU/g), respectively. The samples coated with polysaccharides in the ratio of core-to-wall 1:1 and MD:RS 1:1, with proteins in the ratio of core-to-wall 1:1.5 and WP:DWP 3:1, and with MRPs in the ratio of core-to-wall 1:1.5 and MD:WP 1:3 had the viability reduction of 0.65 log (CFU/g), 0.12 log (CFU/g), and 0.28 log (CFU/g), respectively after treatment in the simulated gastric fluid for 3 hours. Moreover, after placement of microcapsules in the simulated intestinal fluid for 6 hours, viability reduction of 0.11 log (CFU/g), 0.35 log (CFU/g), and 0.52 log (CFU/g) of the viability of viable cells was determined in the cases of coating with polysaccharides in the ratio of core-to-wall 1:1 and MD:RS 3:1, with proteins in the ratio of core-to-wall 1:1 and WP, or with MRPs in the ratio of core-to-wall 1:1.5 and MD:WP 1:3, respectively.

The production of fortified apple juice was successfully performed using probiotic microcapsules coated with polysaccharides, proteins, and MRPs. Application of microcapsules coated by polysaccharides in apple juice, the fortification method resulted in higher viability compared to fermentation. The comparison of the highest viability of probiotics coated by three kinds of coating materials to produce fortified and fermented apple juice after storage for 8 weeks at 4°C and 25°C are listed in **Table 6**. When stored at 4°C, the highest cell number of fortified apple juice produced by probiotics coated by polysaccharides, proteins, and MRPs are 8.43 log (CFU/g), 9.24 log (CFU/g), and 9.44 log (CFU/g), respectively. While the juice produced by the fermented method is 8.43 log (CFU/g), 9.24 log (CFU/g), and 9.44 log (CFU/g) when stored at 4°C.

Table 6. Summary table of the highest viability, viability reduction and final cell number of probiotics coated by three kind of coating materials to produce fortified and fermented apple juice store at 4°C and 25°C for 8 weeks

		parameters	polysaccharides	proteins	MRPs
4°C	fermented	ratio of core-to-wall	1:1	1:1	1:1.5
		ratio of wall materials	MD	WP:DWP 1:1	MD:WP 1:1
		highest cell number	8.26 log (CFU/ml)	8.97 log (CFU/ml)	9.27 log (CFU/ml)
	fortified	ratio of core-to-wall	1:1	1:1	1:1.5
		ratio of wall materials	MD	DWP	MD:WP 1:3
		highest cell number	8.43 log (CFU/ml)	9.24 log (CFU/ml)	9.44 log (CFU/ml)
25°C	fermented	ratio of core-to-wall	1:1.5	1:1	1:1.5
		ratio of wall materials	RS	WP:DWP 1:1	MD:WP 3:1
		highest cell number	7.39 log (CFU/ml)	8.44 log (CFU/ml)	5.10 log (CFU/ml)
	fortified	ratio of core-to-wall	1:1	1:1	1:1
		ratio of wall materials	MD:RS 1:1	WP:DWP 1:3	MD:WP 1:1
		highest cell number	7.67 log (CFU/ml)	8.47 log (CFU/ml)	6.04 log (CFU/ml)

Table 7. Summary table of characteristics of microcapsules

parameters		polysaccharides	proteins	MRPs
samples	ratios of core-to-wall	1:1	1:1	1:1.5
	ratios of wall materials	MD:RS 1:1	WP	MD:WP 3:1
viability changes (8 weeks)	4 °C/25 °C if different	4 °C better	4 °C better	4 °C better
	4 °C	-1.23 log (CFU/g)	-0.20 log (CFU/g)	-0.43 log (CFU/g)
	25 °C	-2.14 log (CFU/g)	-1.17 log (CFU/g)	-1.81 log (CFU/g)
viability changes in juices (8 weeks)	fortified/fermented if different	fortified better	fortified better	fortified better
	4 °C	0.15 log (CFU/ml)	0.16 log (CFU/ml)	-0.30 log (CFU/ml)
	25 °C	-0.93 log (CFU/ml)	0.94 log (CFU/ml)	0.78 log (CFU/ml)
Via-bility	SGF	-0.65 log (CFU/g)	-0.33 log (CFU/g)	-1.30 log (CFU/g)
	SIF	-0.94 log (CFU/g)	-0.35 log (CFU/g)	0.27 log (CFU/g)
viability after microencapsulation		11.19 log (CFU/g)	11.09 log (CFU/g)	12.39 log (CFU/g)
final viabi-lity		8.53 log (CFU/g)	11.21 log (CFU/g)	11.71 log (CFU/g)

MD: maltodextrin; RS: resistant starch; WP: whey protein; DWP: denatured whey protein

Similar results were found for those stored at 25°C. The highest viability of fortified apple juice

produced by probiotics coated by polysaccharides, proteins, and MRPs was 7.67 log (CFU/g), 8.47 log (CFU/g), and 6.04 log (CFU/g), respectively. The highest viability of fermented apple juice produced by probiotics coated by polysaccharides, proteins, and MRPs was 7.39 log (CFU/g), 8.44 log (CFU/g), and 5.10 log (CFU/g), respectively. In addition, the highest viability of probiotic apple juice with the same coating materials for microcapsules was also found to be higher when stored at 4°C compared to 25°C.

In summary, these results (**Table 7**) provided crucial information for the development of microcapsules systems with effective protection ability and potential application.

