Microencapsulation of *Lactobacillus reuteri* DPC16 using spray-drying

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Abstract

Probiotic microorganisms and the products containing the beneficial microorganisms are popular due to their ability to confer health benefits on consumer health. The majority of probiotics are delivered in liquid media which limit their shelf life and they are not convenient for the modern lifestyles. Thus, in this study, different wall materials for the microencapsulation of *Lactobacillus (L.) reuteri* DPC16 were investigated in Stage 1. The shelf-life tests of selected spray-dried powders were carried out in Stage 2 with different packaging materials.

In Stage 1, *L. reuteri* DPC16 was encapsulated in 10% reconstituted skim milk (RSM), 10% gum Arabic, 10% maltodextrin, and 4:1 mixed wall material (2.5% whey protein isolate/ 2.5% gum Arabic/ 2.5% inulin/ 2.5% sucrose), (w/w) then spray-dried at 160 ℃/80 ℃ inlet/outlet temperatures. The spray-dried DPC16 microcapsules were characterised for viable cells of the probiotic, water activity and morphology. Viable cell counts were measured using standard plate count method, water activity using a water activity meter (AquaLab, Series 3, New Zealand) and the morphology of the powder particles was scanned by the electron microscope (FEI Electron Optics, Quanta 200, The Netherlands). Results of Stage 1 showed that at the inlet/outlet temperatures of 160 ℃/80 ℃, the RSM as an encapsulation wall material had the highest cell counts (98.06%±0.86%) with 0.284±0.005, water activity followed by the mixed wall material which contained cells of 93.97%±1.49% log CFU/g with water activity of 0.196±0.010. The powder made from gum Arabic had the lowest viable cells (90.63%±3.08%) with 0.170±0.005, water activity. Thus, RSM showed good potential to maintain high cell viability during spray-drying although the water activity was higher than the expected range of <0.25. For all the treatments, particle sizes of the powders were well below 100 μm which is ideal for addition to food products as they do not affect mouthfeel. Most of the powder particles were spherical with variable sizes and dented surfaces. Thus, RSM and the mixed wall materials were selected for encapsulating DPC16 in the storage trials.

In stage 2, DPC16 were encapsulated using selected wall materials (RSM and the mixed wall material) and vacuum-packed in PET/EVOH/PE co-ex topweb FOC films (Multivac New Zealand Ltd) and aluminium foil bags (ALFW5-18, PBAG, China), then stored at 25 ℃ and 55 ℃ for four weeks. During storage, viable cells of the DPC16, water activity, colour, moisture content, and morphology of the powder were determined. Colour was measured by the Minolta Colourimeter (Minolta, Japan), moisture content was determined by the oven-dry method, bulk density was determined by the measuring cylinder method and the other characteristics of the powders were determined as previously described. The survival of DPC16 cells encapsulated in skim milk and vacuum-packed in aluminium bags were higher and more stable during storage at 25 ℃. Water activity, moisture content, bulk density, colour and morphology of the powder were all relatively more stable than in other treatments. Water activity (mean) and moisture content (mean) were within the expected ranges for the product. When stored at 55 ℃, the viable cell counts of DPC16 encapsulated in RSM and vacuum-packed powder in PET/EVOJ/PE co-ex topweb FOC film decreased to $<10^6$ CFU/g by end storage which was below the FAO/WHO, 2003 recommended level. The moisture content (0.0246±0.0003) was also below recommended levels (0.028 – 0.056), although water activity (0.102±0.007) was within expected levels (<0.25). Low moisture levels are critically important for the survival of encapsulated spray-dried probiotic microorganisms. High moisture initiates chemical reactions within the carrier materials leading to cell death and also affects colour stability. However, storage temperature is also important to cell survival.

In conclusion, the present study showed that spray-drying encapsulated *L. reuteri* DPC16 in 10% RSM at 160°C/80°C, followed by vacuum-packaging in aluminium bags showed potential to maintain cell viability during storage (25 °C) for four weeks. It is desirable to check the performance of the encapsulated DPC16 powders in the simulated gastrointestinal tract and its ability to target-release the cells in the colon.

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Abbreviation

a_w	water activity
AL	aluminium
ANOVA	Analysis of variance
CFU	colony forming units
DE	Dextrose equivalent
EE	encapsulation efficiency
EVOJ	a new material contain Ethylene-vinyl
FAO/WHO	Food and Agriculture Organisation of the United Nations and World Health Organisation of the United Nations
FOC	free of charge
FOS	fructooligosaccharide
g	gram
GA	gum Arabic
GIT	gastrointestinal tract
GLM	general linear model
h	hour
HPMC	hydroxyl methylcellulose
L.	*Lactobacillus*
LAB	lactic acid bacteria
LDPE	low-density polyethylene
MD	maltodextrin
min	minute
mL	millilitre
MRS	De Man, Rogosa, Sharpe
MWM	the mixed wall material
OD	optical density
PE	Polyethylene
PET	Polyethylene terephthalate
rpm	revolutions per minute
RSM	reconstituted skim milk
sec	second
SEM	standard error of the mean
SCFA	short chain fatty acid
VCC	viable cell counts
w/w	weight/ weight
× g	gravity

Chapter 1 Introduction

The concept of functional food has now moved to gastrointestinal health and its relationship with the gut bacteria. This new concept might be caused by the pervasiveness of gastrointestinal diseases as diet is an important factor that affects the activities of indigenous microbiota (Anandharamakrishnan & Ishwarya, 2015). This demand for food supplementary functional health products led to the concept of probiotics.

Probiotics originated from the Greek word “pro bios”, whose meaning is “for life” (Anandharamakrishnan & Ishwarya, 2015) and are a dietary supplement. A bacterium may be described as a probiotic only when it can be proved to be alive when it is consumed in adequate amount to confer health benefits (FAO/WHO, 2002; Sahin et al., 2007). In order to confer health benefits to the host, probiotics must also reproduce in the gastrointestinal tract (GIT), be non-toxic, have antagonism effect with pathogens and be genetically stable (Havenaar & Marteau, 1994; Lee & Salminen, 1995).

Prebiotics are non-digestible food products which can specifically stimulate the growth and the activity of beneficial bacteria in the gut but restrict the proliferation of the harmful ones (Sekhon & Jairath, 2010). It is therefore recommended to use combinations of probiotics and prebiotics, which are known as synbiotics, in both products and GIT (Fooks & Gibson, 2002).

For a probiotic functional food, the product needs to contain more than 10^6 living cells per gram or milliliter at the time of consumption (FAO/WHO, 2003). However, during processing and storage, products may be exposed to moisture, oxygen, shear, light, and heat that contribute to an increased death of the cells. All these challenges suggest that the use and application of probiotics in food are still very limited (Kailasapathy, 2002). Therefore, there is need to develop innovative methods to protect probiotic microorganisms during processing, storage and handling. Several strategies have been proposed to enhance the viabilities of probiotics such as strain selection, strain adaption in the GIT or food matrix, packaging system and addition of probiotic-promoting (prebiotics) compounds (Terpou et al., 2019). Several technologies can be used to achieve the protection of probiotic cells during processing and storage including plain freeze-drying and microencapsulation. The latter (microencapsulation) has been proved successful on the superior stability of cells even in the GIT using different materials and methods, and has become the main modern solution to preserve probiotic viability (Călinoiu et

al., 2019). Microencapsulation is a technology which can wrap solids, liquids as well as gaseous components thereby protecting the contents (Champagne & Fustier, 2007). In this process, individual particles of active components are wrapped in a shell by coating with a continuous outer layer to protect the inner components.

Several processes have been used for microencapsulation, such as spray-drying, freeze-drying, extrusion, emulsion and fluid-bed drying (Burgain et al., 2011; Champagne & Fustier, 2007; Thantsha et al., 2009). Spray-drying is recommended for application in industry because it is relatively economical, easy to scale up (Prüsse et al., 2008), does not need the use of poisonous solvent, and the powder produced does not need refrigeration. However, spray-drying tends to cause damage to cells due to the high temperatures during the drying process. Thus, a proper biopolymer must be used to protect the cells. Natural gums (gum Arabic, alginates, carrageenan, etc.), proteins (milk or whey protein, gelatin) and carbohydrates (maltodextrins with different dextrose equivalent) (Gharsallaoui et al., 2007) have all been used for microencapsulation by spray-drying. This present study investigated the survival of *L. reuteri* DPC16 encapsulated by spray-drying during processing and storage at various temperatures.

Aim

The aim of the study was to select suitable encapsulation wall materials for the protection of *L. reuteri* DPC16 using spray-drying.

The specific objectives were:

a) To select suitable wall materials for the encapsulation of probiotic *L. reuteri* DPC16 using spray-drying;

b) To select suitable inlet-outlet temperatures for spray-drying of encapsulated DPC16;

c) To determine the appropriate materials for packaging of spray-dried DPC16 microcapsules;

d) To evaluate the stability of the most promising treatment during storage (25 ℃ and 55 ℃) by measuring physical characteristics (water activity, colour, bulk density, particle size and morphology of powders) and, analyzing viable cells and water content.

Chapter 2 Literature review

2.1 Probiotics

2.1.1 Definition and common physiology of probiotics

Probiotics are defined as live microorganisms that confer health benefits to the host by their activities in the human gut (Guarner & Schaafsma, 1998; Perdigón & Alvarez, 1992), or more accurately *"live microorganisms, which when administered in adequate amounts confer a health benefit on the host"* (FAO/WHO, 2002).

There are numerous microorganisms in the human gut, but only a few have probiotic features (Gibson & Roberfroid, 1995). According to Conway (1996), the generally agreed selection criteria for obtaining functional probiotic strains are the origin from the human host, capability of surviving the harsh condition in the GIT and reach required dosage at targets, ability to colonize and actively defeat the pathogens, stability during production and distribution for commercial use, safety.

Probiotics can be classified into three types, i.e. lactic acid bacteria (LAB), non-LAB and yeasts. LAB include *Lactobacillus*, bifidobacteria and *Lactococcus lactis* (Mutukumira et al., 2015). Thus, probiotics can be either prokaryotic or eukaryotic microorganisms.

Prokaryotic probiotics are differentiated according to their morphology, spore-forming ability, method of energy, nutritional requirements, as well as their reaction to the Gram-stain. Well-known probiotic prokaryotes belong to the genera *Bifidobacterium* as well as *Lactobacillus* which include *Streptoococcus thermophiles* and *Enterococcus faecium*. The two genera are preferred because they have beneficial effects on human health (Bielecka et al., 2002). About 56 of the 106 species are *Lactobacillus* that have probiotic potential, while about 8 of 30 species are in *Bifidobacterium* (Otieno, 2011).

Eukaryotic probiotics include algae (e.g., *Chlorella*, *Spirulina* species), fungi (e.g., *Aspergillus*, *Penicillum* species) and yeasts (e.g., *Saccharomyces*, *Candida*, *Kluyveromyces*, *Pichia*, *Torulopsis* species). They have been consumed by human and animals for centuries and are

used as single cell protein or parts of food starters (Nayak, 2011).

In order to confer function of properties in the gut, probiotic bacteria need to survive the harsh conditions in stomach. Further, they need to outcompete pathogens in the colon. Orally ingested bacteria are always challenged in the stomach with acids and bile salts/enzymes in the initial part of the intestine, thus protection of probiotic bacteria cells is necessary (Boylston et al., 2004). Therefore, cell numbers have to be high before consumption and more than 10^6 live cells per gram or per milliliter of products are suggested to exert beneficial functions (FAO/WHO, 2003).

2.1.2 Functions of probiotics on human gut health

There has been a greater recognition of beneficial effects of probiotics on the human gut health and, the maintenance and promotion effects are significant since more than 70% of human immune system is located in the gut (Fung et al., 2011). One major function of probiotics in the gut is the alleviation (i.e. the prevention and treatment) of diarrhea including acute diarrhea, antibiotic-associated diarrhea, as well as radiation-induced diarrhea by increasing the propagation of inherent microorganisms (Lye et al., 2009).

Some LAB, such as *Lactobacillus, Bifidobacterium*, and *Streptococcus* can utilize lactose. They can metabolise lactose into galactose and glucose, thus they can alleviate the symptoms of lactose intolerance. Hence, they are widely used in dairy products to aid the digestion of lactose (Rolfe, 2000). *Lactobacillus* and *Bifidobacterium* are the two most preferred genera because they have beneficial effects on human health such as preventing the growth of harmful bacteria, stimulating immune functions, anti-tumour functions, reducing cholesterol, helping digest, absorbing minerals and synthesizing vitamins (Bielecka et al., 2002; Gibson, 1998). Stress, antibiotics, diseases and poor diet are reported to cause the depletion of the probiotics in the gut which reduce the proliferation of pathogens (Madison & Kiecolt-Glaser, 2019). The growth of biomass in the gut is greatly affected by the type of substrate available to microorganisms and colonic residence time, which are dependent on food (Cummings & Macfarlane, 1997). Therefore, in order to maintain the balance in the gut, probiotics contained in dietary supplements and functional food have been introduced.

Probiotics can also prevent colon cancer (Ewaschuk et al., 2006), relieve short bowel syndrome,

inflammatory bowel diseases, irritable bowel syndrome, visceral hypersensitivity and altered gut motility. It can also alleviate the overproduction of hydrogen caused by food malfermentation (Fung et al., 2011).

According to Mandal and Mandal (2011), probiotics have immunomodulatory effects, hypocholesteromic effects, and antihypertensive properties. They can also alleviate postmenopausal symptoms, protect against lung emphysema and have antiallergic effects. In addition it has been reported that except for their effect on the intestines, they can also bring benefits to skin due to their specific properties such as the production of acids, antimicrobial substances, and beta-defensins, which can reduce or inhibit the growth of harmful skin bacteria such as acne episodes (Al-Ghazzewi & Tester, 2010; Krutmann, 2009; Lambers et al., 2006; Mauro, 2006; Oh et al., 2006; Spigelman & Ross, 2008; Sullivan et al., 2009). It can thus be predicted that a more promising market of probiotic products are coming forth.

2.1.3 Antimicrobial activities of probiotics

To keep the viability and integrity of probiotic cells in products, knowledge on the mechanism is desirable. There are mainly two mechanisms for a probiotic to take effect. One is through the stimulation of immune systems, while the other one is via the competitive inhibition for the adhesion sites on the surfaces of intestinal epithelium (Fung et al., 2011) Probiotics can form a protective biofilm via adhering to human intestinal cells, thereby preventing the enterocytes from the invasion of diarrhea-causing microorganisms. They can also produce some inhibitors to pathogens. For example, the antimicrobial reuterin produced by *L. reuteri* can restrain the urease-yielding bacteria, thereby inhibiting rotavirus infection (Shornikova et al., 1997).

2.1.4 Current markets of probiotics

From a global perspective, the current market of probiotics is bright. Probiotics have been incorporated into a variety of food products, such as yogurt, cheese, ice cream, cereals, juice, and sausages or as supplements (Ziemer & Gibson, 1998). The prevalence of functional foods and beverages has also stimulated the growth of probiotic fortified foods. In addition, obesity, which is a growing epidemic, can be controlled using probiotics (Rouxinol-Dias et al., 2016).

All these provide a foundation for the growing interest in probiotics.

