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ENCAPSULATION OF PROBIOTICS FOR INCREASED THERMAL STABILITY FOR STORAGE AND INCREASED VIABILITY PASSING THROUGH THE GASTRO-INTESTINAL TRACT

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SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

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SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

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Master of Engineering

2020

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Abstract

Probiotics are defined as living bacteria that, when administered in adequate amounts, confer a health benefit on the host. (Hill, Guarner, Reid, Gibson, & Merenstein, 2014) Therefore, more and more people are beginning to explore the use of probiotics supplements for their proposed health benefits. However, probiotics being living bacteria, are susceptible to temperature and environmental fluctuations. In order to be able to provide a certain health benefit to the host, these live bacteria should maintain a high enough level of viability throughout the food processing procedures, during the storage duration and while passing through the gastrointestinal tract. (Chávarri, Marañón, & Villarán, 2012) One of the most common food processing methods for inactivation of harmful pathogens is through the use of heat. At present, there are few probiotics that are stable at high temperatures, thereby increasing the urgency to improve their heat resistance either through identification of new heat-stable strains or to develop novel delivery systems to protect them. (Solanki et al., 2013) Hence, much interest has been gathered on using encapsulation as a means of delivery and protection of the probiotics to able survive the food processing procedures and eventually be viable in the gastrointestinal tract.

In this study, the use of Maillard conjugation of whey protein with citrus pectin as encapsulants is explored for the encapsulation of *Lactobacillus plantarum* (DSM 12028) for increased thermal stability for storage and increased viability passing through the Gastrointestinal Tract. Viability of *L.Plantarum* encapsulated in 2% whey protein and 1% pectin (WP1) in room temperature (25°C) storage after 60 days was found to be 7 log units higher than free *L.Plantarum* cells. The encapsulation of WP1 also resulted in an increase in viability through the upper gastrointestinal tract with a log (CFU/g) of 8.15 compared to 4.90 in free unencapsulated *L.Plantarum* cells. WP1 also showed a high heat tolerance in pasteurization with only a 0.33 log units reduction after 30 minutes at 85°C.

1. Introduction

1.1 Hypothesis/Problem Statement

Probiotics have been consumed in many forms over the last several centuries across many different civilizations for their proposed health benefits. However, probiotics being living bacteria, are susceptible to temperature and environmental fluctuations. In order to be able to provide a certain health benefit to the host, these live bacteria should maintain a high enough level of viability throughout the food processing procedures, during the storage duration and while passing through the gastrointestinal tract. (Chávarri et al., 2012) One of the most common food processing methods for inactivation of harmful pathogens is through the use of heat. At present, there are few probiotics that are stable at high temperatures, thereby increasing the urgency to improve their heat resistance either through identification of new heat-stable strains or to develop novel delivery systems to protect them. (Solanki et al., 2013) Hence, much interest has been gathered on using encapsulation as a means of delivery and protection of the probiotics to able survive the food processing procedures and eventually be viable in the gastrointestinal tract. Many different methods of encapsulation of probiotics has since been explored; forming of probiotic emulsions using different encapsulants,(Qi, Liang, Yun, & Guo, 2019) forming microcapsules and microgels using extrusion method,(Heidebach, Först, & Kulozik, 2012) freezing drying of probiotic cells,(Chalat Santivarangkna, Kulozik, & Foerst, 2007) and spray drying of probiotic cells.(De Castro-Cislaghi, Carina Dos Reis, Fritzen-Freire, Lorenz, & Sant'Anna, 2012) Although much research has been done in the field of probiotic encapsulation many of the studies still present some inherent problems:

- The inability to overcome both the problem of thermal stability and navigation of gastrointestinal transit
- Difficulty for incorporation into existing food production due to the shape, size, form etc. of encapsulated particles
- Difficulty for scale up production for commercialization

Hence in this study, the use of Maillard conjugation of whey protein with citrus pectin as encapsulants is explored for the encapsulation of *Lactobacillus plantarum* (DSM 12028) for increased thermal stability for storage and increased viability passing through the Gastrointestinal Tract.

1.2 Objective and Scope

- Encapsulation of *Lactobacillus plantarum* (DSM 12028) using Maillard conjugation of whey protein and citrus pectin
- To test the conjugation efficiency of whey protein and citrus pectin conjugation
- To test the viability of *Lactobacillus plantarum* (DSM 12028) after encapsulation
- To test the viability of encapsulated *Lactobacillus plantarum* (DSM 12028) in storage at room temperature (25°C)
- To test the viability of *Lactobacillus plantarum* (DSM 12028) after pasteurization at (85°C)
- To test the viability of encapsulated *Lactobacillus plantarum* (DSM 12028) after passing through gastrointestinal tract

2. Literature Review

2.1 Probiotics

The term probiotics is developed from the Greek phrase “pro bios” which means “for life”. This would mean that the food or substance promotes improved health in the host. Since ancient times, people have been consuming probiotics in the form of fermented food across many different civilizations. Calpis (Japanese), Kaymak (Turkish), Kefir (Russian), Kimchi (Korean) and Tofu (Southeast Asian) are some examples for such food that have been consumed. (Bagchi, 2014) It was not until the early 1900s that Elie Metchnikoff hypothesized that lactic acid bacteria (LAB) can offer health benefits such as promote longevity through the modification of gut microbiota and replacing harmful gut microbes with useful ones. Following that, Henry Tissier postulated that oral administration of useful microbes (*Bifidobacteria*) to patients with diarrhoea can help improve the conditions through restoring of the gut flora. (Merenstein & Salminen, 2017) Since there, much more research has been done with regards to probiotics and in 2001, Food and Agriculture Organization of the United Nations (FAO) together with World Health Organization (WHO) came up with a definition that is widely used and recognized today: “Live microorganisms which when administered in adequate amounts confer a health benefit on the host”. (Morelli & Capurso, 2012) Through the years of research, scientist have found that probiotics have the potential to provide many different health benefits. Some of these health benefits could include : anti-pathogenic, anti-cancer, anti-inflammatory, anti-diabetic and even angiogenic activities. (Kerry et al., 2018) Many of these benefits however, are specific to the different genus, species and strains.

2.1.1 Health Benefits of Probiotics

Probiotics coming from fermented food such as kefir, kimchi, tofu, etc. have been ingested by humans for many years in the belief that they can provide health benefits. In recent years, as more research has been done into understand our gut microbiota, many studies have postulated that probiotics are playing crucial roles in modulation of gastrointestinal immunological, and respiratory functions. (Floch et al., 2011) Due to the constant scientific evaluation in this area, there is now strong evidence that the use of probiotics can help in treating and preventing certain diseases. (Boyle, Robins-Browne, & Tang, 2006) Probiotics have been said to provide a number of health benefits which includes: a) prevention of gastrointestinal pathogens through the maintenance of a healthy intestinal flora, ; b) strengthening of the immune system; c) lowering of blood pressure and reducing blood cholesterol; d) having anti-carcinogenic properties; e) improved utilization of nutrients; f) prevention of inflammation; g) production of antimicrobial compounds; h) treatment and prevention of diarrhoea; i) improvement of lactose tolerance; j) treatment of urinary tract infections and respiratory infections have also been documented (D'Aimmo, Modesto, & Biavati, 2007; Kechagia et al., 2013; Marco, Pavan, & Kleerebezem, 2006; Parvez, Malik, Ah Kang, & Kim, 2006; Tripathi & Giri, 2014)

2.1.2 Probiotics Mechanism of Action

Over the years, there have been many studies done to try and understand the mechanisms of action of probiotics. However, the exact mechanisms by which how the effects are exerted on the host is not completely understood. (Harish & Varghese, 2006) According to (Hemaiswarya, Raja, Ravikumar, & Carvalho, 2013), how effective the probiotics is on the host depends strongly on their adherence and how well they can colonize of the gut in order to improve the host’s immune system. Different species of probiotics differ significantly in their mechanism of action; None of the probiotic species found till date is able to exhibit all mechanisms of action to prevent or treat all the different diseases. (O'Hara & Shanahan, 2007; Oelschlaeger, 2010) Some of the proposed mechanisms of probiotic actions consist of: a) the production of bacteriocins which provides an antimicrobial effect; b) the production of lactic acid, and short-chain fatty acids which brings down the pH in the gut lumen to inhibit pathogens colonization; c) the activation of tight junction proteins for maintenance of the epithelial barrier to prevent the development of a leaky intestine; d) the prevention of inflammation and apoptosis of the lining intestinal epithelial cells (Harish & Varghese, 2006; Michail, 2005; Ng, Hart, Kamm, Stagg, & Knight, 2009; Sarowska, Choroszy-Król, Regulska-Ilow, Frej-Madrzak, & Jama-Kmiecik, 2013; Sherman, Ossa, & Johnson‐Henry, 2009)

2.1.3 Lactic Acid Bacteria

In order for the probiotic strains to be used, they are required to be identified by their genus, species and strain designation and deposited into an international culture collection. Till date, there have been many different probiotics strains including different types of bacteria and even yeast. (Anadón, Martínez-Larrañaga, Ares, & Martínez, 2016)