6. CONCLUSIONS AND RECOMMENDATIONS

In my Ph.D. research, microencapsulation with three types of coating materials in different ratios of core-to-wall and ratios of wall materials as well as industrial application potential were studied for the development of microcapsules as delivery systems with good protection for probiotic *Lp. plantarum* 299v strain. Three types of microcapsules coated with polysaccharides, proteins, and MRPs were developed successfully. Yield and efficiency of the encapsulation process as well as cell number and bulk density of the microcapsules were influenced by the types of coating materials, the ratios of core-to-wall, and the ratios of wall materials used. Among the investigated coating materials, the MRPs were the bests, because microcapsules coated with them resulted in significantly higher resistance to SGF and SIF than with the two other ones. Additionally, the viability of probiotic cells in microcapsules during storage was dependent on the nature of coating materials, the ratios of core-to-wall, the ratios of different wall materials, the storage temperature, and the storage time. The probiotic microcapsules were ready to apply in the fortification of apple juice, but the developed probiotic drink should be stored at 4°C temperature. Overall, this study provided valuable insights into the development of effective probiotic delivery systems through microencapsulation, and the newly developed microcapsules have high application potential in the fortification of foods.

There are several directions that could be pursued in future research:

(1) Optimization of microencapsulation process with MRPs as coating materials
(2) Study of control release properties of probiotic microcapsules
(3) Evaluation of the administration efficiency of probiotics
(4) Assession of the viability of probiotic microcapsules in the *in vivo* systems.

7. NOVEL CONTRIBUTIONS

1. Probiotic microcapsules were produced by encapsulation with different coating materials, ratios of core-to-wall, and ratios of different wall materials. The highest viabilities in the cases of polysaccharides (ratio of core-to-wall 1:1.5 and MD:RS 3:1), proteins (ratio of core-to-wall 1:1 and WP:DWP 3:1), and MRPs (ratio of core-to-wall 1:1 and MD:WP 1:1) were 11.93 log (CFU/g), 11.29 log (CFU/g) and 13.75 log (CFU/g), respectively.
2. The particle state and the surface morphology of probiotic microcapsules are different depending on the nature of different coating materials, ratios of core-to-wall, or ratios of wall materials. The cells of *Lp. plantarum* 299v strain were homogenously encapsulated and covered in all microcapsules.
3. Different models were developed and used for monitoring the changes in the viability of probiotic cells during storage at different temperatures. The probiotic microcapsules coated with MRPs in the ratio of core-to-wall 1:1 and MD:WP 1:1 showed both the highest cell number of the probiotic microcapsules stored at 4 °C and 25 °C after 8 weeks of storage with 12.59 log (CFU/g) and 13.11 log (CFU/g), respectively.
4. The highest cell number of probiotic cells was obtained to the tolerance of SGF and SIF tests in the case of the microcapsules coated with MRPs with a ratio of core-to-wall 1:1.5 and MD:WP 1:3 with 10.79 log (CFU/g) and 11.02 log (CFU/g), respectively.
5. The application of probiotic microcapsules in apple juice was achieved successfully through fortification and fermentation methods. The highest cell number of fortified apple juice in the cases of polysaccharides (core-to-wall 1:1 and MD), proteins (ratio of core-to-wall 1:1 and DWP), and MRPs (ratio of core-to-wall 1:1.5 and MD:WP 1:3) that stored at 4 °C after 8 weeks storage were 8.43 log (CFU/g), 9.24 log (CFU/g) and 9.44 log (CFU/g), respectively. The fortified apple juice should be stored at 4 °C. The microcapsules coated with MRPs with the mentioned conditions with the fortification method and stored at 4 °C have the highest cell number in the application of apple juice.

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1. **Sun, W.**; Nguyen, Q.D.; Sipiczki, G.; Ziane, S.R.; Hristovski, K.; Friedrich, L.; Visy, A.; Hitka, G.; Gere, A.; Bujna, E. Microencapsulation of *Lactobacillus plantarum* 299v Strain with Whey Proteins by Lyophilization and Its Application in Production of Probiotic Apple Juices. *Appl. Sci.* 2023, *13*, 318. https://doi.org/10.3390/app13010318

2. Pham, T.M.; **Sun, W.**; Bujna, E.; Hoschke, Á.; Friedrich, L.; Nguyen, Q.D. Optimization of Fermentation Conditions for Production of Hungarian Sour Cherry Spirit Using Response Surface Methodology. *Fermentation* 2021, *7*, 209. https://doi.org/10.3390/fermentation7040209

3. **Sun, W.**; Nguyen, Q.D.; Süli B. K.; Alarawi F.; Szécsi A.; Gupta V. K.; Friedrich L.; Gera A.; Bujna E. (2023). Microencapsulation and Application of Probiotic Bacteria *Lactiplantibacillus plantarum* 299v Strain. *Microorganisms* (Under reviewing process)

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1. **Sun, W.**; Bujna, E.; Nguyen, Q.D. (2021). Stability of apple juice fermented and fortified by microencapsulated *Lactobacillus plantarum* 299v during storage. *The 4th International Conference on Biosystems and Food Engineering*. Hungarian University of Agriculture and Life Sciences, Budapest, 4th June. http://www.biosysfoodeng.hu/

2. Pham, T.M.; **Sun, W.**; Bujna, E.; Hoschke, Á.; Nguyen, Q.D. (2021). Application of response surface methodology for fermentation optimization of cherry by *saccharomyces cerevisiae*. *The 4th International Conference on Biosystems and Food Engineering*. Hungarian University of Agriculture and Life Sciences, Budapest, 4th June. http://www.biosysfoodeng.hu/

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