Probiotics have been increasingly significant in paediatric healthcare. Some paediatric gastroenteric diseases such as acute infectious diarrhoea, nosocomial diarrhoea, infantile colic can be prevented by some specific strains or mixture of strains. Probiotics can affect pathogens by competitively adhering to the epithelium and mucosa, strengthening the barrier of epithelium while improving the immune system by adding normal intestinal microbes, producing intestinal mucin or some bacteriocins, or through other mechanism (Cruchet et al., 2015; Indriyani et al., 2012).

In the last decades, few studies on probiotics has focused on the development of health-enhancing strains which can be applied to the dairy industry (Crittenden et al., 2005). The main bacteria of interest are *Lactobacillus* and *Bifidobacterium*.

2.2 *Lactobacillus reuteri*

2.2.1 Source of *L. reuteri*

Lactobacillus reuteri was isolated from human faecal and bowel samples by a German microbiologist Gerhard Reuter who subsequently separated *L. reuteri* from *L. fermentum (Reuter, 2001)*. *L. reuteri* was eventually classified as a distinct species in 1980 according to its genetic and phenotypical features (Kandler et al., 1980).

Lactobacillus reuteri is one of the few indigenous lactobacilli in host intestine (Reuter, 2001) and can be isolated from the intestine or faeces of almost all hosts including humans, monkeys, and other domestic animals (Casas & Dobrogosz, 2000; Mitsuoka, 1992). It is the main constituent of *Lactobacillus* species and is able to build symbiotic relationships with all hosts (Casas & Dobrogosz, 2000). Hence, all species of *L. reuteri* are presumed to have probiotic efficacy.

2.2.2 Morphology of *L. reuteri*

According to Kandler and Weiss (1986), *L. reuteri* is Gram-positive bacteria and can produce lactic acid. The bacteria range 0.7-1 μm in length, 2.0-3.0 μm in width and have an irregular shape, curved rods with rounded ends. The bacterium can exist in different forms, either in a single cell, in pairs or in small clusters.

2.2.3 Metabolism of *L. reuteri*

Lactobacillus. reuteri can ferment glucose alone, and also metabolise glycerol to produce reuterin. The antimicrobial substance reuterin is useful to the pharmaceutical industry. *L. reuteri* is an obligate heterofermentative species and utilizes carbohydrates via the phosphoketolase-based metabolic pathway (Axelsson & Ahrné, 2000; Casas & Dobrogosz, 2000). Glucose alone can be fermented by *L. reuteri*, produce lactate, ethanol, and carbon dioxide as end products (El-Ziney et al., 1998; Lüthi-Peng et al., 2002; Talarico et al., 1988). The most significant feature of *L. reuteri* is its ability to utilize glycerol and thus produce reuterin, i.e. 3-hydroxypropionaldehyde (3-HPA), as one of its end-products (Talarico et al., 1988). However, it was postulated that *L. reuteri* cannot metabolize glycerol alone without carbohydrates (Talarico et al., 1990). Therefore, glycerol can only be utilized after the fermentable carbohydrates are metabolised.

2.2.4 Antimicrobial activity of *L. reuteri*

Lactobacillus reuteri has been proved to have strong probiotic efficacies, especially its antibiotic activities. Its specific binding mechanisms and its specialized surface proteins allow for its strong adherence to the gastrointestinal epithelia of the host (Roos et al., 1999; Wadstroum et al., 1987). This strong adhesion ensures the competitiveness of *L. reuteri* when attaching to sites on the GIT against other microorganisms but also delivers effects of its metabolites on the enterocytes and the immunocytes related to the gut. Thus, *L. reuteri* can positively modulate the host's mucosal defenses.

According to Talarico et al. (1988), the metabolism of glycerol by *L. reuteri* produces lactate, acetate and a series of short chain fatty acids (SCFA) along its heterofermentative pathway. Among these, reuterin, reutericin and reutericyclin are *reuteri*-specific substances, although the last two substances could only be found in some particular strains (Höltzel et al., 2000; Kabuki et al., 1997). Reuterin has strong antimicrobial activity. About 15-30 μg/mL of reuterin can effectively suppress the growth of bacteria, fungi, yeasts, and protozoa. If the concentration is higher, LAB even including *L. reuteri* itself can be killed (Axelsson et al., 1989; Casas & Dobrogosz, 2000; Chung et al., 1989). Thus, the application of this species is broad and promising.

2.3 Previous studies on *L. reuteri* DPC16

An *in-vitro* study was carried on which confirmed the validation of the probiotic concept of this strain, where the antimicrobial activity of its supernatant as well as the culture safety against gastric mucus and normal microflora in GIT, was investigated (Bian, 2008). To deliver this probiotic strain to a target site of the colon and help it resist the harsh conditions in the GIT without any deterioration in physiological characteristics, a novel delivery system was developed by encapsulating the cells into calcium alginate beads (Zhao, 2012). Before that, the cell integrity of *L. reuteri* DPC16 during microencapsulation using emulsion method (Chen, 2007) and freeze-drying method (Chang, 2006; Joshi, 2005; Yin, 2006) was studied to determine its best processing and storage conditions. However, this strain has not been encapsulated by spray-drying and the shelf life of it in a powder form was unknown.

2.4 Microencapsulation

Microencapsulation is the technology to pack solids, liquids or gases in small and sealed capsules. The materials inside the capsule should be able to be released at controlled speed under a certain condition (Anal & Stevens, 2005). Encapsulation has wide applications in the food industry, such as stabilizing materials, controlling release of materials, masking flavors, odors, or colors, and extending the shelf life (Anal & Singh, 2007).

2.4.1 Commonly used microencapsulation materials for the protection of probiotics

In the past the selection of wall materials for the encapsulation of bioactive compounds was largely based on experience (Anandharamakrishnan & Ishwarya, 2015). In the wet encapsulation such as the emulsion method, the emulsification and gelation properties are key criteria when selecting wall material, whereas in the dry method such as spray-drying, emulsification property, film-forming ability, viscosity, glass transition temperature, and degree of crystallinity need to be considered (Anandharamakrishnan & Ishwarya, 2015). However, for the encapsulation of probiotics, other properties such as acid-resistant and controlled-release are also vital and must be considered. Therefore, the wall materials for encapsulation of bacteria cells using the wet method should be considered first as candidates when using the dry method. This section proposed some general wall materials for the encapsulation of probiotic bacteria.

2.4.1.1 Carbohydrates

Calcium alginate beads are commonly used form for the entrapment of LAB (Rowley et al., 1999). Calcium alginate beads can be made by extruding cell-alginate solution into calcium chloride solution (Nigam et al., 1988). However, these capsules are sensitive to acid and are easily decomposed in the stomach (Mortazavian et al., 2007). In contrast, a combination of xanthan gum and gellan gum has been used for the encapsulation of probiotics and it showed a strong ability to resist the acidic conditions (Sultana et al., 2000).

Chitosan is positively charged and has inhibitory effects on various LAB. Therefore, chitosan is normally used for coating alginate beads (Groboillot et al., 1993) to deliver non-LAB cells.

Another widely used carbohydrate for probiotic encapsulation is starch, despite the swelling of amylose in aqueous conditions. Resistant starch can resist digestion in the small intestine but it is digestible in the colon, thus it is suitable for the delivery of probiotic cells (Basit, 2005). Besides starch, the mixture of amylose and ethyl cellulose can achieve the targeted release of the capsules to the colon rather than the stomach or small intestine in vitro (McConnell et al., 2007; Tuleu et al., 2002) and *in vivo* (Basit et al., 2004).

2.4.1.2 Proteins

Gelatin is a protein that can make a thermo-reversible gel for the encapsulation of probiotics. It collaborates very well with anionic polysaccharides such as gellan gum and alginate. When the pH of their environment is below 6 which is the isoelectric point of gelatin, the two polymers have strong interaction with each other, however, at pH above 6, they repel each other (Anal & Singh, 2007; Krasaekoopt et al., 2003). However, gelatin is easily hydrolyzed by intestinal proteolytic enzymes. Thus, when probiotics are encapsulated in the mixture of gelatin and alginate, a continuous release of probiotics has been reported (Cannan & Muntwyler, 1930; Li et al., 2008). This negatively affected the encapsulation effect.

Milk proteins are also widely used for probiotic encapsulation. Milk proteins such as caseins have good gelation properties which are effective for encapsulating probiotic bacteria, and the outstanding buffering capacity of milk proteins also provides good protection for the microorganisms against the harsh conditions in the GIT (Heidebach et al., 2009a, 2009b). Whey protein is the liquid phase of milk after the milk precipitates at pH 4.6, and it has been used in combination with gum Arabic to form beads to deliver probiotic cells to the area near the small intestine (Lambert et al., 2008). Whey protein can well protect freeze-dried *L. rhamnosus* R011 cells against acidic, alkaline, heating and freezing conditions (Reid et al., 2007). It can also protect many other strains (Akalin et al., 2007).

Soy protein has also been used in microencapsulation for the delivery of drugs (Wongkanya et al., 2017; Zheng et al., 2007) and probiotics (Liu et al., 2018) because of its biodegradability, high availability, non- cytotoxicity in addition to high thermal stability. Soy proteins can hold water, bind oil, and possess emulsifying attributes. Thus, soy proteins are considered as a substance to fortify alginate when delivering drugs because alginate has very poor emulsifying ability (Chan, 2011). Soy proteins cannot be applied to microencapsulation alone because it has a lot of constraints such as their limited solubility in water and high viscosity when dispersed at high solid content (Volić et al., 2018).

2.4.1.3 Other polymers

Other polymers such as hydroxyl methylcellulose (HPMC), Eudragit S and skim milk for the immobilization of bacteria cells have also been used as materials for probiotic microencapsulation. HPMC is an emulsification and thickening agent which can be directly added to food and can form a matrix with phytowax to immobilize bacteria cells (Sahoo et al., 2008).

Eudragit S is the short form for the polymethacrylic methyl methacrylate ester co-polymer, a polymer dissolves above pH 7. It can therefore achieve the goal of targeted delivery of probiotics to the lower small intestine (Tyagi et al., 1998).

Skim milk is commonly used as a protective agent during freeze-drying (Khoramnia et al., 2011). It can enhance the strength of alginate due to the contribution of calcium in milk (Ross et al., 2008) and lactose which can provide nutrient for the growth of bacteria (Ross et al., 2008).

2.4.2 Commonly used microencapsulation methods

Various materials and methods can be used for the encapsulation of probiotic bacteria. The methods involve hard capsules, film coating, hot-melt coating, matrix cell entrapment and compression coating (Huckle & Zhang, 2011). Most techniques for encapsulation are mentioned in this section including emulsion, extrusion, freeze-drying, fluidized bed drying and spray-drying (Alhnan & Basit, 2011; Anal & Singh, 2007).

2.4.2.1 Emulsion

The emulsion technique utilizes hydrocolloids to encapsulate active probiotic cells. In emulsion technology, a water-in-oil emulsion can be formed by adding a cell-polymer suspension into a vegetable oil (Burgain et al., 2011). Then a cross-linking agent is added to the solution to form solidified beads while stirring. Thereafter, a protective coating layer of the beads can be formed by introducing the beads into another polymer solution (Burgain et al., 2011). The emulsion

technique is relatively simple to scale up and the viability of the cells is high. However, the size of the beads formed have a diameter of 200 to 1000 μm, which are too large to create a smooth mouthfeel as a supplement in food products (Burgain et al., 2011; Capela et al., 2007).

2.4.2.2 Extrusion

Extrusion is a relatively easy and economical encapsulation technique whereby a cellular solution is projected into a hardening solution such as calcium chloride by a nozzle at high pressure (Burgain et al., 2011). The size of the capsules is determined by the diameter of the nozzle (Vos et al., 2010). The biggest advantage of this technique is that anaerobic microorganisms can be better protected because the extrusion device can operate under conditions where oxygen is replaced by nitrogen (Vos et al., 2010).

2.4.2.3 Freeze-drying

Freeze-drying has been widely used in the food industry (Beer et al., 2009) as it guarantees a stable shelf life, reduces the cost of transportation, and globally facilitates trade. It is commonly used to preserve bacteria cells (Tsen et al., 2007). Freeze-drying consists of several steps including loading, freezing, primary drying, secondary drying, and unloading. In the primary drying stage, the frozen water is removed by sublimation and in the secondary drying stage, any unfrozen water can be removed by desorption (Tsen et al., 2007). However, freeze-drying without the encapsulation technique has its drawbacks. Firstly, the equipment used for freeze-drying is costly; secondly, the freeze-drying process takes a long time; thirdly, the low temperature (-50 ℃ to -80 ℃) for freeze-drying or dehydration may lead to loss of cell survival and materials have a short shelf life (Bolla et al., 2011). Another factor is poor buffering capacity (Laulund, 1994) and is sensitive to humidity (Laulund, 1994). According to Castro et al. (1997), temperature changes, phase changes, and dehydration can damage cell membranes, cell walls and ribosomes DNA. The nature of a strain is of vital importance which determine whether a high viability can be obtained or not during freeze-drying. There are also other factors which are reported to be critical to cell survival. These involve initial cell mass, the conditions of growth, the composition of the media used for probiotic growth and drying, as well as rehydration conditions (Carvalho et al., 2004). Freeze-dried LAB show good survival

under storage at low temperature (4 ℃), however, the buffering capacity of probiotic powder is poor, which means that very limited amounts of bacteria can reach the colon (Laulund, 1994). In addition, freeze-dried LAB are also sensitive to humidity (Laulund, 1994) if they are mishandled by consumers, such as exposing the powders to atmospheric conditions, or scooping the powders using a wet spoon (Huckle & Zhang, 2011). However, the cells can be protected from the acidic condition by encapsulation (Huckle & Zhang, 2011).

So far freeze-drying is still a preference among all probiotic preservation techniques. However, mainly due to the high cost and a large time-consumption of freeze-drying, spray-drying is one of the most prevailing drying technique in the dairy industry.

2.4.2.4 Fluidised bed drying

A fluidised bed drying process involves drying, cooling, agglomeration, granulation as well as coating of certain materials. The temperature of the process can be adjusted so that the method can be applied to encapsulate both thermo-sensitive and non-thermo-sensitive materials (Joshi & Thorat, 2011). It has been proved to be efficient during encapsulation of probiotic microorganisms (Stummer et al., 2010).

2.4.2.5 Spray-drying

Spray-drying is a relatively quick and economical microencapsulation technique, and it is easy to scale up. It converts a feed solution containing bacteria into powder by hot air (Møller et al., 2009). The rapid encapsulation process can prevent the dehydration inactivation of entrapped cells by dehydration (Perdana et al., 2014). Therefore, spray-drying was adopted for probiotic encapsulation in this project.

The key step of spray-drying is to spray the liquid solution into fine droplets (normally 10 - 150 μm) and is pumped into the dry and hot air flow (normally 150 ℃ - 250 ℃) (Anandharamakrishnan & Ishwarya, 2015). Problems and solutions regarding the spray-drying of probiotics will be discussed in the subsequent sections.

2.5 Encapsulating probiotics using spray-drying

2.5.1 Challenges associated to powder products containing probiotics

The main challenges associated to powders containing probiotic bacteria are maintaining their viability during industrial production process, storage, and usage (Liao et al., 2017). Also, bacteria have to survive the harsh conditions in GIT. However, several factors can affect the viability of cells such as oxygen, moisture, heat, and acid. Therefore, good protection must be provided to probiotic cells for a long shelf life and their successful delivery in the colon.