Probiotic Microorganisms	Species Involved	References
Lactobacillus	*L.Plantarum, L.paracasei, L.acidophilus, L. casei, L. rhamnosus, L. crispatus, L. gasseri, L.reuteri, L.bulgricus*	(Dixit, Wagle, & Vakil, 2016; Rault, Béal, Ghorbal, Ogier, & Bouix, 2007)
Streptococcus	*S. thermophilus, S. salivarius, S. sanguis, S. oralis, S. mitis*	(Arora, Singh, & Sharma, 2013)
Bifidobacterium	*B. longum, B. catenulatum, B. breve, B. animalis, B. bifidum*	(Kurmann & Rasic, 1991; Sakata et al., 2002)
Bacillus	*B. coagulans, B. subtilis, B. laterosporus*	(Nguyen et al., 2016)
Lactococcus	*L. lactis*	(Eid et al., 2016)
Enterococcus	*E. faecium*	(Lund, Adamsson, & Edlund, 2002)
Pediococcus	*P. acidilactici*	(Sornplang & Piyadeatsoontorn, 2016)
Propionibacterium	*P. jensenii, P. freudenreichii*	(Zárate, 2012)
Peptostreptoccus	*P. productus*	(Kontula et al., 1998)
Escherichia coli	*E. coli Nissle*	(Ukena et al., 2007)
Saccharomyces	*S. boulardii*	(Chen et al., 2013)

Table 1: Microorganisms used as probiotics

Presently, the most common and widely researched probiotic microbes are Lactic Acid Bacteria (LAB) which include *Lactobacillus*, *Streptococcus* and *Bifidobacterium.* (Vanderhoof, 2000) These bacteria produce Lactic Acid as one of the major end products. Amongst those, *Lactobacillus* is known to be the largest genus of the group. *Lactobacillus* comes from the phylum *Firmicutes*, class of *Bacilli*, order of *Lactobacillales* and family of *Lactobacillaceae*. They are rod shaped bacteria which are anaerobic, Gram positive and non-spore forming. (M. De Angelis, 2016)

2.1.4 Lactobacillus Plantarum

One of the most studied species of the genus would be *Lactobacillus plantarum* as many strains within the species have found to have distinct probiotic properties. They are said to produce many desirable health benefits including having antipathogenic properties, antiallergic properties, ability to reduce cholesterol and even improving brain function in autistic children. (Adlerberth et al., 1996; Kumar et al., 2016; Puertollano et al., 2008; Umbrello & Esposito, 2016; Zendeboodi, Khorshidian, Mortazavian, & da Cruz, 2020) Given the myriad of beneficial properties, this allows for *Lactobacillus plantarum* to be a promising candidate for use as probiotics. However, the guidelines from FAO/WHO emphasized the need for probiotic strains to be viable throughout the gastrointestinal tract to be able to confer the proposed health benefits upon entering the large intestine, regardless the delivery mode applied. (Bosnea, Moschakis, & Biliaderis, 2017; Morelli & Capurso, 2012)

2.2 Gastrointestinal Tract

As it is critical to ensure that probiotics are viable through to their destination of action in order to provide the proposed benefits, understanding of the barriers and harsh conditions they face through the gastrointestinal transit becomes critical. After being ingested, probiotics face many different harsh conditions throughout the Gastrointestinal Tract; which includes the mouth, the stomach as well as the small intestine.

In the mouth, probiotics are mainly affected by mineral salts, mucin and salivary amylase. However, as the pH within the mouth is relatively neutral, pH 6-7, which is similar to that of the colon where the probiotics are conditioned for. (Yeung, Arroyo-Maya, McClements, & Sela, 2016) The transit time within the mouth is also short, hence the harm caused to the probiotics is not as significant.

As they proceed into the stomach, the gastric fluids which are highly acidic, reduces the pH as low as pH 1-3 causing the conditions to be increasingly challenging. (Lankaputhra, 1995)

This low pH causes a disruption in the cytoplasmic pH of the probiotics and in turn reduces the glycolytic enzyme activity and reduces the survivability of many probiotics under these conditions. (Cotter & Hill, 2003; Sarao & Arora, 2017) Within the stomach, there are also other factors such as high salt content, enzymatic activity and mechanical churning which also reduces the viability of probiotics. (González-Ferrero, Irache, & González-Navarro, 2018; Surono, Verhoeven, Verbruggen, & Venema, 2018; Yeung et al., 2016)

Upon reaching the small intestine, probiotics are affected by the digestive enzymes and bile acids present. According to Hamner et. al (2013), (Hamner, McInnerney, Williamson, Franklin, & Ford, 2013) bile acids possess some antibacterial properties which could disrupt cell membranes and damage bacteria DNA. (Begley, Hill, & Gahan, 2006) mentioned however, that some probiotics could circumvent this through production of bile salt hydrolases (BSHs) to unconjugated bile acids. However, the exact mechanisms in which probiotics and bile acids interact remains unclear and further research needs to be done to better understand these interactions. (Jia & Xie, 2018)

According to the guidelines from FAO/WHO, even before the probiotics weather the conditions of the gastrointestinal tract, there should be a "minimum therapeutic" level of live probiotic microbes in a food product of at least 10^6 CFU/g of live cells during the entire shelf-life of the product. (Morelli & Capurso, 2012) This means to say that, to account for the loss in the gastrointestinal tract as well, the conditions for an effective probiotic product would be much stricter. Many reports have also mentioned, that probiotics survivability in many products which uses free probiotic cells is very poor. (de Vos, Faas, Spasojevic, & Sikkema, 2010) Some of these probiotic products in the market encapsulate them in a form of supplement such as tablet or capsules. However, although these formulations are large enough to encapsulate a large amount of probiotics, they are too large to past directly through the pyloric sphincter which leads to the small intestine. Hence, when the encapsulated probiotics are released into the stomach, they become susceptible to the unforgiving environmental conditions there. (Yao et al., 2020) Hence, in order to ensure that all the necessary conditions for probiotic effectiveness are met, methods to protect the probiotic strains through the food processing procedures, storage conditions and the harsh environment of the gastrointestinal tract have been actively researched. One of the most common and widely researched methods would be to encapsulate the probiotics.

2.3 Encapsulation

Encapsulation is known as either a mechanical or physiochemical process in order to encase a substance, which could be a functional or bioactive compound, in a material in order to protect it, or for further delivery and release. (de Vos et al., 2010) These particles produced could have diameters ranging from a few nanometres to a few millimetres. These results in small particles that are capsules which form a shell or coating to contain active agents or core material.(Chávarri et al., 2012) This technique has seen increased research and interest, due to its applications in the pharmaceutical industry for drug and vaccines delivery. It is also been increasingly used in the food industry for the delivery of bioactive molecules (e.g. carotenoids, fatty acids, vitamins and antioxidants) or live cells such as probiotics. (Nedovic, Kalusevic, Manojlovic, Levic, & Bugarski, 2011) Encapsulation is a versatile technique that could be used for many purposes. It can be used to mask undesirable taste, odour or colouration which could affect the product performance. It can also be used to prevent interactions and reactions between the active food ingredients to be delivered and other ingredients or food components. Apart of that, it can also be used for controlled release of the food ingredient. (Sobel, Versic, & Gaonkar, 2014) In the case of probiotics, encapsulation is utilized to protect them from several factors:

- Processing conditions (temperature, oxidation, shear, etc.)
- Storage conditions (oxygen, moisture, temperature, packaging, etc.)
- Degradation by Gastrointestinal Tract (enzymes, low pH, bile acids, etc.)

This allows the probiotics to be released in a biologically active and viable state in the colon, (Picot & Lacroix, 2004) (Ding & Shah, 2007) while keeping crucial factors such as preserving the aroma and taste, maintaining the nutritional value, and keeping product appearance consistent. (Parra Huertas, 2010) There are several methods of encapsulation. These methods include extrusion, emulsification, coacervation and spray drying. (Rathore, Desai, Liew, Chan, & Heng, 2013) Among those, the most commonly used microencapsulation technique in the food industry due its flexibility, cost effectiveness and ability to be scaled up easily, would be spray drying. (Pu, Bankston, & Sathivel, 2011)

2.3.1 Spray Drying

Spray drying relies on the use of rapid dehydration to transform a feeding suspension, emulsion, solution, or dispersion into small dried particles by spraying the feed onto a heated surface or medium. Depending on the properties of the feed to be sprayed, the dried particles could appears as powders, granules or agglomerates. (Patel, Patel, & Suthar, 2009) Since the

1940s, pharmaceutical industry has successfully utilized spray drying for production of drug substances such as antacids, vitamins, antibiotics and analgesics. Then, from the late 1950s onwards, spray-drying was picked up by the food industry to encapsulate various oils to prevent them from degradation and oxidation and to produce powders from liquids for easier storage and extending shelf-life. (Phisut, 2012) Spray drying is presented as a viable method for drying of heat-sensitive active biological substances, such as live cells, proteins and even enzymes, with very minimal activity losses. (Idham, Muhamad, & Sarmidi, 2012) The process of microencapsulation by spray drying begins with the formation of an emulsion or suspension of coating or shell material with the core material. Then the emulsion or suspension is fed into the atomizer where it is sprayed out as tiny droplets into the drying chamber. This chamber is circulated with hot dry air which rapidly dehydrates and evaporate the droplets leaving the core material entrapped in the coating material as small dried particles. The dried particles are then sent through the cyclone and collected in a container. (Khuenpet, Charoenjarasrerk, Jaijit, Arayapoonpong, & Jittanit, 2016)

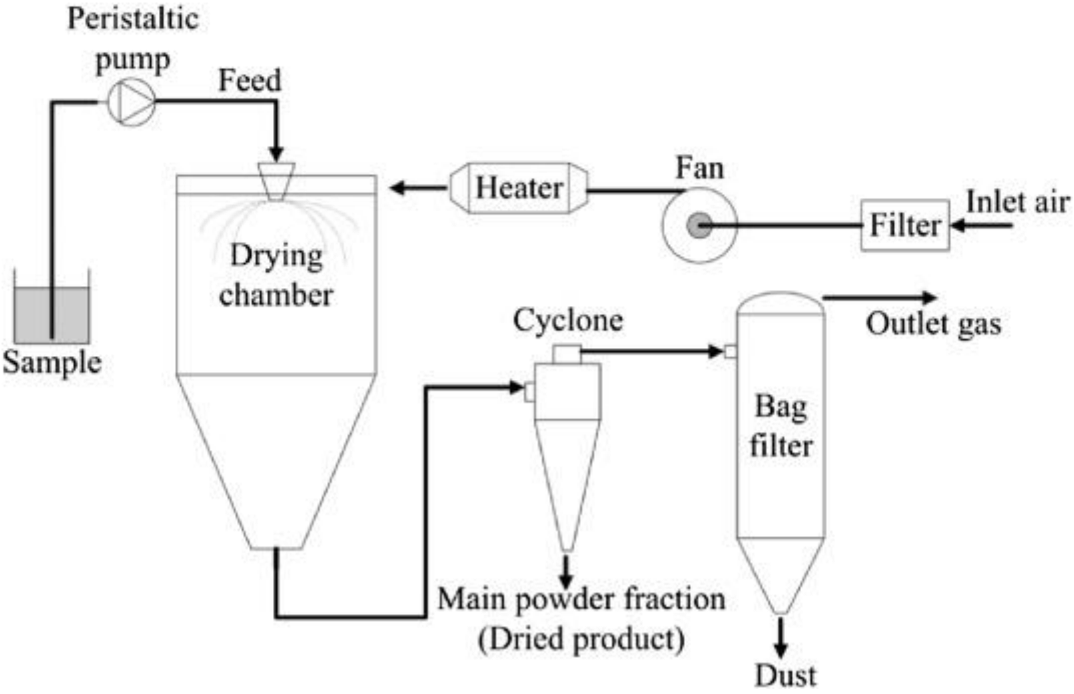


Figure 1: Schematic Diagram of a Spray Dryer (Khuenpet et al., 2016)

The utilization of spray drying for probiotic bacteria in the food industry has been around for decades and is used predominantly by the dairy industry. (Huang et al., 2017) The technique involves dispersing the probiotic cells into the encapsulating polymer solution and atomized into the spray drying chamber to evaporate the solvents to form the encapsulated particles. (De Castro-Cislaghi et al., 2012) In spray drying, bacterial cultures usually die from exposure to heat, oxidative and osmotic stress, resulting from the high temperature during the dehydration. (Paéz et al., 2012) According to (Peighambardoust, Tafti, & Hesari, 2011), the main reasons for the loss of viability of probiotic cells is due to the combination of temperature and time, and that the inlet air temperature has little impact on probiotic death. (C Santivarangkna, Kulozik, & Foerst, 2008) proposed that outlet air temperature is the key parameter which affects the survivability of probiotic cultures during spray drying, and this parameter is dependent on the other parameters such as air flow rate, inlet air temperature, aspiration droplet size, and feed pump rates. (Chalat Santivarangkna et al., 2007) This claim that lower outlet air temperature results in an increased survival of probiotic cells is also supported by other researchers. (Ananta, Volkert, & Knorr, 2005; Desmond, Ross, O'callaghan, Fitzgerald, & Stanton, 2002) During a study done on several strains of probiotics isolated from kefir, (Golowczyc, Silva, Teixeira, De Antoni, & Abraham, 2011) found that *L.Plantarum* exhibited the highest survival rate across different outlet temperatures (70, 75, 80, 85°C). A similar results was found by (Barbosa et al., 2015; Iaconelli et al., 2015) and they concluded that sensitivity to different drying methods is bacterial strain specific.

2.3.2 Encapsulation materials

The selection of a wall coating material for microencapsulation is of paramount importance to obtain a stable and effective encapsulation. Encapsulation materials are typically selected based on the physicochemical properties such as melting/glass transition temperature, crystallinity, solubility, molecular weight and emulsifying properties. (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007) Many researchers such as (Agnihotri, Mishra, Goda, & Arora, 2012; Anandharamakrishnan & Ishwarya, 2015) emphasize the need to select appropriate wall materials as they determine the physical and chemical properties of the resulting microcapsules. Some of the materials commonly used to encapsulate probiotic cells include different polysaccharides (alginates, pectin, xanthan gum, gum arabic, carrageenan, chitosan, starch, etc.) oligosaccharides (maltodextrin, sucrose and corn syrup), proteins (soy protein, whey protein, casein, albumin and gluten), and lipids (waxes, paraffin, diglycerides, oils, fats, etc) (Das, Ray, Raychaudhuri, & Chakraborty, 2014; De Castro-Cislaghi et al., 2012; Serna-Cock & Vallejo-Castillo, 2013)

2.3.3 Pectin

Pectin is biopolymer which is increasingly used as an encapsulation material. This is due to several desirable properties it has, like superior gelling properties, great binding abilities the ability to stabilize emulsions, (Assadpour, Jafari, & Maghsoudlou, 2017; Gharehbeglou, Jafari, Hamishekar, Homayouni, & Mirzaei, 2019) Given that it is typically obtained from fruits, it is can also be considered as a food safe ingredient. (Colodel, das Graças Bagatin, Tavares, & de Oliveira Petkowicz, 2017) Different types of pectin exhibit different hydrophobicity. Unlike low methoxylated pectin which is usually hydrophilic, high methoxylated pectin is known to be very hydrophobic and interacts well with hydrophobic molecules. This enables pectin to play an important role in the incorporation of hydrophobic drugs, such as fluoroquinolone which is a type of antibiotics, into the encapsulation matrix to allow for controlled release. (Cacicedo et al., 2018) Other than that, pectin is also commonly used for the encapsulation of bioactive ingredients (such as polyphenols, carotenoids etc.) through several methods, such as spray drying, forming of emulsions and coacervation. (Fathi, Martin, & McClements, 2014)

2.3.4 Whey Protein

Whey proteins which are derived from whey, In the food industry, whey proteins are one of the most used food ingredients given their desirable functional properties. They are typically used as stabilizers, in gelling, texturizing and even as emulsifiers for various oil-in-water emulsions. (de Castro et al., 2017; Foegeding, Davis, Doucet, & McGuffey, 2002) The main components in whey protein, β-lactoglobulin and α-lactalbumin, are known to be able to form protective films (wall system), allowing them to be optimal wall material for encapsulation. Whey protein isolate (WPI) may also be formulated into nanoparticles, which could be used to as a medium of encapsulation for several different types of bioactive compounds, such as carotenoids, vitamins, and isoflavonoids etc. (Abbasi, Emam-Djomeh, Mousavi, & Davoodi, 2014; Flores, Singh, & Kong, 2014) (Schuck, Dolivet, Mejean, Hervé, & Jeantet, 2013) also found that through the use of sweet whey as the drying medium, probiotics can be spray dried with close to 100% survival rate. However, the use of whey protein also comes with some drawbacks. Within the food industry, the main limitations for the use of whey protein as a high value ingredient include: a) having poor protein solubility under low pH conditions, which causes phase separation and turbidity of the mixture (Akhtar & Dickinson, 2007); b) once the proteins are hydrolysed, they exhibit poor emulsification properties which limits its utility by causing challenges such as poor emulsion formation ability, instability, and becoming very sticky, resulting in difficulty in spray drying (Singh & Dalgleish, 1998); c) the proteins also tend to be physical instable, causing sedimentation, creaming and aggregation during processing, storage in highly charged environments as well as during thermal processing.(Yadav, Parris, Johnston, Onwulata, & Hicks, 2010) In order to prevent undesirable effects such as whey protein aggregation, the conjugation of whey proteins to different polysaccharides was explored as a viable method.(Akhtar & Dickinson, 2007; Xu, Wang, Jiang, Yuan, & Gao, 2012; Zhu, Damodaran, & Lucey, 2010) Conjugation is able to successfully modify the functional properties of a myriad of milk protein/peptide-based ingredients, which includes whey protein ingredients as well. In particular, (Setiowati, Saeedi, Wijaya, & Van der Meeren, 2017) found that conjugation of whey protein isolate and low methoxyl pectin significantly improved heat stability.