2.5.2 Intrinsic factors affecting the survival of probiotics during spray-drying

To protect probiotic cells, the maintenance of the structure of the cell membrane is critical as it is the most susceptible site of probiotic cells during spray-drying. The membrane may change from crystal state to gel state when exposed to high temperatures. In addition, peroxidation may happen on the lipid membrane bilayer during and after atomization process while most probiotics are obligate anaerobes (Talwalkar & Kailasapathy, 2004; Teixeira et al., 1995).

Meanwhile, the most important intrinsic factors that affect the survival of encapsulated bacteria are the growth stage of cells at harvesting time, their adaptation to thermal and osmotic pressure, and the strain types and species of the probiotic bacteria. In this study, cells were harvested at the logarithmic stage, as cells at logarithmic are usually strong enough (Anandharamakrishnan & Ishwarya, 2015).

2.5.2.1 Mechanisms of bacterial fatality during spray-drying

In order to protect probiotic cells, it is also vital to understand the mechanism of bacterial fatality. During the spray-drying process, the damage of cells is mainly caused by cell conformation, dehydration, and phase transition (García, 2011). The death of cells during spray-drying is relevant to the damage to the cell wall and cytoplasmic membrane which

contain lipids, and spray-drying changes the configuration/ profile of cellular lipids. The damage is reported to be caused by the oxidation of unsaturated fatty acids as well as lipolysis (Teixeira et al., 1996). Thus, cell membrane would be well-protected if cellular lipids are preserved.

2.5.2.2 Mechanisms of bacterial fatality during storage

Protection must be provided for probiotic cells during storage because cells cannot survive under the normal environmental storage conditions. Factors affecting the survival of bacteria during storage include the reaction of cells with oxygen, light, moisture, contamination and higher storage temperatures (Morgan et al., 2006). The most likely reason for the death of cells during storage is still the oxidation of fatty acids of cellular membrane lipids (Teixeira et al., 1996). The increased lipid oxidation of cellular membrane during storage can lead to some adverse physical changes in membrane structure and function. The best storage stability can be achieved by encapsulating the probiotic cells using proper wall materials before storing the cells at low oxygen environment and low temperature (Chávez & Ledeboer, 2007). More strategies for the protection of probiotic cells using spray-drying method are discussed later.

2.5.3 Potential solutions to improve the viability of probiotics in powders produced by spray-drying

2.5.3.1 Exposure to sub-lethal stress

Normally the cells intended for encapsulation are harvested at the logarithmic or stationary stages (Anandharamakrishnan & Ishwarya, 2015). The spray-drying of probiotics can be a strain-independent method. Although it is challenging to customize a process of spray-drying for each strain, it is quite achievable to adapt probiotic cells to spray-drying conditions by subjecting them to pre-treatment like nonlethal heat treatment. Some certain kinds of wall materials can also be employed. The most commonly used materials are whey protein isolate, skim milk, maltodextrin, gum Arabic, gelatin, chitosan, calcium/sodium alginate or the

combinations. (Anandharamakrishnan & Ishwarya, 2015)

It has been found that exposing the cells to a sub-lethal stress such as oxygen (Kosin & Rakshit, 2010), heat (Kosin & Rakshit, 2010) or acid (Sánchez et al., 2007) can improve cell viability during processing and the passage through GIT. Sub-lethal stress aims to arouse the inherent adaptive stress response of cells so that they can be prepared for the coming harsh environment.

Preheating can improve cell survival during exposure at high temperatures of the spray-drying process and even during the subsequent storage time. This is because increasing the temperature by 10 °C beyond the optimum growth temperature of the microbes can cause thermal shock, which further leads to the expression of stress resistance proteins in cells before drying (Teixeira et al., 1994). Paéz et al (2012) exposed various *Lactobacillus* strains to 52 °C for 15 minutes and showed increased survival during spray-drying of some strains including *L. casei* Nad and *L. plantarum*. Angelis and Gobbetti (2004) have reported that the heat resistance caused by pre-treatment can last for at least 4 h before spray-drying and can also extend the storage period. They also reported that the pre-treatment was ideal for *Lactobacillus* strains. However, in this research, preheat treatment was not applied to DPC16 cells to prevent the loss of cells caused by the fluctuation of temperature because of the use of ice bath. The preheat treatment is considered as an optional treatment when the encapsulation rate of all wall materials is low.

2.5.3.2 Effects of feed formulation on the survival of probiotic cells

Feed formulation is a significant parameter in alleviating destructive effects of both dehydration inactivation and thermal inactivation, which are the two main mechanisms which can explain the susceptibility of microbes (Janning & Veld, 1994). It can be optimized by determining proper total solid concentration, optimum core-to-wall ratio, and adding certain functional additives. The viability of probiotics inside the microcapsules is reported to be strongly dependent on the concentration and type of wall material (Lian et al., 2002).

The concentration of the feed solution should be neither too high nor too low. When the concentration of the wall material increases, the viscosity may also be higher, which can cause larger particle size, longer drying time and more heat damage, thus the loss of viability will increase (Santivarangkna et al., 2007). When the concentration of the wall material decreases,

the economic efficiency may be too low.

An alternative method for manipulating the feed solid concentration could be the use of wall materials or protective adjuvants that can protect cells from thermal effect as previously discussed (Anandharamakrishnan & Ishwarya, 2015). For wall materials which have thermoprotective properties, with the exception of RSM and trehalose, the best examples include gum Arabic and pectin which can stabilize phospholipid membrane by forming hydrogen bonds (Salar-Behzadi et al., 2013; Schutyser et al., 2012), as well as gelatin, which can stabilize the cell membrane by forming a thermo-reversible gel (Salar-Behzadi et al., 2013).

2.5.3.2.1 Milk proteins

Reconstituted skim milk can maintain better viability of spray-dried probiotic cells during storage (Corcoran et al., 2008). Skim milk, trehalose, and maltodextrin have been used as wall materials to encapsulate *L. casei* LK-1 by spray-drying and the protective effect during the drying process, storage period as well as in vitro digestion were evaluated (Liao et al., 2017). The importance of the matrix was proved and skim milk has been reported as an almost perfect wall material for spray-drying encapsulation. Skim milk was better than trehalose and maltodextrin not only during drying process and storage but also in either gastric or intestinal juice, which conformed with the conclusion reported by Pinto et al. (2015), who showed that skim milk protein and trehalose can replace the water molecules because of their low molecular weights. Therefore, skim milk protein and trehalose can maintain the integrity of structure and function of the cellular membrane of bacteria during exposure to high temperature, while skim milk has better performance.

Whey protein is an excellent candidate for probiotic encapsulation due to the emulsification, gelation in addition to its film-forming properties (Perez-Gago & Krochta, 2001). Higher tensile property, as well as lower oxygen permeability, which can protect the probiotics from harsh conditions in GIT, can be achieved by denaturing the whey protein (Perez‐Gago & Krochta, 2001; Rajam et al., 2012).

The addition of milk proteins can generally improve the survival of LAB during their exposure to simulated gastric juice (Charteris et al., 1998). This result conformed with that of Kos et al. (2000) who reported that the addition of casein, skim milk, mucin or whey protein concentrate

could all substantially increase the survival of *L. acidophilus* M92 during their exposure to simulated gastric juice of pH 2. The effect of protection of such materials is mostly caused by their buffering capacity (Huckle & Zhang, 2011).

2.5.3.2.2 Prebiotics

Substances that can be used with other carrier materials and protect probiotics against mainly thermal inactivation are referred to prebiotics (Anandharamakrishnan & Ishwarya, 2015). These are also food ingredients which are non-digestible but have a beneficial effect on the host through stimulating the activity and/or growth of bacteria in the colon to improve the well-being of the host (Gibson & Roberfroid, 1995). To be classified as a prebiotic, the ingredient must not be digested, adsorbed or absorbed by the host, but should be able to be fermentable by the microflora in the GIT and thus selectively stimulate the activity and/or growth of microflora in the GIT. When prebiotics and probiotics are used together at the same time, the product is called a symbiotic product (Schrezenmeir & de Vrese, 2001). In recent years, scientists have focused on producing symbiotic products (Homayouni et al., 2008; Ooi & Liong, 2010). The reason for their protective effect during spray-drying can be ascribed to their high demand for activation energy during the drying process, which provides better resistance to oxygen diffusion through drying microcapsules (Pérez-Alonso et al., 2003; Rodríguez-Huezo et al., 2007). The activation energy of protectants can be calculated using thermogravimetry analysis where the mass of a sample is measured over time as the temperature changes, and the application of Fick's second law (1), which predicts how diffusion causes the concentration to change over time (Pérez-Alonso et al., 2003).

$$\frac{\partial \varphi}{\partial t} = D \frac{\partial^2 \varphi}{\partial x^2} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

Where $\boldsymbol{\varphi}$ is the concentration (amount of substance/m^3)
t is time (s)
$\boldsymbol{\partial}$ is the diffusion coefficient (m^2/s)
x is the position (m)

Non-digestible polysaccharides and oligosaccharides, such as inulin, fructooligosaccharides (FOS) and resistant starch have prebiotic properties (Anandharamakrishnan & Ishwarya, 2015). Prebiotics can improve probiotic viability not only during spray-drying, but also during subsequent storage period (Ross et al., 2005). The application of prebiotics can reduce the moisture content in addition to water activity, contributing to better storage stability of powders (Tonon et al., 2009).

Fructooligosaccharide (FOS) is an inulin-type fructose with prebiotic function. The FOS can reduce glass transition temperature of the matrix, which is not ideal for cell survival, however, it can improve the encapsulation efficiency (up to 98.63%), storage stability and protection of cells in harsh conditions of the GIT when combined with denatured whey protein isolate at 1:1 core-to-wall ratio (Rajam & Anandharamakrishnan, 2015). This is due to the ability of cellular water replacement and cellular coating formation of FOS (Adhikari et al., 2009), in addition to its ability of gel network formation (Parthasarathi et al., 2013).

However, the preserving effects of prebiotics on one strain of bacterium may not necessarily work on another strain, even though the external conditions are similar, because of their variances in intrinsic factors such as heat tolerance of the strain, osmotic stress, and mechanical stress (Chen et al., 2011). The high amount of oligosaccharide such as FOS may lead to an increase in osmotic stress, which may harm the survival of cells (Ivanovska et al., 2015). Therefore, whether the addition of prebiotics could provide better protection for DPC16 during spray-drying and storage still needs to be investigated.

2.5.3.2.3 Low (small) molecular weight sugars

Low molecular weight sugars can also be used as wall materials or adjuvants to alleviate inactivation by dehydration. Glucose could be an excipient compound (Ying et al., 2012). Although it can reduce the glass transition temperature, it still has a positive effect on the protection of cells during spray-drying. This may be caused by the water (in cellular proteins and enzymes) replacement ability of small sugars (Castro et al., 1997; Leslie et al., 1995). During long-term storage, both the glassy state maintenance and the involvement of small molecular weight sugars such as glucose in feed materials are necessary for optimum cell survival of probiotic in spray-dried powders (Ying et al., 2012). However, whether this effect of small sugar is strain-related still needs to be investigated as the study by Ying (2012) only

tested *L. rhamnosus*.

2.5.3.3 Outlet temperature of the spray-dryer

As previously discussed, thermal damage is one of the main mechanisms of probiotic inactivation during the spray-drying process (Perdana et al., 2013). An outlet temperature above 70°C significantly decreased the residual viability (Perdana et al., 2015). During the spray-drying process, the survival ratio of probiotics was found to be dependent on the time-temperature combination (Santivarangkna et al., 2008). The thermal inactivation is not predominant at the beginning when there is constant drying rate and wet bulb protection on cells, but thermal effect is apparent in the falling rate period. Therefore, the outlet temperature has great impact on cell survival, while the inlet temperature has an indirect effect (Santivarangkna et al., 2008). It has been suggested that, for most strains which are thermosensitive, 70-80°C should be adopted as the outlet temperature (Ananta et al., 2005; Ying et al., 2012). It could be concluded that though the outlet temperature is important, it is dependent on several parameters including inlet temperature, air flow rate, feed rate, wall-to-core ratio, total solid content as well as atomized droplet size (Boza et al., 2004; Santivarangkna et al., 2007; Santivarangkna et al., 2008).

To increase the viability of microcells, another approach is to increase the feed flow rate, which can in turn, reduce the outlet temperature, resulting from the variations in heat and mass transfer at the interface between air and solid (Behboudi-Jobbehdar et al., 2013). When the feed flow rate is increased, the surface temperature of droplets will decline, which may lead to changes in heat and water diffusivity and therefore increase the survival rate of cells (Barbosa-Cánovas et al., 2005). However, the feed flow rate is not always settable depending on the design of each spray-dryer. In this research, only inlet/outlet temperatures could be set.

2.5.3.4 Adjustment of other parameters

Other parameters that have a great influence on the viability of probiotics during spray-drying process includes shear rate, atomization pressure (extensional and shear stress), type of atomizer (eg. rotary wheel atomizer works better than nozzle type), level of oxygen exposure

(eg. the presence of ascorbic acid in feed materials, filling in nitrogen in drying chamber or the adoption of vacuum spray-dryer) (Anandharamakrishnan & Ishwarya, 2015).

2.5.3.4.1 Level of oxygen exposure

A previous study reported on the decrease of cell survival as the shear rate increases (Ghandi et al., 2012). In addition, at the same shear rate (10^3 sec^{-1}), the survival rate of *L. lactis* was ranging from about 45% to 62% respectively, in the presence of air, air with ascorbic acid, nitrogen, and nitrogen with ascorbic acid. The study also showed a 30% reduction in survival loss when rotary wheel atomizer rather than nozzle type atomizer was used, however in the presence of ascorbic acid was compulsory. To control the level of oxygen exposure, the spray-dryer equipment needs to have good air-tight.

2.5.3.4.2 The nozzle-type atomizer

As for the nozzle type atomizer only, both shear rate and atomization pressure have an impact on the viability of cells. A decrease of atomization pressure from 100 to 50 kPa led to increased viability of *L. acidophilus* (Lievense et al., 1994). Similarly, a decrease of spray-dryer pressure from 200 to 100 kPa increased survival of *L. bulgaricus* (Riveros et al., 2009). This may due to the low pressure leading to low shear force and reduced stress on the bacteria (Riveros et al., 2009).

2.6 Probiotic powder preservation

The effectiveness of probiotics on human health depends on their viability, therefore, it is of vital importance to ensure their viability during both spray-drying process and storage period (Fritzen-Freire et al., 2012). Storage temperature, relative humidity, and physical state are determinants for the survival of probiotics during storage time (Jiménez et al., 2015). Common phenomenon that may happen on powder products of probiotics during storage is discussed later.

2.6.1 Water sorption and crystallization

Water activity is a vital parameter for the quality of powder during their shelf life. All food powders, not only probiotic powder, are sensitive to heat, moisture, oxygen and light, these factors are the most important factors which determine shelf life of food (Hedegaard & Skibsted, 2013). Most frequently, the shelf life of food powders is controlled by the temperature and availability of water which causes both physical and chemical changes. Water activity is important for the stability of storage and impacts the rate of physical deterioration such as stickiness and caking. To maintain the food powders in a glassy state is also vital as this can prevent the powders from crystallization, non-enzymatic, browning and oxidative deterioration (Hedegaard & Skibsted, 2013). The oxidation of lipid and protein, as well as non-enzymatic browning reactions may cause off-flavors, discoloration, and nutrition loss. These reactions are also based on water activity and are accompanied with a physical change in a complicated way (Hedegaard & Skibsted, 2013).

Water may act as a reactant, product or solvent in a deterioration reaction in food products. Increasing water activity with constant water content when the storage temperature increased is critical for the storage and transport of food powders, especially at high temperature in closed containers (Hedegaard & Skibsted, 2013). Depending on the water activity of food powders and relative humidity of the environment, food powders may absorb or desorb water to facilitate or prevent the deteriorative reactions. In food powder, the behavior of water sorption is grossly affected by changes in the physical state of ingredients, for example, the crystallization of sugars can lower water binding capacity and ΔH_{DESORP}, thus there will be an increase in water activity despite of constant water content (Hedegaard & Skibsted, 2013).

The optimum storage conditions of probiotic powders in the industry are now recommended to be determined by a T/a_w phase diagram, which combines information of isotherms and extended phase diagram, and shows regions of stable glassy state where temperature and water activity are the representative axes. Figure 2.1 is a T/a_w phase diagram which shows stable glassy-state regions of freeze-dried LAB cultures in a sucrose or lactose matrix. The viability of LAB indicated that a glassy state provided better protection for bacteria compared to non-glassy state, and sucrose as a non-reducing sugar gave better protection to bacteria compared to lactose which is a reducing sugar (Kurtmann, Carlsen, Skibsted, et al., 2009). As lactose is

an abundant component in skim milk powder with a content of about 50% to 53% ("Chapter 3 Lactose content of milk and milk products," 1988), it is possible to predict the physical state of lactose, which is the main physical state of skim milk powder, at a given combination of humidity and temperature (Hedegaard & Skibsted, 2013). It is also possible to calculate the required water activity that the milk powder needs to be dried according to this phase diagram. In order to confront 40 ℃, the water activity of milk should be below 0.2, and a water activity of 0.4 may lead to the collapse of milk powder at around 20 ℃.

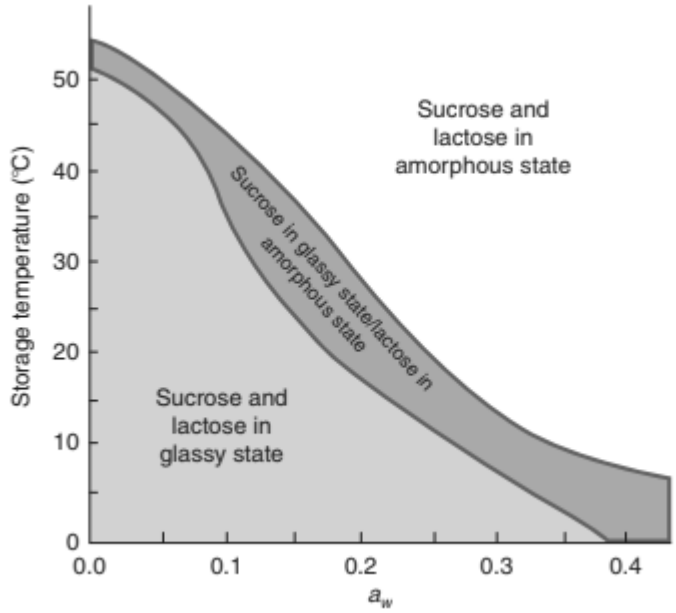


Figure 2. 1 Temperature/water activity phase diagram of freeze-dried LAB cultures in a sucrose or lactose matrix
(Kurtmann, Carlsen, Skibsted, et al., 2009).

The glassy state is always a preferred state for dehydrated food products. In spray-dried milk powder, maintaining the glassy state of lactose, i.e. keeping the storage temperature below T_g for longer periods or avoiding severe fluctuations of the storage temperature, is important for a prolonged shelf life since the lactose in glassy state forms a matrix where proteins, lipids, and air bubbles are diffused and where water is constrained to lactose and proteins (Thomsen et al., 2005). Water is released when lactose transforms from amorphous state to crystalline state (Jouppila & Roos, 1994). This improves the mobility of components in milk powder and thus accelerates the crystallization (Thomsen et al., 2005). The crystallization rate of lactose increases as the water activity increases even when the water content does not change because of the plasticizing effect of water, which lower the glass transition temperature of lactose

(Thomas et al., 2004). For commercial milk powder, the initial water activity of milk powder is about 0.2 (Jouppila & Roos, 1994) and the T_g is about 40 °C (Thomsen et al., 2003). As the water activity of 0.4 is the threshold that separates glassy and non-glassy states as the main form of lactose at room temperature (Jouppila & Roos, 1994), the water activity of 0.2 is far below the threshold value 0.4 and helps to keep the glassy state of lactose. When the water activity of commercial milk powder is above 0.3, its crystallization tends to happen from the amorphous glassy state and thereafter, browning reactions will be initiated, which is an indication of the deterioration of the food quality (Thomsen et al., 2003).

To prevent the lactose and also other sugars in both frozen products and food powders from crystallization, the addition of sugars has an inhibition effect. For lactose, although the addition of sucrose can lower the T_g a bit, it can prolong the induction time of lactose for crystallization (Thomsen et al., 2006).

In conclusion, the water activity of the powder, glass transition temperature and storage temperature are important parameters affecting the quality of powders containing probiotic bacteria cells as well as other types of food powders. The three factors affecting each other can determine the physical state of the powder, and therefore have great influence on the viability of bacteria cells. The deterioration of food powders is triggered by changes in the physical state, for example, the collapse of glassy state may cause crystallization, which further enhances deterioration caused by chemical reactions such as non-enzymatic browning and the oxidation of lipid and proteins (Hedegaard & Skibsted, 2013). In dehydrated food such as milk powder, T_g is influenced generally by the components and the water content in food in addition to the rate of temperature change. The quality of a dehydrated food is most influenced by carbohydrates because of their low T_g, although the main components in such foods are carbohydrates, proteins, and lipids.

For the powder products containing probiotic cells, water activity 0.25 significantly increase the mortality rate of bacteria by stimulating metabolism due to possible higher molecular mobility in the water (Albadran et al., 2015). Whereas a low relative humidity can also affect the cells due to the removal of water (Castro et al., 1995). It is therefore important to keep water activity at intermediate levels (Okuro et al., 2013). It was reported that residual water content ranging from 2.8% to 5.6% can prolong the storage time of powders containing probiotics (Khem et al., 2016) and 4.0% is regarded an optimum level for the storage of bacteria (Ananta et al., 2005; Masters, 1985). Prebiotics lower the moisture content and water activity in

microcapsules, thus increasing powder stability and prolonged storage time (Tonon et al., 2009).

In addition, the glassy state is suitable, however, it is not a pre-requisite factor for storage stability, because presence of small molecular sugars can also enhance storage stability (Liao et al., 2017). When evaluating long-term viability, both storage temperature (T) and glass transition temperature (T_g) need to be considered. This is a parameter known as T-Tg, and is independent of the protective agent, but has a direct impact on the shelf life of bacteria (dos Santos et al., 2014).

For freeze-dried cells, the number of live cells decreases during storage, however, higher viability is reported during lower temperature storage (Huckle & Zhang, 2011). Further, subsequent storage conditions, such as temperature, atmospheric composition, light exposure and humidity are all critical for the recovery of feeze-dried cells (Huckle & Zhang, 2011). A study by Castro et al. (1996) showed that the lipid profile of freeze-dried *L. bulgaricus* changed during storage, which suggested that oxidation happened in cell membrane lipids. To prevent or reduce lipid oxidation during storage, cell powders should be stored under vacuum conditions, or under an environment where water activity is controlled.

2.6.2 Maillard reactions and oxidation reactions

The term Maillard reactions refers to the chemical reactions that are initiated by condensation of reducing sugar and an amino group in proteins or peptides then followed by a series of reactions producing several intermediates such as aroma components and brown polymers (Nursten, 2005). Presence of brown colour is undesirable in milk powder and probiotic powders. However, the large amount of lactose and proteins that contain lysine makes milk and milk products with intermediate water activity (0.5 - 0.7) sensitive to Maillard reactions (Labuza, 1970). This is induced by heat (Morales et al., 1997). Therefore, it is vital to produce a powder with low water activity and store such products under low temperature conditions because the destruction of amino acids and the cross-links among chains of protein reduce the solubility and digestibility of proteins (Hurrell, 1990).

Oxidation of membrane lipids, destructions of proteins and changes in cell wall and DNA can all lead to the death of cells during drying and storage of bacteria cultures (Hedegaard & Skibsted, 2013). Maillard reactions can not only damage proteins and sugar matrix but also

cause lipid oxidation indirectly. The water generated at the first step of Maillard reaction affects the water activity of a dry powder (Hodge, 1953), which makes the food material, irrespective of its glassy or amorphous state, more susceptible to crystallization (Jouppila & Roos, 1994). Crystallization of lactose under stable water content leads to a high increase in water activity (Vuataz, 1988), and Maillard reactions in powders are also reported to increase as well as the water activity. The increase of water activity catalyses lactose crystallization and Maillard reactions, thereby producing more water which affects lipid oxidation (Hedegaard & Skibsted, 2013). Lipids are contained in cell membranes; therefore, cell viability may also be affected.

Browning may be an indicator of survival loss of probiotic powders and a relation between discoloration and survival loss of cells has been reported (Carvalho et al., 2007; Kurtmann, Carlsen, Risbo, et al., 2009; Kurtmann, Carlsen, Skibsted, et al., 2009), especially when cells are trapped in the matrix of reducing sugar. This may be caused by the condensation of reducing sugars and proteins during Maillard reactions, which leads to the collapse of the glass-state matrix of reducing sugars. During Maillard reactions, reducing sugars and proteins or peptides condense and thus the glass-state matrix of reducing sugar collapses. Thus, non-reducing sugars such as sucrose are recommended for the encapsulation of probiotic cells. The mechanism of the protective effect of sugars can be explained by two hypotheses. One is the ability of sugar to form a glassy matrix where bacteria can be embedded. The other is that sugars can form hydrogen bonds with bacteria when water is evaporated during drying, which is also called water-replacement hypothesis (Higl et al., 2007).

According to Higl et al. (2007), the survival of dried probiotic cells may markedly decrease if they are stored under unsuitable storage conditions or have poor product formulations despite the removal of water. The physical changes of product formulations and chemical reactions within the components causes the deterioration of a probiotic powder product. The deteriorative reactions, for example, fusion and leakage, are rather slow when bacteria are stored below T_g, where bacteria can be trapped in the gassy-state-sugar matrix, while they are accelerated when temperature is above T_g (Crowe et al., 1997; Sun et al., 1996). Thus, this has been an active research area to optimize the matrix that is used for cell protection and seek the optimum storage conditions for the probiotic powder products.

2.6.3 Packaging of probiotic powder products

The strains that are normally used in probiotic products are anaerobic and microaerophilic, thus oxygen in the packages of probiotic products should be as low as possible to avoid the accumulation of toxins and cell fatality (Cruz et al., 2007). To minimize the oxygen in the packages, the involvement of antioxidants like ascorbic acid and the removal of peroxide producing strains were considered (Champagne et al., 2005). Research on the packaging of probiotic products in this decade has focused on active packaging and gas-impermeable film (Cruz et al., 2007). Although it has been found that the glass bottle preserves probiotic cells better than plastic packages due to the low gas permeability (Jayamanne & Adams, 2004), it is inappropriate to be applied in industry because of the high cost and hazards of glass. Powder products should also be stored under dark conditions to prevent the oxidation of the lipids in cell membrane. In addition, the packages need to be water-impermeable to maintain a stable water content inside the packs as it affects the glassy state of the matrix protecting the cells.

Previous research has been conducted on the shelf life model of powdered infant formula. It was reported that oxygen below 2% and a temperature below 25 ℃ can significantly extend the shelf life of the powder (An et al., 2018). For microencapsulated probiotic powder, Hsiao et al. (2004) found an improved cell viability of bifidobacteria when an oxygen absorber and desiccant is included in the polyester bottles, although the best survival was obtained in glass bottles. However, not many studies applied modified atmosphere packaging (MAP) to probiotic powder preservation, although it is an even cheaper way for probiotic powder preservation. The MAP is the enclosure of food in a package where the atmosphere is vacuumed, and if necessary, replaced by gas such as carbon dioxide, nitrogen or oxygen (Farber, 1991). The modified atmosphere composition was reported to influence the growth of pathogens and spoilage bacteria markedly. The 100% carbon dioxide was found to inhibit the growth of a lot of microorganisms (Fedio, 1994). In this research, the only consideration is to remove the oxygen because purity of culture in powders was confirmed before storage and all packaging materials were sterilised before use. As the oxygen susceptibility of probiotics may be strain-independent and the research on the interaction between oxygen and probiotics is inadequate (Talwalkar & Kailasapathy, 2004), in this research the lowest vacuum value that can obtain is adopted for the packaging of DPC16.

2.6.4 Key properties of probiotic powders

2.6.4.1 Cell viability

Cell viability is the most important property for a probiotic product as the product cannot be regarded as functional food if the viability of probiotic cells is lower than 10^6 CFU per gram or per millilitre during consumption (FAO/WHO, 2003). Factors that affect the survival of probiotic cells during storage include the reaction with oxygen and moisture, high storage temperature, light, and contamination by other bacteria (Morgan et al., 2006).

2.6.4.2 Water activity

The concept of water activity was introduced by Scott (1953) to define the impact of the moisture content in food on the microbial response. Microbiologists have noticed that it is the water activity rather than the moisture content that had great influence on the response of microorganisms, sporulation, and toxin-production (Jay et al., 2008).

2.6.4.3 Colour

Colour is a characteristic of a food material. It is of vital importance for the identification and evaluation of the quality of the product. Whey protein isolate and skim milk powder, which are excellent oxygen and aroma barriers (Maté & Krochta, 1996; McHugh & Krochta, 1994; Miller et al., 1998) had been adopted as wall materials for the encapsulation of probiotic cells. However, it is well-known that powders of milk proteins can undergo Maillard reactions at high temperature during storage, which leads to the yellowing and loss of nutritional value of powder products (Miller et al., 1997). Labuza and Saltmarch (1982) reported that the formation of brown pigments increased with the storage temperature (25 °C to 45 °C) and water activity (0.33 to 0.65). To predict the colour change of powders during storage, stability of the colour of powders as compared to their original colour should be determined. Several colour scales have been used to describe colour, among which the most commonly used are the CIE system,

the Hunter *L, a, b* system and the Munsell colour solid (Giese, 2000). The first two systems measure the degree of lightness (*L*), redness of greenness (*a*), and yellowness or blueness (*b*). The Munsell colour-order system shows the relationships among colours. All colours have three attributes, value, chroma and hue, which are also called Munsell notations. All colours lie within a specific region of Munsell colour space, which is called the Munsell colour solid (Giese, 2000).

2.6.4.4 Moisture

The moisture content is of vital importance for the understanding of the interactions between water and non-aqueous components in food (Kaymak-Ertekin & Gedik, 2004). Water is an active component in food which controls the biochemical reactions, texture and overall physical properties of food (Doporto et al., 2012). The moisture sorption isotherms describe the relationship between water activity and the equilibrium moisture content at a given storage temperature (Sahin & Sumnu, 2006), the information of which can be used to assess food processing operations, for example, drying, mixing, packaging and storage (Debnath et al., 2002). Thus, the moisture content is measured to gain information to optimize the storage condition, packaging systems, maximize the retention of colour, texture property, nutrients and biological stability (Debnath et al., 2002).