2.4 Maillard Reaction

Over the years, researchers have explored means of altering properties and improving the functionality of proteins for different industrial applications. One such method is through the use of Maillard Reaction (MR). Maillard reaction has been widely researched for altering and improving the properties of whey protein significantly. (Spotti et al., 2013) Maillard Reaction is a reaction between proteins or amino acids and carbohydrates. The ε-amino groups in the proteins covalently bonds with the end carbonyl groups of polysaccharides under heated conditions. This covalent crosslinking of the proteins and carbohydrates results in highly desirable properties for food processing such as increased heat stability and better emulsifying properties. (Kato, 2002; Pellegrino, Van Boekel, Gruppen, Resmini, & Pagani, 1999) According to (Hodge, 1953), Maillard Reaction divided into three stages; namely the early, intermediate and advanced stages.

In the early stage, the free amino group from the protein is bonded to the reducing sugar group in the carbohydrate to form a Schiff base and produces a H_2O molecule. The Schiff base is unstable and would undergo spontaneous rearrangement to form either an Amadori (Aldose) product or a Heyn’s (Ketose) product depending on the reducing sugar. (Wrodnigg & Eder, 2001) Then, during the intermediate stage, the Amadori/Heyn’s product will undergo several reactions such as dehydration, isomerization, cyclization and condensations, which results in sugar degradation and fragmentation, and amino acids will undergo degradation as well. (Koubaa et al., 2018) The advanced stage on the other hand become much more complex. The reactions that occur become variable and highly dependent on environmental conditions. Although some studies mentioned that some of the products from advanced stage Maillard Reaction have beneficial properties such as antihypertensive antimicrobial and antioxidant properties, (Nooshkam, Babazadeh, & Jooyandeh, 2018; Nooshkam, Varidi, & Bashash, 2019; H.-Y. Wang, Qian, & Yao, 2011)others stated that products can lead to diseases like diabetes and Alzheimer.(Silván, Assar, Srey, Del Castillo, & Ames, 2011)

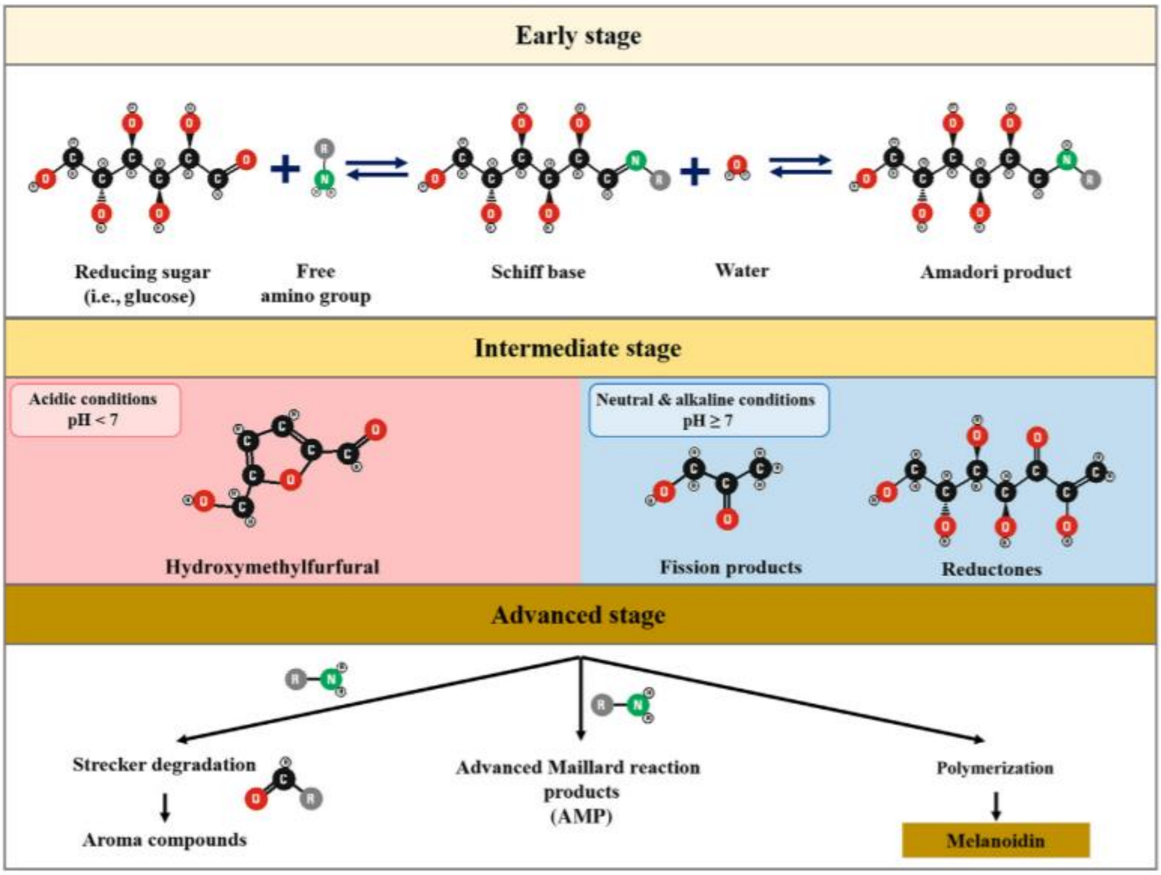


Figure 2: Schematic diagram of Maillard Reaction (O'Mahony, Drapala, Mulcahy, & Mulvihill, 2017)

Hence, it is advisable to use more superficial Maillard conjugation methods, which is sufficient to improve protein properties such as increased solubility and enhanced thermal stability, to prevent any complications. (de Oliveira, Coimbra, de Oliveira, Zuñiga, & Rojas, 2016) It can be categorized into two different processes; wet and dry heating, both having their own sets of advantages and disadvantages. During dry heating, the rate of reaction can be easily controlled owing to the lower moisture diffusion resulting from having a lower water content. (Nasirpour, Scher, & Desobry, 2006). However, dry heating is usually more tedious and needs a considerably long reaction time with harsher conditions. During the process, formation of insoluble substances might occur as well. Apart from relying on dry heating to conjugate whey protein and polysaccharides, researchers have also managed to form complexes through the wet heating method as well. (W.-q. Wang, Bao, & Chen, 2013) In the wet heating process, there is a retardation of the rate of reaction which is caused by the dilution of the water-soluble reactants given the higher water content. (Nasirpour et al., 2006) The wet heating process is also much faster as it is much less laborious. Similar to the dry heating process, it is also possible to control the reaction degree of wet heating effectively.

However, these might also result in addition interactions such as electrostatic or hydrophobic interactions. (Perusko, Al-Hanish, Velickovic, & Stanic-Vucinic, 2015)

3. Materials and Methods

3.1 Culture of Probiotics

Bacterial strain Lactobacillus plantarum (DSM 12028), obtained from the Leibniz Institute DSMZ German Collection of Microorganism and Cell was inoculated into 5mL MRS broth (Sigma Aldrich) and incubated at 37 °C for 48 h which serves as the stock culture. After which, the stock culture was inoculated into a 250mL Erlenmeyer Flask at an optical density of 0.2 at 600nm wavelength, which corresponds to 10^8 cells/mL (Ramos, Thorsen, Schwan, & Jespersen, 2013) and incubated at 37 °C for 48 h. When harvesting, cells were initially centrifuged at 5000g at 4°C for 10 minutes and washed thrice with milliQ water, then the cells were kept at 4°C to be used for encapsulation.

3.2 Enumeration of Viable Cells

Enumeration of *L. Plantarum* was done using the spread plate method. 100 µL of each cell suspension was taken and serially diluted in 900 µL of milliQ water. 100 µL of each dilution (10^{-6}–10^{-9}) was added into sterile petri dishes which has 15 mL of MRS agar poured into each dish that was cooled and set. A L-shaped spreader is then used to spread the 100 µL suspension across the entire agar plate and incubated anaerobically at 37°C for 48 h in BD GasPak™ EZ container systems and the colonies were counted between 15 and 300 and the number of colony-forming units (CFU) in 1 mL of dilution was determined (CFU = no. of colonies × dilution factor).

Figure 3: *L. Plantarum* incubated anaerobically in BD GasPak™ EZ container system

3.3 Maillard Conjugation of Encapsulation materials

The citrus peel pectin was obtained from Sigma Aldrich, USA and the 15% protein content sweet whey powder was obtained from Jinan Meishubao Biotechnologies, China. The pectin powder was added to 500mL of milliQ water at 2% w/v and mixed for 30 minutes at 60°C until fully dissolved. The sweet whey powder was diluted in 500mL of milliQ water to obtain a 2% w/v whey protein solution. The method for wet heating was adapted from the method proposed by Gao F et. al, 2019 (Gao et al., 2019). Briefly, the whey protein and pectin solution were added together to obtain 2:1, 1:1 whey protein:pectin ratio solutions (WP2, WP1) respectively. Both solutions were adjusted to pH 7.0 and constantly stirred magnetically and maintained at a temperature of 85°C for 48h to allow Maillard conjugation to occur. The solutions were then rapidly cooled to room temperature for use in encapsulation.