2.6.4.5 Bulk density

The bulk density of a powder is the mass of the powder divided by its bulk volume (Abdullah & Geldart, 1999), which depends on the compactness of the powder. To measure bulk density, a certain amount of powder is transferred into a graduated cylinder and then powders with different bulk densities have different volumes of free space for packages of similar shape (Robertson, 2013). The volume of free space affects the rate of oxidation. A large volume of free space, which is a low bulk density, in addition to a large package surface area, are undesirable as these can cause more chances for the powder to be exposed to oxygen (Robertson, 2000).

2.6.4.6 Particle size

Particle size is one of the most important properties of powder particles because it is influenced by several aspects of bulk behaviour of particles (Allen, 1997, 2013), such as flowability, explosibility, compressibility and fluidisability. Particularly, particle size influences the surface area of powder particles which further determine the degree of interactions between the powder particles and between particles and surrounding liquids. Equivalent spherical diameters are always adopted for defining the particle sizes. The equivalent spherical diameter is the diameter of a particle whose value of property is the same as that of the particle, such as volume, mass, surface area and projected area (Fitzpatrick, 2013).

2.6.4.7 Surface structure and components

The surface of particles is rarely smooth. It can be cracked, shrivelled or dented based on the production processes of the powder, and the surfaces are broken structures. The chemical bonds on the surface of powder particles are unsaturated because of the breaking of the chemical bonds with other molecules. Thus, the surface of powders is more susceptible to the reactions with gases and water vapour. This causes more oxidation and other undesirable changes of powders. The absorption of water vapour at powder surface influences the cohesiveness of the powder particles. The increased cohesiveness of powders at high moisture content reduces the flowability of powders (Fuji et al., 2006). During the drying process, new surfaces are formed. Food products normally contains protein, fat and carbohydrates. Compared to the bulk composition, the surface contains more proteins and fat. High precision equipment such as the X-ray photoelectron spectroscopy, atomic force microscopy and the scanning electron microscope can measure the surface microscopy of powders accurately. The migration of surface-active proteins to the surface of powder particles has been reported by several groups of researchers (Fyfe et al., 2011; Shrestha et al., 2007). While the increase of fat on the particle surface can cause reduced wettability and flowability, the increase of protein on the surface results in the change in surface morphology of powder particles. When the surface is covered by a high content of proteins, it becomes more wrinkled (Xu et al., 2012). In addition, decreases in the hygroscopicity and encapsulation efficiency were found when the particles were covered by high molecular weight compounds (Fang & Bhandari, 2012). The increase of protein

concentration and the density of surface structure always occurs during drying. The powder with crust surface is poorly soluble (Mimouni et al., 2010), but the degree of surface smoothness varies based on composition. The powders at nanometre scale with compounds in low molecular weight were observed to have smoother surface of powders than that at micrometre scale with higher molecular weight compounds (Bhandari, 2013).

2.7 Release of encapsulated probiotic cells in GIT

Regardless of GIT, bacteria cells in dried dormant mode are reported to be revitalized initiated by an increase in a_w (Sapru & Labuza, 1993). Except for the viability and stability of probiotics during spray-drying, storage time and passage through the GIT, the core release characteristics, which are affected by the encapsulating wall matrix as well as core-to-wall ratio, should be considered (Lee & Rosenberg, 2000). The cells must be easily released when they come across the colon so that they can colonize before being excreted. The most commonly used principles for the release of cells is triggered by changes in pH, enzymatic activities or osmotic pressure. Release of cells can also be triggered by the presence of certain chemical components in the colon (Gouin, 2004; Mortazavian et al., 2007).

2.8 Conclusion

Products containing probiotics can be produced by several methods which include emulsion, extrusion, freeze-drying, fluidised bed drying and spray-drying. At present, the majority of probiotics are produced by freeze-drying which is expensive. Therefore, there is need to search for more economical methods to produce the bioactive products. Very little research has been conducted on the production of probiotics using the spray-drying method. This study investigates the potential of several materials for the encapsulation of the probiotic strain *L. reuteri* DPC16 by spray-drying, selects the suitable inlet/outlet drying temperature, determine the appropriate packaging materials and evaluate the stability of the spray-dried probiotic powders during their storage periods.

Chapter 3 Materials and Methods

3.1 Description of the experiments

The investigation of the optimal conditions for the microencapsulation of *L. reuteri* DPC16 by spray-drying was conducted in three integrated phases, comprising (1) activation and preservation of the DPC16 cells; (2) selection of wall materials and spray-drying conditions for the microencapsulation of DPC16; (3) testing the storage stability of the selected formulations of the powder. All the experiments were repeated twice, and duplicate samples were analysed for each treatment. The chemicals used in the experiments were of food grade or analytical grade. All microbiological media used were sterilized at 121 ℃ for 15 minutes in an autoclave (AMA040, Astelle Scientific, UK), according to the supplier's instructions. Glassware and other equipment were sterilised by dry heat at 105 ℃ for 3 h using the hot air oven (Soil drying oven, Unitemp, Queensland, Australia).

3.2 Activation and preservation of the DPC16 cells

3.2.1 Phase 1 Cell activation and preservation

The frozen *L. reuteri* DPC16 probiotic culture was supplied by Bioactive Research New Zealand Ltd (Auckland, New Zealand). The culture was activated in sterile MRS broth (Merck, Darmstadt, Germany) and incubated for 18 h at 37 ℃. After incubation, a loopful of the grown culture was streaked on solidified MRS agar (Oxoid, Thebarton, Australia) plate, and incubated at 37 ℃ for 48 h under anaerobic environment using anaerobic sachet (Oxoid, Thebarton, Australia). To examine the purity of the grown colonies, one isolated colony was streaked on another solidified MRS agar plate several times until a pure culture was obtained. The purity of the grown colonies on MRS agar plates were confirmed by Gram staining and examination under the light microscope (Axiostar Plus, Zeiss, Germany). The morphology of DPC16 was also compared to previous studies (Kandler & Weis, 1986). *L. reuteri* strains are Gram-positive LAB, with slightly irregular cell shape, curved rods and rounded ends. The bacterium has a length of 2.0 - 3.0 μm and a width of 0.7 -1.0 μm. Cells may occur singly, paired, or in small

clusters.

Single colonies from the pure culture were inoculated into individual 9 mL sterile MRS broth. After incubation at 37°C for 18 h, DPC16 cells were harvested by centrifugation at 3200 × g (Sigma 6 -16 KS, Sigma, Germany) for 10 min, then suspended in cryo-vials (Microbank^{TM}, Pro-Lab Diagnostics, UK). The vials were left at room temperature (20 °C) for 2 minutes to allow glycerol to interact with the cell walls of the bacterium to form a protective layer. The glycerol stock cultures were stored at -18 ℃ until required for further studies.

3.2.2 Examining the growth of the DPC16 using the plating method and the optical density method

The Microbank^{TM} Manual (Protection of Microorganism Preservation System, Microbank^{TM}, Pro-Lab Diagnostics, UK) was used for the recovery of the frozen cells with minor modifications. To recover the cells, the frozen vial was retrieved from the freezer and one bead was withdrawn from the cryo-vial using a sterile needle, inoculated into 9 mL sterilised MRS broth, then incubated at 37 ℃ for 18 h. Then, 1.2 mL of the culture was inoculated into 120 mL fresh MRS broth. The 120-mL sub-cultured broth was incubated at 37 ℃ for 18 h.

During incubation, 2 mL of sub-cultured broth was withdrawn to measure the optical density at 595 nm and 1 mL was used to enumerate the growth of the DPC16 by the pour-plate method. Sampling of the incubated culture was conducted hourly with duplicated samples. The data were used to generate a growth curve using the mean of log viable cell counts [log (CFU/mL)] obtained at each sampling interval against the incubation time (h). The turbidity (optical density, OD) of each hourly sample was measured using a spectrophotometer (Novaspec III, Amersham Biosciences, UK) at 595 nm. A correlation curve between the OD_{595nm} and the concentration of viable cells [log (CFU/mL)] was generated. The graphs were generated by Microsoft Excel (Microsoft 2010, Washington, USA), showing the mean and standard deviation of four samples at each time interval. The correlation was also expressed as an equation with a fitting Pearson's correlation coefficient R^2 of the regression line on the graph.

The correlation curve was used to estimate the concentration of the viable cells in the liquid suspension according to its optical density before it was mixed with a wall material solution (Sutton, 2006) as described in section 3.3.3 and section 3.4.2. The growth curve was used to

estimate the incubation time required to propagate cells to obtain the desired (cell) concentration before encapsulation. The cells in the logarithmic phase were used for the encapsulation because they were considered stronger and healthier (Zhao, 2012).

3.3 Phase 1 Selection of the optimal conditions for the microencapsulation of DPC16 by spray-drying

3.3.1 Description of microencapsulation materials

The wall materials used for the encapsulation of DPC16 were selected based on previous studies (Anandharamakrishnan & Ishwarya, 2015; Fritzen-Freire et al., 2012; Gul, 2017; Ivanovska et al., 2015; Lakkis, 2007; Liao et al., 2017; Ying et al., 2012). The materials used were:

- Gum Arabic (Caldic, New Zealand Chemical Supplier, New Zealand);
- Maltodextrin (Interchem, New Zealand Chemical Supplier, New Zealand);
- Anchor^{TM} skim milk powder containing 0.62% milkfat (Fonterra, New Zealand dairy company, New Zealand) was purchased from Pak'n'Save Supermarket, Auckland, New Zealand;
- Inulin (Caldic, New Zealand Chemical Supplier, New Zealand);
- Whey protein isolate (Caldic, New Zealand Chemical Supplier, New Zealand); and,
- Sucrose (Crescendo, New Zealand Chemical Supplier, New Zealand).

The wall materials used in this study were 10 % (w/w) RSM, 10% (w/w) gum Arabic, 10% (w/w) maltodextrin and the mixed wall material (MWM) containing 2.5% (w/w) whey protein isolate, 2.5% (w/w) GA, 2.5% (w/w) inulin, and 2.5% (w/w) sucrose. The inlet/outlet temperatures used were 160 ℃/80 ℃ and 180 ℃/100 ℃, respectively (Anandharamakrishnan & Ishwarya, 2015; Fritzen-Freire et al., 2012; Gul, 2017; Ivanovska et al., 2015; Lakkis, 2007; Liao et al., 2017; Ying et al., 2012).

Table 3. 1 Experimental setup of the conditions for the microencapsulation of DPC16 by spray-drying

Experiment	Wall material	%Concentration (w/w)	T_{inlet} (°C)	T_{outlet} (°C)
1	RSM	10	160	80
2	Gum Arabic	10	160	80
3	Maltodextrin	10	160	80
4	MWM	10	160	80
5	RSM	10	180	100

Notes: RSM =reconstituted skim milk; MWM=mixed wall material comprising 2.5% (w/w) whey protein isolate/2.5% (w/w) gum Arabic/2.5% (w/w) inulin/2.5% (w/w) sucrose; T_{inlet} =inlet temperature; T_{outlet} =outlet temperature.

3.3.2 Description of the spray-dryer

The Saurin SL-10 pilot scale spray-dryer (Saurin Enterprises Pty, Australia) was used to spray-dry a liquid suspension containing viable cells of the DPC16 with each respective encapsulating material. The dryer consisted of a twin-fluid nozzle atomization system (Figure 3.1a, b) with a liquid outlet (Ø = 0.8 mm). The drying chamber was 1.4 m high with a Ø of 0.8 m.

The nozzle cross section (Figure 3.1b) shows the direction of flow of the air stream and stream of the feed solution. The configuration allows rapid evaporation of water with spontaneous encapsulation of the probiotic cells (Gharsallaoui et al., 2007; Rajam & Anandharamakrishnan, 2015).

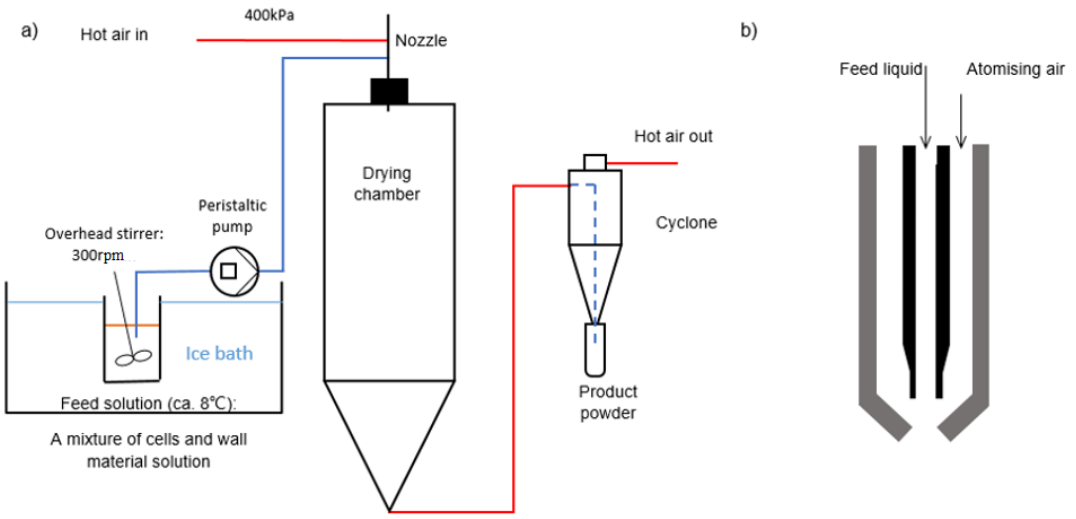


Figure 3. 1 (a) Schematic diagram of the single effect spray-dryer, (b) Cross-section of a pneumatic (twin fluid) nozzle

Notes: Figure 3.1 was drawn using Microsoft Paint (Microsoft office 2013, Microsoft, US), based on the Saurin SL-10 Operating manual (SL- 10, Saurin, Australia).

3.3.3 Preparation of liquid suspension containing DPC16 (the feed solution) for spray-drying

The method for the recovery of cells has been described in 3.2.2. One bead was withdrawn from the frozen vial and inoculated into 9 mL MRS broth, followed by incubation for 18 h at 37 °C. After incubation, 0.4 mL were inoculated into 40 mL fresh MRS broth and then incubated for another 18 h at 37 °C for sub-culturing. Sub-culturing aims to boost the metabolism of the cells (Lye et al., 2013). DPC16 cells were harvested by centrifugation (Sigma 6-16 KS, Sigma, Germany) at 3200 ×g for 10 min at 4 °C. The cell pellets were washed twice by suspending in 0.1% peptone water (w/w) (Merck, Darmstadt, Germany) and gently swirled before centrifugation. The supernatant was discarded, then the optical density (595 nm) of the cell suspension was adjusted to an absorbance of 0.5 (giving $\approx 10^8$ CFU/mL) using peptone water in a spectrophotometer (Novaspec III, Amersham Biosciences, UK). About 10 times dilution (compared to the volume of the growth media, MRS broth) was required to achieve an absorbance of 0.5 using peptone water. Thus, approximately 400 mL peptone were needed in

the screening stage and 3 L in the storage stage. Then the cell suspension was centrifuged at 3200 × g for 10 minutes and the supernatant discarded, leaving the cells attached to the wall of the centrifuge tube to be mixed with the wall material solution. To maintain consistency of the initial cell numbers for encapsulation by spray-drying, cells were mixed with 400 mL wall material colloids at the screening stage and 3 L at the storage stage (Table 3.2) to form the feed solutions.