3.4 Conjugation Efficiency

Using the O-phthaldialdeyde (OPA) method modified from (Davidov-Pardo, Joye, Espinal-Ruiz, & McClements, 2015), the conjugation efficiency was inversely determined through analysing the free amino groups. By determining the difference in free amino groups, we can determine the change in conjugation. The OPA reagent used was obtained from Sigma Aldrich, USA and the UV Spectrophotometer used was from the NanoDrop 2000 from Thermo Scientific. Briefly, 200 μL of sample solution was mixed with 4 mL of OPA reagent and then heated through a water bath at 35 °C for 2 min. Then, using the UV

Spectrophotometer, the absorbance of the reacted solution was taken at a wavelength of 340 nm. Conjugation Efficiency (CE) was back calculated from the change in the amount of unconjugated free amino groups using the following equation:

$$CE=(1- A_{After}/A_{Before})\times 100\%$$

Where A_{After} and A_{Before} were the amount of unconjugated amino groups found in the solution before and after conjugation respectively. A control group of 2% whey protein solution, was also included.

3.5 Size distribution of whey protein-pectin conjugates

Size distribution of the whey protein-pectin conjugates were measured through dynamic light scattering using the Malvern Zetasizer Nano SZ, UK. and the mean particle size was expressed as d50.

3.6 Encapsulation through spray-drying of *L.Plantarum*

200mL of both WP2 and WP1 solutions were inoculated with *L.Plantarum,* and stirred constantly for 30 minutes with the CFU in each at 7.6 x 10^9 CFU/mL of WP solution. A control group with 7.6 x 109 CFU/mL of *L.Plantarum* in 200mL of milliQ water was also added. BUCHI Mini Spray Dryer B-290, Switzerland was used to process samples at inlet temperature of 120°C, air flow rate of 35m^3/h, compressed air pressure of 6 bar, feed rate of 7.5mL/min and outlet temperature of between 55-60°C. All the solutions were stirred constantly throughout the spray drying process to ensure even mixing.

Figure 4: BUCHI Mini Spray Dryer B-290

3.7 Shelf-life testing of microencapsulated *L.Plantarum*

The powder was collected after spray-drying and stored at 25°C for 60 days and enumerated fortnightly. To enumerate the surviving *L.Plantarum* bacteria, 200mg of encapsulated *L.Plantarum* powder was dissolved in 20mL of milliQ water and mixed for 30 minutes. The solution was then serially diluted and plated according to the enumeration method mentioned in 3.2 above.

3.7 Scanning Electron Microscopy (SEM) imaging

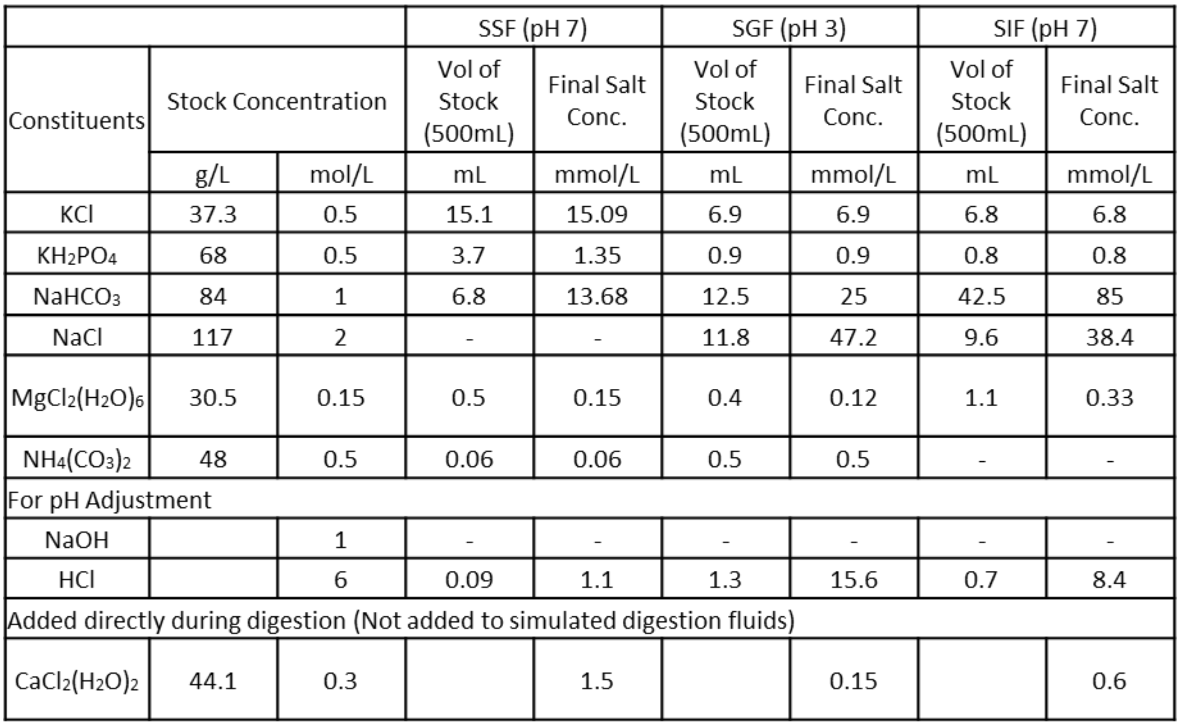
Scanning Electron Microscopy imaging of the encapsulated samples was done using the JEOL JSM 6701F scanning electron microscope from Japan. The accelerating voltage was 6.0kV. Trace amount of samples were added to a double sided adhesive carbon tape before sputtered coated with platinum layer using the JEC-3000FC Auto Fine Coater to inhibit charging.

3.8 Viability of *L.Plantarum* after pasteurization

The pasteurization testing method was done in accordance to the method proposed by (Mao, Pan, Hou, Yuan, & Gao, 2018). 200mg of encapsulated *L.Plantarum* powder was dissolved in 20mL of milliQ water and mixed for 30 minutes. Thereafter, the mixture was then heated in a water bath at 85 °C for 15 minutes. Then, the samples were cooled to room temperature and the surviving bacteria was enumerated according to the method described in 3.2.

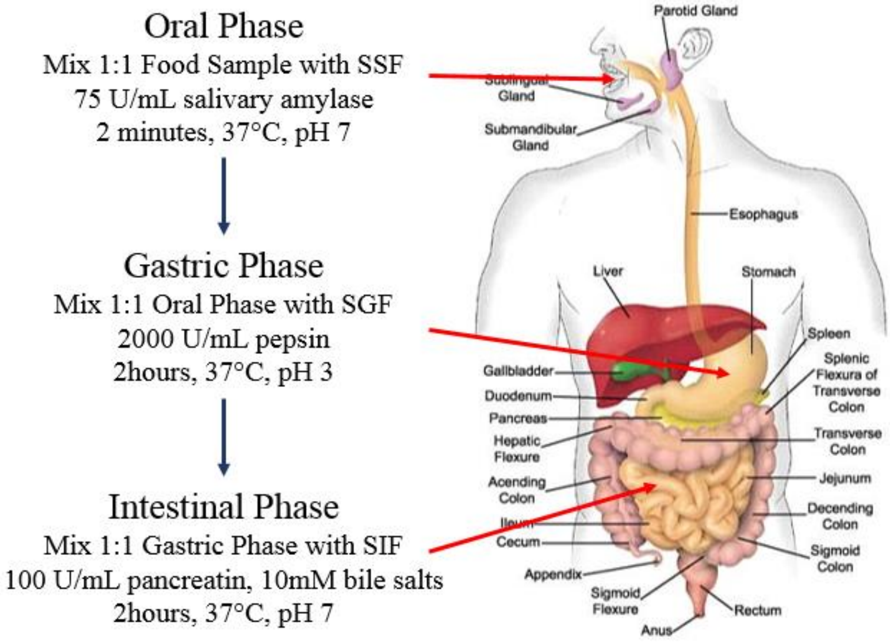
3.9 In-vitro Gastrointestinal Digestion

All the reagents and enzymes used where obtained from Sigma Aldrich, USA. Stock solutions of Simulated Salivary Fluid, Simulated Gastric Fluid and Simulated Intestinal Fluid were prepared according to the table below:



Constituents	Stock Concentration		SSF (pH 7)		SGF (pH 3)		SIF (pH 7)	
			Vol of Stock (500mL)	Final Salt Conc.	Vol of Stock (500mL)	Final Salt Conc.	Vol of Stock (500mL)	Final Salt Conc.
	g/L	mol/L	mL	mmol/L	mL	mmol/L	mL	mmol/L
KCl	37.3	0.5	15.1	15.09	6.9	6.9	6.8	6.8
KH_2PO_4	68	0.5	3.7	1.35	0.9	0.9	0.8	0.8
$NaHCO_3$	84	1	6.8	13.68	12.5	25	42.5	85
NaCl	117	2	-	-	11.8	47.2	9.6	38.4
$MgCl_2(H_2O)_6$	30.5	0.15	0.5	0.15	0.4	0.12	1.1	0.33
$NH_4(CO_3)_2$	48	0.5	0.06	0.06	0.5	0.5	-	-
For pH Adjustment								
NaOH		1	-	-	-	-	-	-
HCl		6	0.09	1.1	1.3	15.6	0.7	8.4
Added directly during digestion (Not added to simulated digestion fluids)								
$CaCl_2(H_2O)_2$	44.1	0.3		1.5		0.15		0.6

Table 2: Stock Solution Composition for Simulated Digestion Fluids (Minekus et al., 2014)



Oral Phase
Mix 1:1 Food Sample with SSF
75 U/mL salivary amylase
2 minutes, 37°C, pH 7

Gastric Phase
Mix 1:1 Oral Phase with SGF
2000 U/mL pepsin
2hours, 37°C, pH 3

Intestinal Phase
Mix 1:1 Gastric Phase with SIF
100 U/mL pancreatin, 10mM bile salts
2hours, 37°C, pH 7

Figure 5: Overview of simulated in vitro digestion system. Adapted from (Minekus et al., 2014)

The gastrointestinal digestion process comprises of 3 parts, namely the oral phase, gastric phase and the intestinal phase.