The wall material colloids were prepared by dissolving each wall material in distilled water to produce a solid-liquid ratio of 10% (w/w). The prepared colloids were kept in ice-bath (≈ 8 °C) with continuous agitation (300 rpm) using an overhead stirrer (RW 20.n, IKA Labortechnik, Malaysia) for at least 30 min to remove any lumps.

Table 3. 2 Preparation of the feed solution (mL) for spray-drying

Stage	Growth medium	Peptone water	Wall material colloid
Screening	40	400	400
Storage	300	3000	3000

Notes. Peptone water was used for adjusting the cell suspension to obtain an absorbance of 0.5 at 595 nm; MRS broth (growth medium) was used for the cell culture; the wall material colloid was used to mix with the harvested cells which formed the feed solution for spray-drying.

After the temperature of the wall material colloids had stabilised at ≈ 8 °C in the ice bath, the sediment of cells in the centrifuge tube (250 mL) to which cells attached was mixed with the wall material colloid. Cells attached onto the wall of the centrifuge tube were dislodged by a pipette and gentle swirling. After mixing, the mixture in the centrifuge tube was poured into the beaker containing the rest of the wall material solution, which constituted the final feed solution. The feed solution was immediately pumped into the dryer (after the spray-dryer had stabilised at the desired levels of inlet/outlet temperatures) with continuous stirring at 300 rpm using an overhead stirrer (IKA RW 20.n, IKA, Malaysia). The flow-chart of the preparation of the feed solution at the screening stage is shown in Figure 3.2.

3.3.4 The spray-drying process

During the spray-drying process, the feed solution was kept in the ice-bath (≈ 8 °C) as was shown in Figure 3.1 (a) to minimize the growth of the cells. Samples for the determination of the cell concentration in the feed solutions were collected in duplicate (from the feed solution) at two time points: before pumping the feed solution into the spray-dryer and just before the end of the feeding process. The feed solution was continuously agitated at 300 rpm by an overhead stirrer and was pumped into the spray-dryer at ≈ 350 mL/min using a built-in peristaltic pump (Verderflex, RS Components, New Zealand) (Figure 3.1 a). Dried capsules were blown down the chamber (Figure 3.1 a) and then up a pipeline in order to fully remove the remaining water droplets before being sedimented by a built-in cyclone. In the cyclone, heavy capsules were accumulated in a steel bottle at the bottom (Figure 3.1 a).

The inlet air temperature was set at 160 °C and the outlet temperature at 80 °C according to Gardiner et al. (2000). An inlet temperature of 170 °C and outlet temperature of 80-85 °C were reported to be optimal for the encapsulation of *L. paracasei* and *L. salivarius* strains by spray-drying, to produce powders with ≈ 4% moisture content, which was desirable (Masters, 1985). For some thermal sensitive strains such as *L. acidophilus*, an increase in inlet temperature from 120 to 160 °C significantly ($P<0.001$) reduced the viability of the cells during spray-drying (Behboudi-Jobbehdar et al., 2013). However, DPC16 is not described as a thermally sensitive strain (Joshi, 2005). Therefore, in this study 160 °C was selected as the inlet temperature to retain relatively high cell viability and an optimum moisture content of the powder.

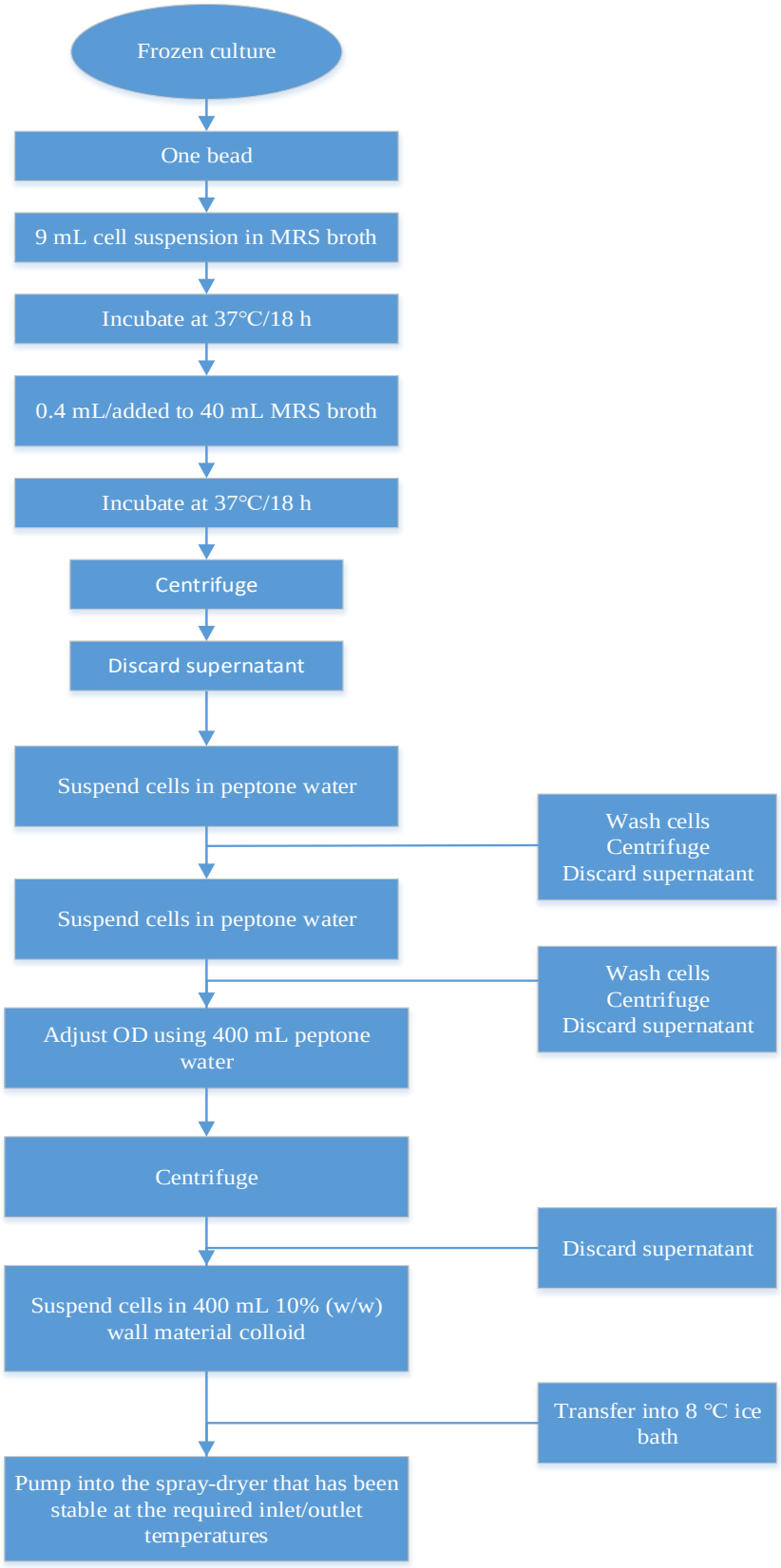


Figure 3. 2 Preparation of feed solution of the wall material at the screening stage

3.3.5 Packaging of the powder product after spray-drying

Following spray-drying, the dry powder (approximately 20 g) was collected and packed in aluminium foil bags (PET/ AL/ LDPE) (ALFW5-18, PBAG, China) and heat-sealed by a foot-operated heat sealer (Hardy Packaging Ltd., New Zealand).

3.3.6 The effect of spray-drying conditions on the microencapsulation of DPC16 cells

3.3.6.1 Determination of encapsulation efficiency

The encapsulation efficiency was calculated using Equation (2):

$$\% \text{ Encapsulation Efficiency (EE)} = \frac{N}{N_0} \times 100\% \text{.....} \quad (2)$$

Where N_0 is the log number of viable cells in CFU/g of a wall material on a dry basis; N is the log number of viable cells in CFU/g of microcapsules (Rajam & Anandharamakrishnan, 2015).

Cell concentration was analysed by the pour plate method within three hours after spray-drying. To analyse the cell concentration of feed solution, suitable serial dilutions were prepared and plated in MRS agar and incubated anaerobically at 37 ℃ for 48 h. For analysis of the cell concentration of the powder, 10 g were dissolved in 0.1% (w/w) peptone water to make a 10% (w/w) solution. The rehydration of powder samples facilitated the release of bacterial cells from the microcapsules (Rajam et al., 2012). Suitable serial dilutions were then plated as described earlier in section 3.2. Incubated plates containing 30 - 300 developed colonies were enumerated as log CFU/g powder.

3.3.6.2 Determination of the water activity of DPC16 microcapsule powders

The water activity of the spray-dried powders was measured by AquaLab Series 3 water activity meter following the supplier's instructions (New Zealand). The water activity (25 ℃) of the DPC16 microcapsule powder samples (2 g) was measured in duplicates immediately

after powder production using the water activity meter. The meter was calibrated at 25 ℃ using the supplied standard samples prior to use.

3.3.6.3 Determination of the morphology of the DPC16 microcapsule powder

Determination of the particle size of the powders

In this thesis, the term 'particle' is synonymous with the term 'microcapsule'. The particle size of the DPC16 microcapsule powder was determined based on the scanned electron micrograph (SEM) captured by the FEI Electron Optics, Quanta 200 (The Netherlands). The method used was based on the report by Tan and Balasubramanian (2017) with minor modifications.

The (probiotic) powder samples for the SEM imaging were prepared in a cool room (22 ℃). The powders were sprinkled on a double-sided tape (Deskwise, Warehouse, New Zealand) on the supplied aluminium sample holder and excess particles of the powder were removed using a rubber suction bulb (60 mL Laboratory Tool, Burry Life Science, UK). The sample was then examined under the scanning electron microscope unit using an accelerating voltage of 10.00 kV. Digital images were captured at ×2000 and ×16000 magnifications.

The particle sizes of the probiotic powder samples were examined using the SEM images with ×2000 magnification as these pictures provided a representative overall distribution of powder particles. Three images were captured for each treatment of powder. The area of five particles in each image was calculated using the ImageJ software (ImageJ2, University of Wisconsin, US). To reduce errors, five particles were chosen for measuring their areas according to their positions on the SEM images. The particles closest to one third of the points, two thirds of points and the middle point of the picture were selected according to Tan and Balasubramanian (2017) with some modification. The equivalent diameter of each particle was calculated according to equation 3:

$$D = \sqrt{4 \times S/pi} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (3)$$

Where *D* was the equivalent area of each particle; *S* was the area of each particle; *pi* was the circular constant ≈3.1416 (Tan & Balasubramanian, 2017).

The particle size of each sample in one batch was the average equivalent diameter of 15 measurements. The final particle size was the average diameter of two batches.

Determination of the surface structure of the DPC16 microcapsule powders

The external (surface) morphology of the DPC16 microcapsule powder samples was examined using the SEM images with ×2000 and ×16000 magnifications.

3.4 Phase 3 Storage stability of the selected formulations of the powder

Powders made from RSM and the MWM were selected for the storage trial. Skim milk had significantly higher EE with higher water activity than the other wall materials. Although the MWM did not show high EE, the MWM microcapsules had lower water activity. In addition, the presence of more smooth-surface particles in MWM microcapsules may facilitate the dissolution of the microcapsules (Reyes, 2018).

3.4.1 Recovery of bacterial cells

The method for the activation of cells was similar to the description given in 3.3.3. One bead was withdrawn from the vial and inoculated into 9 mL MRS broth, followed by incubation at 37 ℃/ 18 h. In this phase, 3 mL of the overnight sub-culture was inoculated into 300 mL fresh MRS broth and then incubated for a further 18 h at 37 ℃.

3.4.2 Preparation of feed material

The method for the preparation of feed material was similar to the description in section 3.3.3, However, in this phase, 3 L of the selected wall material (skim milk powder or the mixed material) colloid (10%, w/w) was mixed with cells to give an initial cell concentration of $\approx 10^8$ CFU/mL after adjusting the cell suspension using peptone water and the spectrophotometer.

3.4.3 The spray-drying process

The spray-drying process was described in section 3.3.4.

3.4.4 Packaging and storage of DPC16 microcapsule powders of the selected samples for the storage trial.

Based on the results of the screening phase, the wall materials with promising results were the 'RSM' and the 'MWM' (Table 4.2).

Table 3. 3 Storage of DPC16 powders for four weeks at two temperatures for 4 weeks

Wall	Packaging	Storage Temperature (°C)
RSM	Film	25
RSM	Film	55
RSM	Aluminium bag	25
RSM	Aluminium bag	55
MWM	Film	25
MWM	Film	55
MWM	Aluminium bag	25
MWM	Aluminium bag	55

Notes: RSM = reconstituted skim milk; MWM = mixed wall material; film = gas-impermeable film; aluminium bag = aluminium foil bag; each duplicate sample weighed 5 ±0.2 g

The storage stability trial was designed as shown in Table 3.3. The powder made from each wall material was packed in two types of packaging materials (aluminium bag and gas-impermeable film), and then stored at two temperatures (25 °C and 55 °C) in incubators (Esco Isotherm, Esco Micro Pte. Ltd, Singapore) for four weeks. Duplicate samples of each treatment were prepared.

Samples were vacuum-packed separately using the modified atmosphere packaging (MAP) equipment (Multivac C300, Multivac, New Zealand) under 2 torr vacuum which was the lowest vacuum level for the equipment. The gas-impermeable film (PET/EVOJ/PE co-ex topweb FOC)

was recommended by the Multivac New Zealand Ltd (Sandra Murphy, personal communication, 23rd, February, 2018) according to the packaging of milk powder. The aluminium foil bags (PET/ AL/ LDPE) (ALFW5-18, PBAG, China) were purchased online (https://www.aliexpress.com/i/472984647.html). Gas-impermeable film was selected because it is inexpensive while the aluminium foil bag was chosen for its excellence in gas-permeability (Tatipata, 2009).

Duplicate samples for each treatment were withdrawn from each incubator at the same time on weekly basis for conducting various analyses. Water activity, cell viability, colour, moisture content and bulk density were determined. The images captured by the scanning electron microscope (FEI Electron Optics, Quanta 200, The Netherlands) were examined to determine the morphology consisting of the particle size and the surface appearance of the DPC16 microcapsule powders.

3.4.5 The effect of encapsulation wall materials and storage conditions on the characteristics of DPC16 microcapsule powders during storage for four weeks

3.4.5.1 Enumeration of microencapsulated *L. reuteri* DPC16 during storage at 25 ℃ and 55 ℃

The viability of microencapsulated *L. reuteri* DPC16 during storage for four weeks at 25 ℃ and 55 ℃ was determined by enumeration on MRS-agar plates as described in section 3.3.6.1.

3.4.5.2 Determination of water activity

Water activity (a_w) of the probiotic powders was measured as described in 3.3.6.2.

3.4.5.3 Measurement of the colour of the DPC16 microcapsule powders

The colour of the spray-dried DPC16 microcapsule powders was measured in triplicate using

the Minolta CR-300 colorimeter (Minolta, Japan) following the manufacturer's instructions (Minolta, n.d.). The colorimeter was equipped with a geometry of 8-mm measuring area, 0° viewing angle and diffuse illumination with d/0°, specular reflection. Before measurement, the colorimeter was calibrated using a standard white calibration plate. The plate was placed on the measuring head which is the tip of the light projection tube of the colorimeter, then the instrument was calibrated by pressing the 'calibrate' button. To measure colour, the experimental powder sample was placed on a supporting glass petri dish with a diameter of 3 cm before being placed on the measuring head. The petri dish together with the light projection tube were covered by a dark box to prevent interference from external light. The colour measurement was initiated by pressing the 'measure' button and triplicate measurements were automatically recorded. The mean value of three colour measurements were calculated automatically and printed out from the instrument, displayed as CIE Yxy, CIE L^* a^* b^*, CIE L^* c^* h, CIE XYZ and Hunter L a b. In this report, the results are reported following the International Commission on Illumination guidelines using the colour values of (CIE) L^* a^* b^*. L^* is lightness from black (0) to white (100). a^* is from green (-) to red (+). b^* is from blue (-) to yellow (+) (International Colour Consortium, 2004).

3.4.5.4 Determination of the moisture of powders

The moisture content (wet basis) of the spray-dried DPC16 microcapsule powders was determined by the hot-air-drying method at 102 ± 2 ℃ to constant weight (Internation Dairy Federation, 1993).

3.4.5.5 Determination of the bulk density of the DPC16 microcapsule powders

The bulk density of the powders, defined as the weight of a specified volume of powder was measured according to the method described by Bae and Lee (2008), with minor modifications. One gram of the probiotic powder was transferred into a 10-mL glass measuring cylinder. The cylinder was tapped on a flat surface by hand to allow the powder to pack to a constant volume for all the samples. Bulk density was calculated by dividing the mass of the powder sample by its volume.

3.4.5.6 Determination of the particle size of the DPC16 microcapsule powders

The particle size of the DPC16 microcapsule powder was determined as described in section 3.3.6.3.

3.4.5.7 Determination of the surface appearance of DPC16 microcapsules

The surface of the probiotic powder particles was examined using the method described in section 3.3.6.3.

3.5 Data analysis

Data were analysed using Minitab statistical software (Minitab 17, Minitab Inc, US). Prior to the analysis, the data were tested for normality using the Shapiro-Wilk test at 95% confidence level. Data on microbial cell counts were $\log_{10}$ transformed for comparative analysis. Raw data and statistical outputs are presented in Appendices 2 to 4.

A. Screening of the optimal conditions for the microencapsulation of DPC16 by spray-drying

The data of EE and water activity were analysed by the Analysis of Variance (ANOVA) using the General Linear Model (GLM) to determine the effect of wall material, batch and inlet/outlet temperatures on the EE of each wall material and water activity of the DPC16 microcapsule powder ($P<0.05$). Significant differences between means were separated using Tukey's Pairwise comparison test (Maciel et al., 2014).

To determine the existence of any significant growth or reduction of DPC16 cells in feed solutions during drying ($P<0.05$), log cell counts/mL at the start and end of the drying process for each wall material solution were analysed by ANOVA-ONEWAY. Significant differences between the mean of log cell counts/ mL at the start and end of the drying process were separated by the Tukey's test ($P<0.05$).

B. Stability of the selected microencapsulated DPC16 powder products during storage

Data were analysed by ANOVA using the GLM to determine the effect of storage temperature,

wall material, packaging material and storage time on cell viability, water activity, colour, moisture content, bulk density and particle size of the powders during storage at 25 ℃ and 55 ℃ for four weeks ($P<0.05$). Tukey's test was used to compare the mean values of the investigated responses at $P<0.05$.

Chapter 4. Screening of optimal wall materials and spray-drying conditions for the encapsulation of *L. reuteri* DPC16 cells

4.1 Introduction

In this chapter, the optimal encapsulation wall materials and spray-drying temperatures for the encapsulation of *L. reuteri* DPC16 were selected. The selection was based on the EE of DPC16 cells, water activity and morphology (including size and surface appearance) of the spray-dried DPC16 microcapsule powders. EE reflects the ability of the wall material to protect cells from high temperatures during spray-drying (Rajam & Anandharamakrishnan, 2015). Water activity significantly affects the survival of microorganisms (Jay et al., 2008). The size, distribution and shape of individual particles in the dehydrated powder influence their flowability, dispersibility, dissolution rate, mouth feel and efficiency of mixing with other ingredients (Dodds, 2013). Particle surface affects function of powder properties such as rehydration, caking, flowability and sticking (Gaiani et al., 2013). The aim of the screening phase of this study was to select the best wall material with high EE, low water activity and optimal powder morphology (size and surface) for further study.

Before spray-drying, cell purity of DPC16 was confirmed by Gram-staining and examination under the microscope. Growth of the DPC16 culture was monitored by the standard plate counts and absorbance$_{595nm}$ readings. A correlation curve between the absorbance and standard plate counts of DPC16 was also created.

4.2 Correlation of the standard plate counts and absorbance readings of grown cultures

A correlation curve of optical density (OD) at 595_{nm} and the concentration of viable cells of DPC16 is shown in Figure 4.1. The data were used to estimate the concentration of viable cells in the wall material colloids before spray-drying. When the OD_{595nm} increased from 0 to 0.5, log viable cell counts (VCC) increased from 5.5 to 8 log CFU/ mL. However, when the OD_{595nm} was between 0.5 and 2.5, the log VCC were between 8 and 9 log CFU/ mL. Thus, to be consistent in the initial cell numbers for encapsulation by spray-drying, all feed solutions were adjusted to OD_{595nm}= 0.5 before being pumped into the drying chamber (Figure 3.1a, Chapter

3), thereby giving an estimated average of 8 log CFU/ mL.

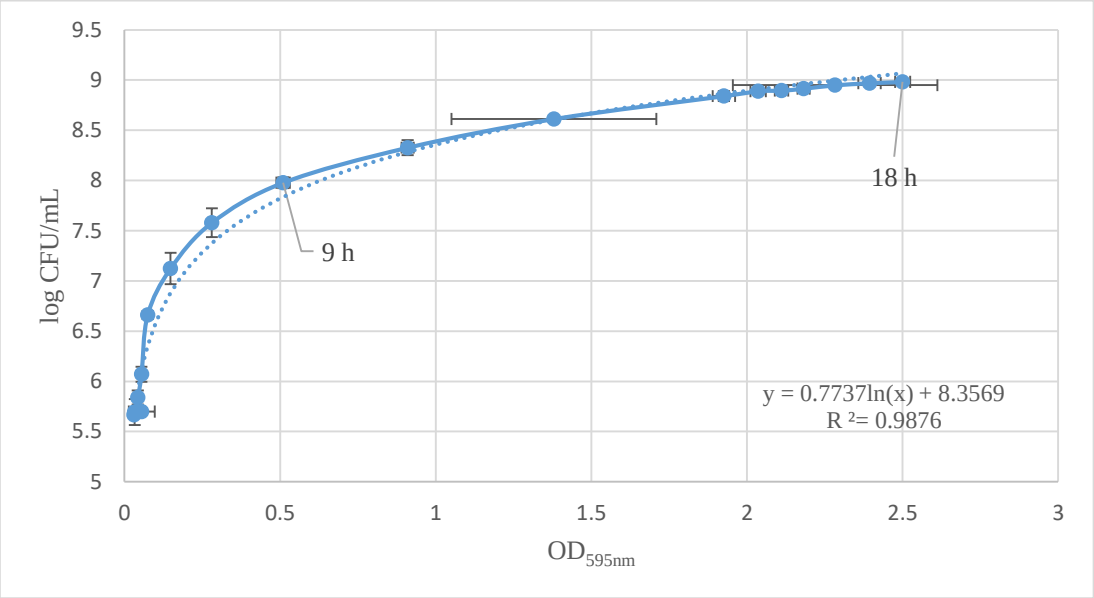


Figure 4. 1 Correlation (R^2 = 0.9876) of OD_{595nm} and the concentration of viable cells of DPC16 during anaerobic incubation at 37 ℃/18 h.

Notes: At 8 h, OD_{595nm} was 0.510±0.021, VCC = 7.98±0.06; At 18 h, OD_{595nm} was 2.500±0.024, VCC was 8.98±0.00; Data points are means of duplicate analyses for both plots for n = 2, with standard deviations expressed as vertical bars for the VCC (log CFU/mL) and horizontal bars for the OD_{595nm}; The mathematical equation with a fitting coefficient R^2 indicated a logarithmic correlation (Appendix 2).

The results show that at 9 h of growth, the log VCC of DPC16 were 7.98±0.06 log CFU/mL at OD_{595nm} = 0.510±0.021. The log VCC were 8.98±0.00 log CFU/mL at OD_{595nm} = 2.500±0.024 at the end of incubation (18 h). These results (correlations) were different from the study by Bian (2008), who reported that the log VCC of DPC16 were 9.00 log CFU/mL at OD_{620nm}= 0.350 at 12 h. In the study by Bian (2008), DPC16 cells began to be grown from 4.00 log CFU/mL, unlike in this study where 5.70 log CFU/mL was the initial log VCC. The discrepancy in the two studies may also be partially explained by differences in the wavelengths used to measure the absorbances. However, the results in the current study were in agreement with Li et al. (2018) who reported VCC of between 8.70 to 9.00 log CFU/mL at an OD_{600nm} of between 0.500 to 2.000 for *L. plantarum* cell suspension.

4.3 Growth of *L. reuteri* DPC16 at 37 ℃ for 18 h

A growth curve of *L. reuteri* DPC16 (Figure 4.2) shows that there was a lag phase of about 5 h followed by an exponential growth phase in the next 7 h. During the exponential phase, the VCC increased from 6.07 ±0.08 to 8.84 ±0.05 log CFU/mL, with OD_{595nm} increasing from 0.055 ±0.005 to 1.926 ±0.036. Thereafter, the growth curve entered the stationary phase which lasted for about 6 h.

This growth curve of *L. reuteri* DPC16 (Figure 4.2) was similar to those of *L. reuteri* CRL1098 and *L. reuteri* MF14-C reported in previous studies (Griet et al., 2018; Hayek et al., 2013). The growth curve (Figure 4.2) based on optical density measurements at 595nm was also similar to the study by Hayek et al. (2013) who reported an exponential phase of 6 h for *L. reuteri* MF14-C when OD_{610nm} increased from 0 to 1.6. A similar trend of growth curve was also reported for *L. acidophilus* cell suspension (Mazzeo et al., 2015). In their study, The VCC increased from 5.00 to 7.00 log CFU/mL as OD_{600nm} increased from 0.000 to 0.500 during incubation for the first 12 h. At OD_{600nm} of between 0.500 and 1.000, the VCC were between 7.00 and 8.00 log CFU/mL. The VCC and absorbance were stable at 8.00 log CFU/mL and 2.000, respectively from the 20th h to the end (the 28th h).

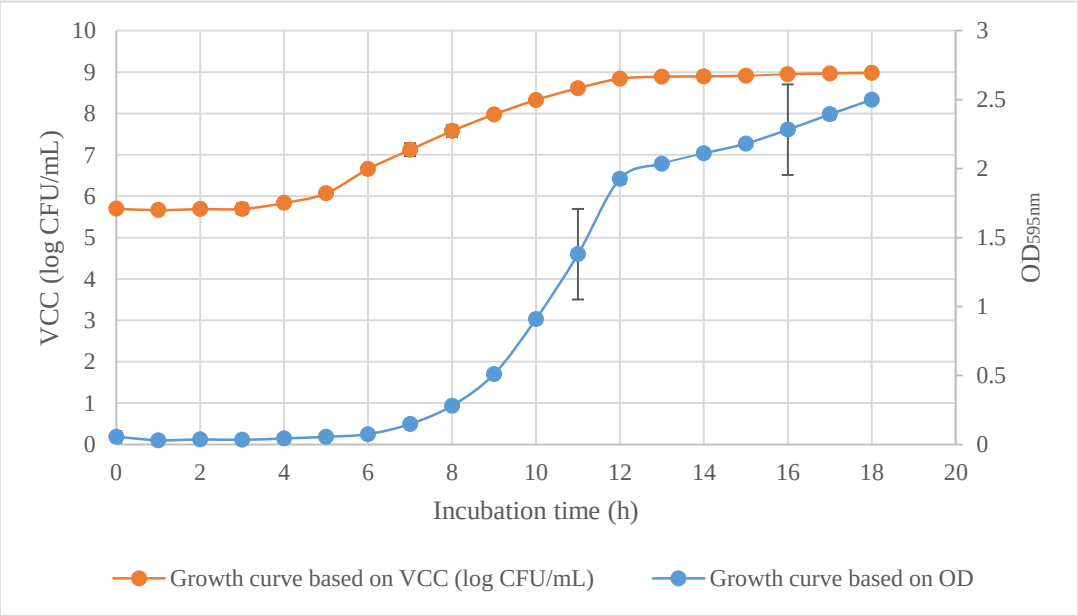


Figure 4. 2 Growth curves of DPC16 in MRS broth during anaerobic incubation at 37 ℃/18 h

Notes: Data points are means of duplicate analyses with standard deviations expressed as vertical bars for the viable cell counts in log CFU/mL.

Figure 4.3 shows the Gram-stains of the DPC16 cell culture used throughout the study. The cells were Gram-positive, curved rods with rounded ends. The cells occurred singly or in pairs, ranging from 2.0 - 5.0 μm long and 0.7-1.0 μm wide. The results were consistent with the description of *L. reuteri* in a previous study (Kandler & Weiss, 1986).

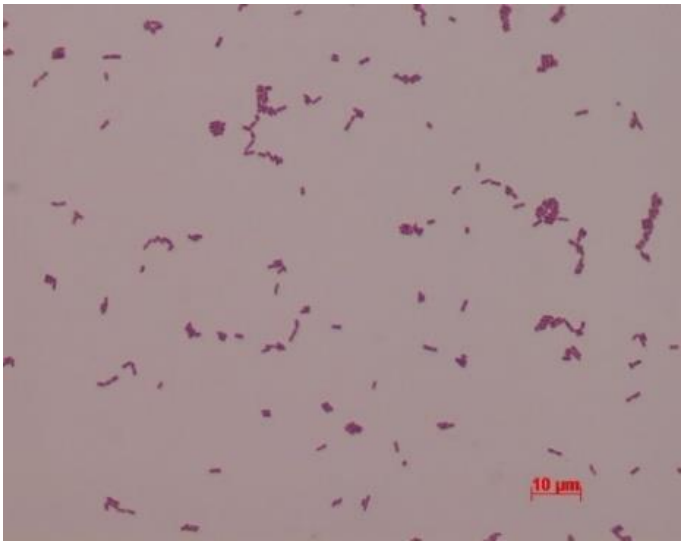


Figure 4. 3 Gram-stained DPC16 (x1000 magnification under oil immersion).

Notes: The image was captured by Axiostar Plus microscope (Axiostar Plus, Zeiss, Germany)

4.4 Concentration of cells in the feed solution (wall material) during the spray-drying process

Table 4.1 shows VCC in the feed solution at different sampling time points during the feeding process during encapsulation by spray-drying. Results showed that cell concentration of DPC16 was stable in feed solutions during the spray-drying processes ($P<0.05$). It was important to reduce any significant differences in the concentrations of encapsulated cells by the wall materials.