Oral Phase

Firstly, 1g of encapsulated L.Plantarum powder was added to a 250mL Erlenmeyer Flask and dissolved in 4 mL of milliQ water and mixed for 10 minutes to obtain 5g of slurry. Then, the slurry is added with 5mL of Simulated Salivary Fluid (SSF). The amylase concentration is ensured at 75 U/mL in the final mix. After which 25µL of 0.3M $CaCl_2$ is added to obtain a concentration of 0.75mM. Then, the pH is adjusted to pH 7. Once done, the mixture is placed in the shaker incubator to react for 2 minutes, shaking at 200rpm and 37°C.

Gastric phase

After that is done, 10mL of Simulated Gastric Fluid (SGF) is added at a ratio of 1: 1 with the oral phase mixture. Ensure that the pepsin concentration is 2000 U/mL in the final mix. (Added to the SGF beforehand) 5µL of 0.3M $CaCl_2$ is added to obtain a concentration of 0.075mM. Then, the pH is adjusted to pH 3 using 6M HCl. Once done, the mixture is placed back into the shaker incubator to react for 2 hours, shaking at 200rpm and 37°C.

Intestinal phase

Finally, 20mL of Simulated Intestinal Fluid is added at a ratio of 1: 1 with the gastric phase mixture. Ensure that the pancreatin concentration of 13.369 mg/mL and a bile salt concentration of 10mM in the final mix. (Added to SIF beforehand and stirred for 30 minutes at 37°C to fully dissolve) 40µL of 0.3M $CaCl_2$ is added to obtain a concentration of 0.3mM. Then, the pH is adjusted back to pH 7 using 1M NaOH. Once done, the mixture is placed back into the shaker incubator to react for 2 hours, shaking at 200rpm and 37°C. After the digestion is completed, the flasks were immersed into an ice bath to stop the digestion reaction. The surviving bacteria is then enumerated using the method described in 3.2.

3.10 Statistical Analysis

All experiments conducted were performed in triplicates and the values were reported as mean ± standard deviation. Significance was determined at $p \leq 0.05$ and analysed using ANOVA.

4. Results and Discussion

4.1 Characterization of Encapsulants

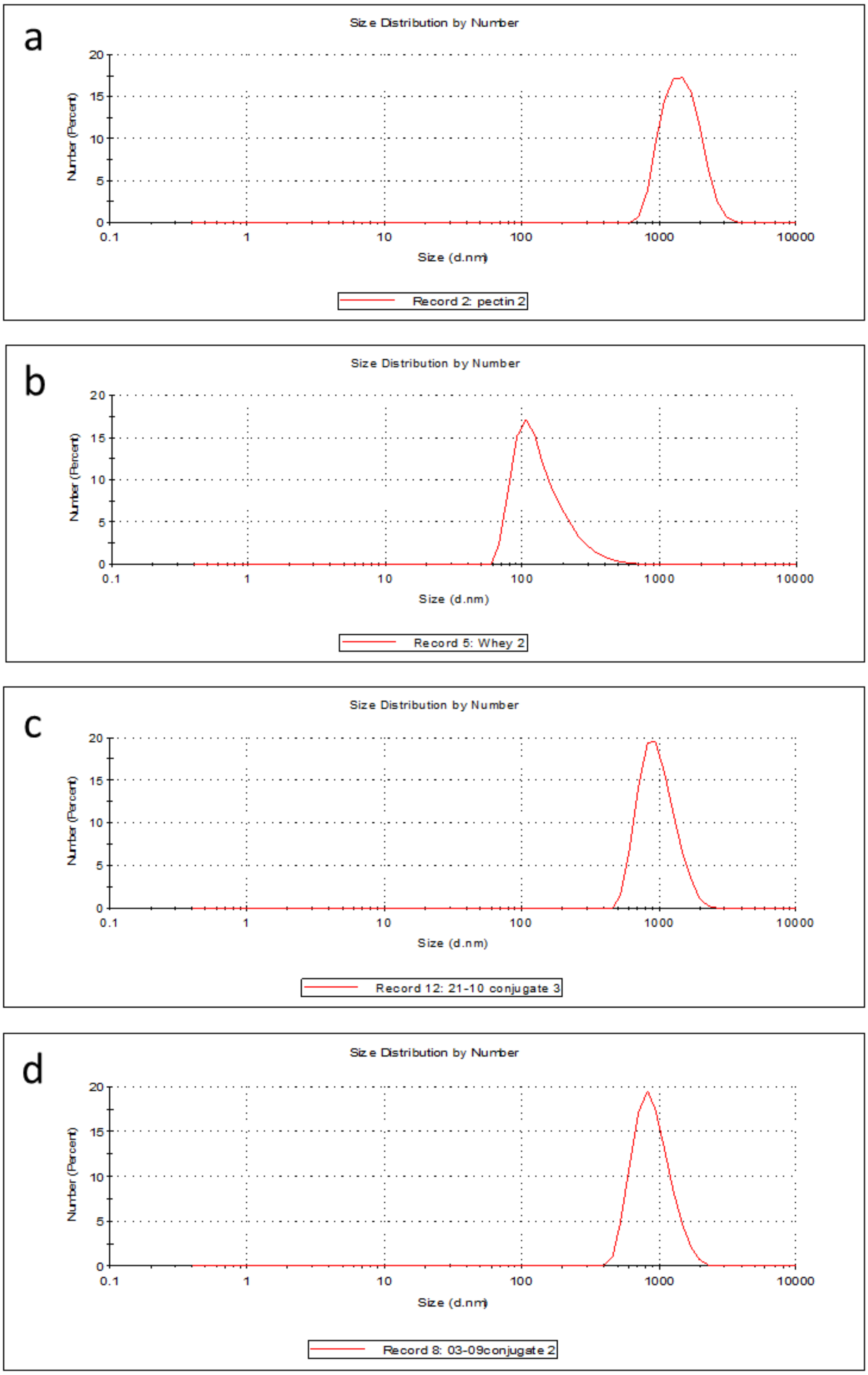


Figure 6: Particle size distribution a: pectin, b: whey protein, c: WP2, d: WP1

Comparing the size distributions in figure 6, it was found that the average size for whey protein is the smallest at about 100nm and pectin is the largest at about 2000nm. Upon conjugation, the average size is reduced to 850-950nm. The change in particle size is indicative of the conjugation that has occurred. The conjugation efficiency test of WP1 and WP2 found a 16.25% and 19.17% respectively. According to (Davidov-Pardo et al., 2015), this low conjugation efficiency could be due to the reduced accessibility to the conjugation sites caused by steric hindrance from the larger polysaccharides, in this case the pectin.

4.2 Effects of Spray Drying on viability of *L.Plantarum*

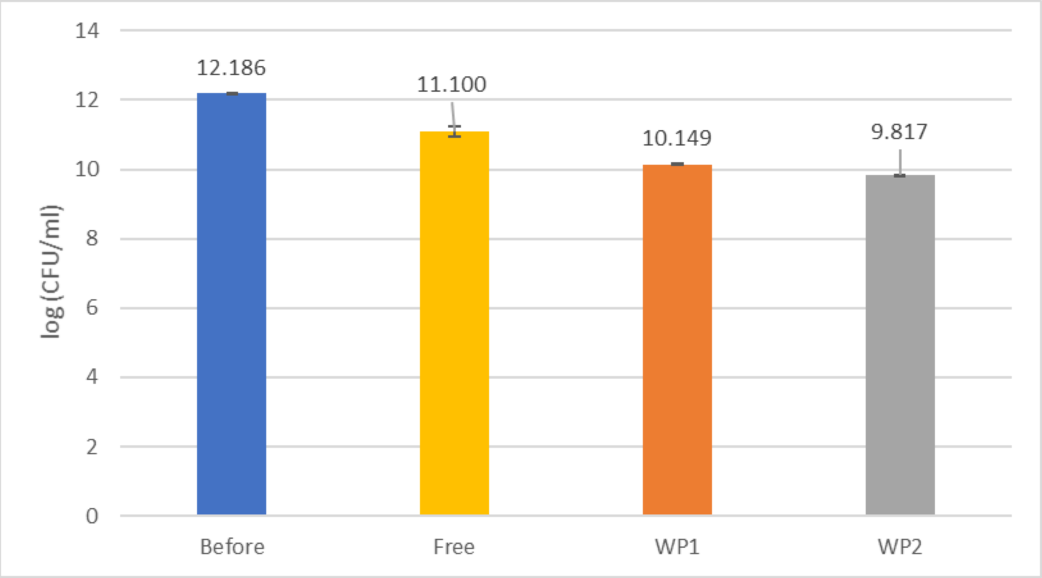


Figure 7: Viability of *L.Plantarum* before and after spray drying

	Total Viable Cell Count	Spray dried powder obtained/ 200ml solution
Before Encapsulation	1.533×10^{12}	
Free *L.Plantarum*	1.044×10^{10}	101mg
WP1	6.035×10^{10}	4.28g
WP2	2.055×10^{10}	3.13g

Table 3: Viable cell count of *L.Plantarum* before and after spray drying

After the *L.Plantarum* was spray dried into powder, the viability of the microencapsulated *L.Plantarum* was compared. According to Fig. 3 above, it might seem that the free *L.Plantarum* viability is higher than those encapsulated in the Maillard conjugates. This is because of the density of the *L.Plantarum* bacteria. Although the free *L.Plantarum* has a higher CFU/ml, the amount of spray dried powder produced is much lesser compared to the encapsulated powder. There was 101mg of free *L.Plantarum* powder per 200ml of solution, compared to 4.28g and 3.13g per 200ml for WP1 and WP2 respectively. Comparing the cell count of the *L.Plantarum* inoculated, it was found that there was a reduction of about 2 log units decrease in all the spray dried powders. This is partly due to the amount of sample lost from powder that was stuck onto the walls of the spray dryer. Although it is expected that both encapsulated powders had a higher viable cell count compared to the free *L.Plantarum* cells, it is interesting to note that the WP1 which has lesser pectin content had a viable cell count of which was 3 fold higher than that of WP2.

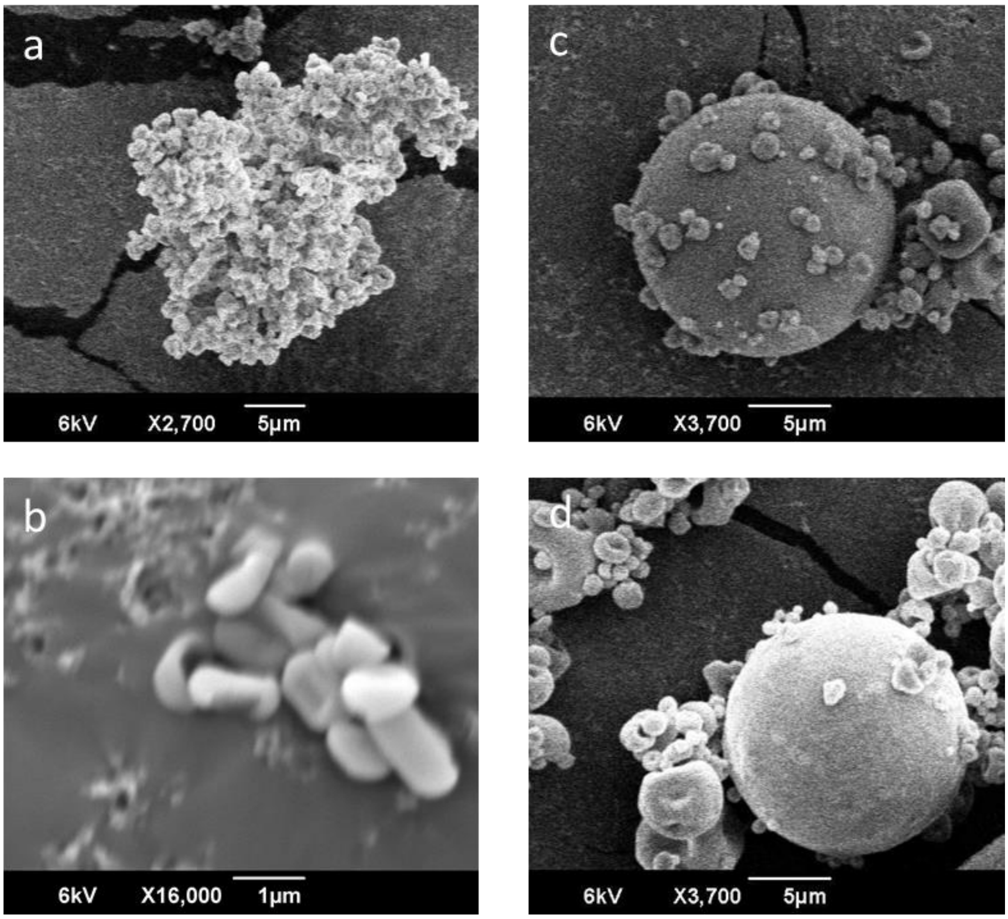


Figure 8: SEM image of encapsulated probiotics. a,b: Free *L.Plantarum*, c: WP1, d: WP2

Examining the SEM images of the particles (Figure 8), we can see that in a and b, the free *L.Plantarum* tend to aggregate together. (Janković, Frece, Abram, & Gobin, 2012) mentioned that aggregation by Lactobacilli is correlated to adhesion. This serves as one of the mechanisms used for colonization of the gut. It is also mentioned that there are two types of aggregation. Autoaggregation, which is the aggregation among the same strain and coaggregation, which is aggregation of different species. It is said that coaggregation is the mechanism that prevents colonization of harmful pathogens. (Kos et al., 2003) Images c and d shows that the encapsulated particles are generally between the size of 3-20 μm have relatively smooth surfaces and are spherical in shape. Comparing this with images from Figure 9, the surfaces of the particles which were kept in storage at room temperature for 8 weeks tend to appear rougher and have started deforming. This is indicative of the disintegration of the particles which results in the loss of viability. It is interesting to note however, that many of the particles have smaller particles within them, suggesting that the encapsulation is a matrix type encapsulation rather than a reservoir type one.

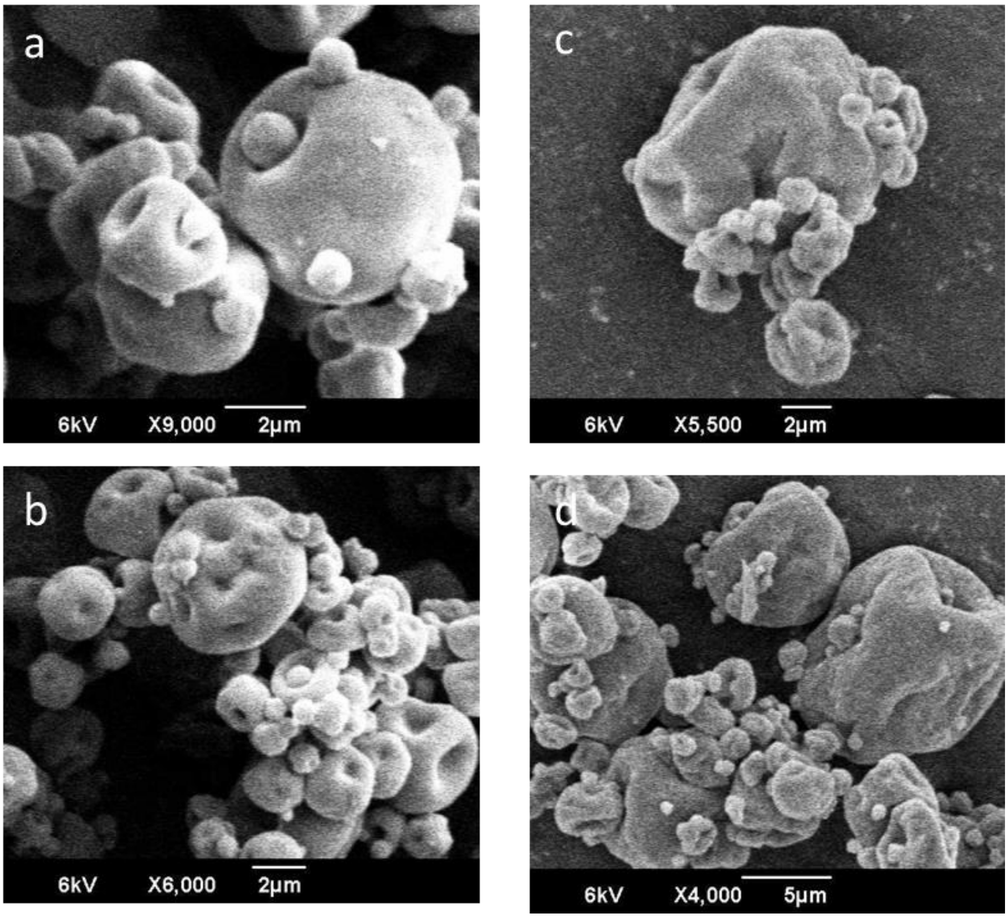


Figure 9: SEM image of encapsulated probiotics at Week 8 of storage. a,b: WP1, c,d: WP2

4.3 Viability of Encapsulated *L.Plantarum* during storage

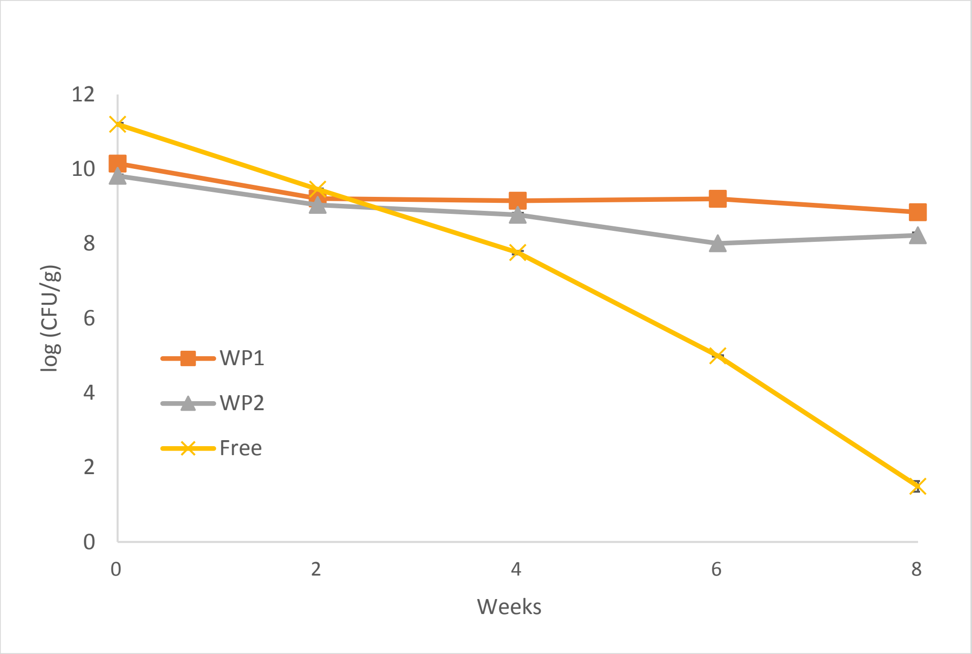


Figure 10: Viability of *L.Plantarum* in storage (Room Temperature)

L.Plantarum encapsulated in WP1 and WP2 were found to have a relatively stable viability over the 8 weeks with an overall log reduction of 1.3 and 1.6 respectively. In comparison, free *L.Plantarum* showed a 9.7 log reduction over the course of 8 weeks. Something interesting to note is that for WP1 and WP2, there was a slight increase in viability during the course of the storage. This is consistent with a finding presented by (Mao et al., 2018) which suggested that this could be due to the prebiotic effects from the encapsulating materials. However, WP2 did not exhibit any significant increase compared to WP1 even though it has a higher amount of pectin, which is a prebiotic. Hence, as the effects are not consistent, more research must be done to identify the exact mechanism behind this occurrence.

4.4 Protective effects of encapsulation on *L.Plantarum* during pasteurization

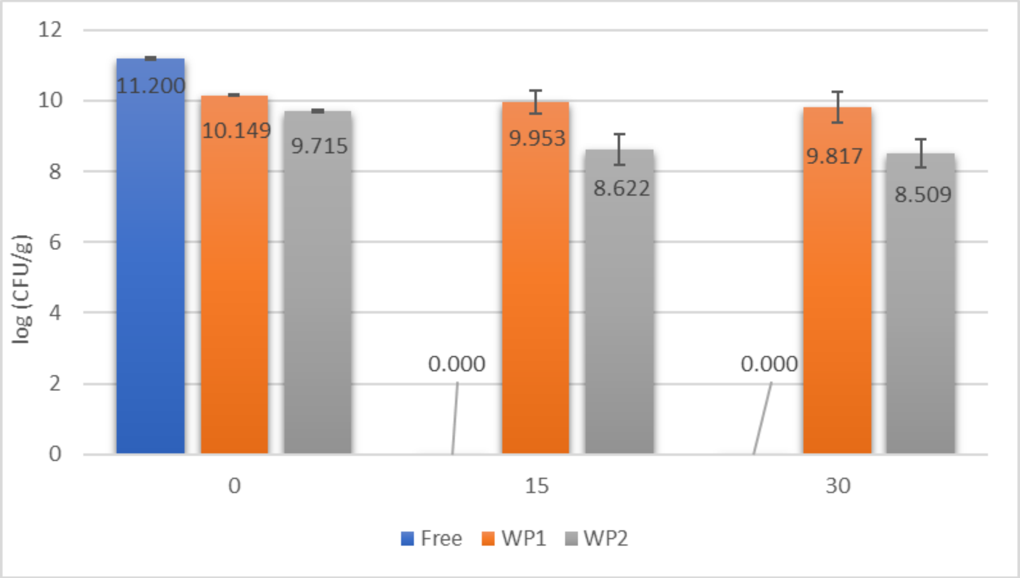


Figure 11: Viability of *L.Plantarum* after 15 and 30 min of pasteurization at 85°C

As seen from figure 11, WP1 and WP2 were also found to have significant protective effect during pasteurization. There was only a 0.3 log reduction for WP1 and 1.2 log reduction for WP2 where viability of the free *L.Plantarum* became indeterminant after 15 minutes. The reason the decrease in viability in WP2 is more significant could be a result of a higher pectin content. According to (Einhorn-Stoll, Kastner, Urbisch, Kroh, & Drusch, 2019; Fraeye et al., 2007), the thermal degradation becomes more significant with increasing temperatures above 60°C. Hence, with a higher pectin content, WP2 is more susceptible to pectin thermal degradation and a lower viability of probiotic cells.

4.5 Viability of Encapsulated *L.Plantarum* after in vitro digestion

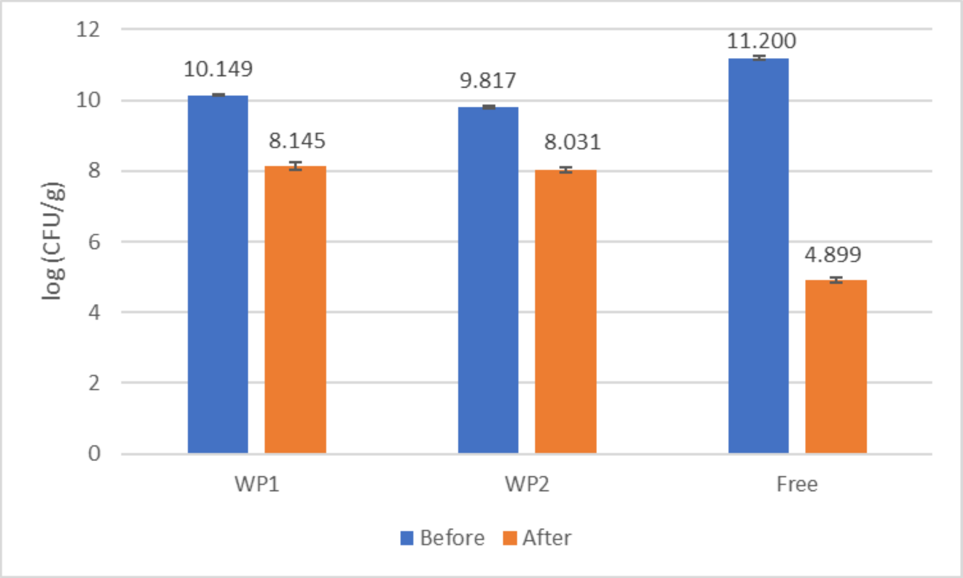


Figure 12: Viability of *L.Plantarum* before and after in vitro digestion

Figure 12 shows the comparison of viability of *L.Plantarum* before and after in vitro digestion. WP1, WP2 and free *L.Plantarum* cells showed a 2, 1.8 and 6.3 log reduction respectively. This different between WP1 and WP2 could be attributed to the amount of pectin present in the encapsulating material as well. As pectin is known to be a dietary fiber and prebiotic, it is not well degraded by the enzymes present in the gastrointestinal tract. (Bang et al., 2018) Therefore, with a higher pectin content, the WP2 particles tend to be more resilient and offer better protection for the *L.Plantarum* bacteria.

5. Conclusion

In summary, from this study, it is shown that whey protein-pectin conjugate proves to be an ideal wall material for encapsulation of probiotics for increased heat stability in storage and surviving the harsh conditions of the gastrointestinal tract. However, more research has to be done in several areas. Optimizing the parameters for spray drying to increase the yield of viable probiotics is necessary to overcome the high percentage loss of viable cells. Other methods of encapsulation could also be explored for an increased yield. The composition of protein to carbohydrate ratio could also be tweaked to improve functionality of the particles. Addition coating such as chitosan could be added for improved functionality. In this study, materials used were commercial material which contributes to the cost. In order to reduce the cost of production further, materials from food side streams such as okara protein isolate or durian seed pectin could be considered to be used as encapsulating material.

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