Table 4. 1 Viable cell counts (log CFU/mL) in the feed solutions at different sampling time points during the feed process

		Cell concentration in the feed solution (log CFU/mL)			
Batch #	Sampling time	RSM	gum Arabic	Maltodextrin	MWM
1	T_{01}	7.64	7.64	7.56	7.65
1	T_{02}	7.69	7.78	7.68	7.76
1	T_{11}	7.74	7.60	7.54	7.68
1	T_{12}	7.71	7.76	7.69	7.59
2	T_{01}	8.19	8.11	7.60	8.26
2	T_{02}	8.13	8.05	7.65	8.54
2	T_{11}	8.08	8.08	7.74	7.95
2	T_{12}	8.08	8.09	7.64	8.33

Notes: concentrations of all feed solutions were 10% (w/w); RSM = reconstituted skim milk; MWM = 10% mixed wall material (2.5% whey protein isolate/ 2.5% gum Arabic/ 2.5% inulin/ 2.5% sucrose) (w/w). T_{01}: sampling time point at start of spray-drying process; T_{11}: at the end of the spray-drying process; T_{02} and T_{12}: a duplicate sample at start of sampling point and end of a spray-drying process, respectively.

4.5 Encapsulation efficiency

At the inlet/outlet temperatures of 180 ℃/100 ℃, about 84% of DPC16 cells survived when they were encapsulated in 10% RSM (w/w) (Data not shown). However, at the inlet/outlet temperatures of 160 ℃/80 ℃, the EE of all wall materials were above 90.00% (Table 4.2). The RSM had the highest EE (98.06 ±0.86%), followed by the powder made from the MWM with an EE of 93.97 ±1.49%. Maltodextrin and gum Arabic had lower EE at 92.50 ±0.37% and 90.63 ±3.08%, respectively. Results showed that both drying temperature and the type of the wall material affected the EE of a wall material ($P<0.05$). Spray-drying process can cause conformation, dehydration and transition of cells from glassy state to rubbery state (Garcia, 2011). Cell death during spray-drying was reported to be mainly caused by changes of the configuration and profile of cellular lipids in the cell wall and cytoplasmic membrane. The unsaturated fatty acids and lipolysis in cells can be damaged by oxidation (Teixeira et al., 1996). The encapsulation wall material which aims to protect cellular membrane lipids has been reported to be a determinant factor for the survival of probiotic cells due to their differences in thermal conductivity and diffusivity (Lian et al., 2002).

The high EE of all wall materials (>80.00%) were in agreement with previous research. Maltodextrin, RSM and gum Arabic have long been used as wall materials to encapsulate

probiotics and have been proven to be effective (Manojlović et al., 2010; Sohail et al., 2013). In our study, the MWM also had a high EE of 93.97 ±1.49% which was in agreement with Ying et al. (2012) who reported that a combination of protein, prebiotic and small sugar can provide good protection to cells during spray-drying. It could even enhance the cell survival during storage.

Table 4. 2 The Encapsulation efficiency (EE) and water activity of spray dried DPC16 microcapsules

Parameter	Maltodextrin	gum Arabic	RSM	MWM
EE (%)	92.50 ±0.30	90.63 ±3.08	98.06 ±0.86	93.97 ±1.49
Water activity	0.237 ±0.010	0.170 ±0.005	0.284 ±0.005	0.196 ±0.010

Notes: concentrations of all feed solutions were 10% (w/w); RSM = reconstituted skim milk; MWM = mixed wall material.

The EE of maltodextrin for DPC16 was 92.50 ±0.37%, which was much higher than *L. casei* LK-1 (34.60%) reported by Liao et al. (2017) using a lower outlet temperature (70 °C). In this study, maltodextrin as a wall material was less effective than skim milk, which also agreed with the results of Liao et al. (2017). According to Pinto et al. (2015), maltodextrin with low dextrose equivalent (DE) has high glass transition temperature but is unable to replace water molecules because of its high molecular weight. In our study, the DE of maltodextrin was 10 which was relatively low. During the spray-drying process, it is vital to maintain the structure and function of bacterial cell membrane when cells are exposed to high temperature. Maltodextrin is therefore less effective to protect the cells during spray-drying (Pinto et al., 2015). In addition, maltodextrin has low emulsifying ability which can result in the leakage of cells (Cano-Higuita et al., 2015). Maltodextrin also tends to penetrate the cell membrane and is less likely to bind to the cell membrane (Semyonov et al., 2010). The slow deposition of maltodextrin in the chamber of the spray-dryer also contributed to the low EE of maltodextrin because cells were exposed at the inlet of the dryer for longer period (Langrish et al., 2007). Therefore, maltodextrin could be used as a secondary wall material and improve the drying properties of capsules.

The EE of 10% gum Arabic (w/w) was 90.63 ±3.08% at the outlet temperature of 80 °C. It was the only wall material that had significant difference of EE from RSM in this study ($P<0.05$).

The relatively low EE of gum Arabic might be caused by its high viscosity and poor thermo-protective effect on cells at lower outlet temperature (<100 ℃) (Desmond et al., 2002).

The EE of (98.06±0.86%) of 10% RSM (w/w) for DPC16 at 160 ℃/80 ℃ was comparatively higher than for *L. casei* LK-1 (92.90%), when an outlet temperature of 70 ℃ at a flow rate of 320 mL/h was used (Gul, 2017). RSM has been reported to have outstanding protective properties for probiotic bacteria and therefore, it is the most frequently used wall-protective material for microencapsulation of bacteria by spray-drying (Fritzen-Freire et al., 2013; Gul, 2017). The higher survival of DPC16 cells encapsulated in RSM compared to those of gum Arabic and maltodextrin during spray-drying at 160 ℃/80 ℃ might be due to the presence of lactose which can replace cellular water molecules during spray-drying thereby maintaining cell structure (Ying et al., 2012). In addition, compared to gum Arabic which was also added to the MWM, RSM had lower viscosity at the same temperature and concentration (Phillips & Williams, 2000; Schmid & Smith, 1992). Thus, lower viscosity of the encapsulation wall material is preferred because it promotes the atomization of feed solution, thereby protecting cells from thermal effect and dehydration by early crust formation (Anandharamakrishnan & Ishwarya, 2015).

With 10% RSM (w/w) as wall material, the EE decreased greatly at elevated inlet/outlet temperatures of 180 ℃/100 ℃ (83.85±1.50%) compared to the results obtained using 160 ℃/80 ℃ (98.06±0.86%). The drying temperatures were thus important factors for the survival of cells, which was in agreement with previous research (Anandharamakrishnan & Ishwarya, 2015; Würth et al., 2018). The viability of *L. paracasei* F19 cells encapsulated in skim milk concentrate decreased when the drying temperature was increased from 75 ℃ to 155 ℃ (Würth et al., 2018). Increase in the outlet temperature was reported to be more fatal to cell viability (Anandharamakrishnan & Ishwarya, 2015).

For the MWM, the EE of the mixed material (93.97±1.49%). According to Ying et al. (2012), the EE of WPI:inulin:glucose for *L. rhamnosus* GG at an inlet/outlet temperatures of 160 ℃/65 ℃ was between 82.00~88.00%, lower than the results obtained in this study. The high EE in the current study may be caused by the thermal resistance of DPC16. In addition, mixing gum Arabic and other wall materials rather than using the gum alone may improve the EE of probiotic cells (Fazilah et al., 2019).

4. 6 Characteristics of spray-dried *L. reuteri* DPC16 microcapsules

4.6.1 Water activity

Table 4.2 shows that the water activity of DPC16 microcapsules made from different wall materials and spray-dried at 160 ℃/80 ℃ followed the order of: gum Arabic (0.170 ±0.005) < MWM (0.196 ±0.010) < maltodextrin (0.237 ±0.010) < RSM (0.284 ±0.005). Water activity of the DPC16 microcapsules made from RSM and spray-dried at 180 ℃/100 ℃ was 0.200 ±0.004 (data not shown in Table 4.2). Results showed that the wall material and inlet/outlet temperatures were both significant factors to the water activity of the spray-dried DPC16 microcapsules ($P<0.05$).

The sequence of water activity of the powder made from gum Arabic < maltodextrin < skim milk powder at 160 ℃/80 ℃ in this study agreed with the result obtained by Kalušević, Lević, Čalija, Milić, et al. (2017). In the study by Kalušević, Lević, Čalija, Pantić, et al. (2017), the wall materials (gum Arabic, maltodextrin and skim milk powder) were used to encapsulate grape skin extract at inlet/outlet temperatures of 140 ℃/ 65 ℃. Water activity ranged from 0.24 to 0.28 following the order of gum Arabic < maltodextrin < skim milk. However, under the similar spray-drying inlet/outlet temperatures, water activity of the powders made from these three wall materials ranged from 0.31 to 0.33 and no significant differences were found ($P<0.05$) when they (wall materials) were used to encapsulate soybean coat extract (Kalušević, Lević, Čalija, Pantić, et al., 2017).

When powders were produced at the inlet/outlet temperatures of 160 ℃/80 ℃, RSM had the highest EE (98.06 ±0.86%) and DPC16 microcapsule made from this wall material had the highest water activity (0.284 ±0.005) (Table 4.2). For powders containing probiotics, water activity <0.25 is recommended to avoid the mortality of bacteria caused by stimulating metabolism due to higher mobility of water molecules (Albadran et al., 2015). However, RSM microcapsules produced in this study were higher than this range. The higher water activity of RSM microcapsules than microcapsules made from gum Arabic and MWM (which also contained gum Arabic) produced at 160 ℃/80 ℃ might be related to the viscosity of the feed solutions. Gum Arabic can increase the viscosity of the feed solution which leads to difficulties in atomization (Anandharamakrishnan & Ishwarya, 2015; Schmidt & Smith, 1992). Thus, our spray-dryer (Saurin SL-10) produced the gum Arabic microcapsules and MWM microcapsules

with at a low flow rate with inlet/outlet temperatures (160 ℃/80 ℃). The decrease in mass flow rate reduces water content in the powders produced due to presence of gum Arabic and MWM droplets at the inlet of the dryer (160 ℃) for a longer time leading to more evaporation of water. The water activity of RSM microcapsules was also higher than the maltodextrin microcapsules, although maltodextrin does not have a relatively higher viscosity (Dokic et al., 1998). This might be caused by the slower deposition of maltodextrin than RSM in the spray-drying chamber which leads to longer drying time of a particle. Langrish et al. (2007) reported that the deposition process is likely to be initiated by the adhesion of particles on the clean surface of the spray-drying chamber. After the chamber is covered by particles, cohesion of particles onto other particles is likely to happen, which control the process of deposition. However, for maltodextrin, the cohesion of particles occurs more rapidly than adhesion of particles to the chamber, thus the deposition (adhesion) of maltodextrin is slow because of a lack of particles on the wall of the chamber.

The water activity of the DPC16 microcapsule powder made from the MWM (10%, w/w) was 0.196±0.010, lower than that of the powder made from skim milk, 0.284±0.005 (10%, w/w). Except for higher viscosity caused by the presence of gum Arabic, the low water activity was probably also attributed to the presence of inulin and sucrose (Avila-Reyes et al., 2014). The shorter chains of inulin and higher number of hydroxyls can bind water molecules that are not removed during the spray-drying process which lower the water activity of powder made from the MWM (Avila-Reyes et al., 2014). Sucrose and sodium chloride are the most commonly used osmotic agents which decrease the water activity of food (Kim & Toledo, 1987).

The water activity of powder made from RSM and spray-dried at 180 ℃/100 ℃ was 0.200±0.004, lower than the sample spray-dried at 160 ℃/80 ℃ (0.284±0.005). This result suggested that the water activity of the powder was related to the drying temperatures. An increase in the inlet/outlet temperatures decreased the water activity ($P<0.05$) which agreed with previous studies (Baysan et al., 2019; Teanpaisan et al., 2012). Water activity is vitally important for the quality of powder during storage because it affects the rate of physical deterioration such as caking and stickiness (Hedegaard & Skibsted, 2013). The reactions such as non-enzymatic browning and the oxidation of lipid and protein in cell membrane which lead to cell death are also based on water activity (Hedegaard & Skibsted, 2013). Thus, low water activity of DPC16 microcapsules is desirable for the storage of powders.

4.6.2 Morphology

For the powders in different treatments, both particle size and particle surface were measured/observed. The particle size of DPC16 microcapsules made from different wall materials (RSM, gum Arabic, Maltodextrin and MWM) ranged from 3.59±0.21 μm to 3.79±0.30 μm (Appendix 2, Table 2.5). However, results showed that there were no significant differences in particle sizes of powders made from different wall materials ($P<0.05$), which partially agreed with previous studies. According to Arslan et al. (2015), the mean diameter of powder particles made from gum Arabic, maltodextrin, and whey protein concentrate were not significantly different.

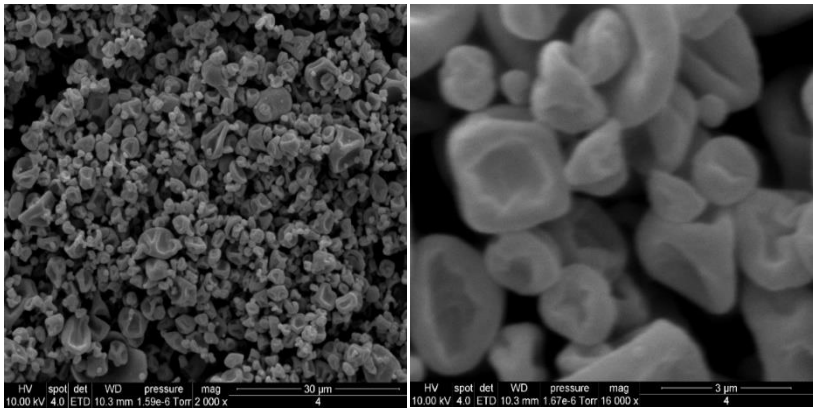


Figure 4. 4 Scanned electron micrograph of DPC16 microcapsules made from 10% skim milk (w/w) and spray-dried at inlet/outlet temperatures of 160 ℃/80 ℃

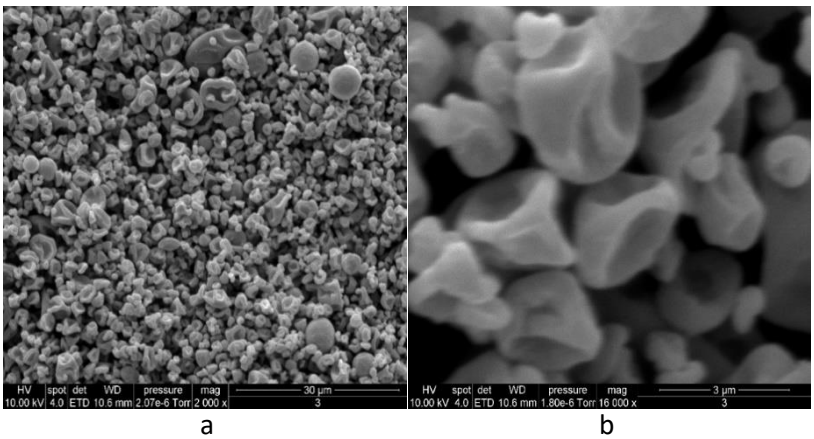
Notes: (a) at ×2,000 magnification, (b) at ×16,000 magnification; The scanned electron micrographs were captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

Description of the particle size of DPC16 microcapsules made from RSM and spray-dried at 160 ℃***/80*** ℃

The the microcapsules of *L. reuteri* DPC16 made from 10% RSM (w/w) and spray-dried at 160 ℃/80 ℃ were evenly distributed. The average particle size was 3.59±0.21 μm, which was much smaller than the results (5 to 15 μm) reported by Desmond et al. (2002) using 20% RSM (w/v) by spray-drying at an inlet/outlet temperatures of 170 ℃/95 ℃. The smaller particle size in this study could have been caused by the reduction in milk concentration in the feed solution during encapsulation by spray-drying (Elversson & Millqvist-Fureby, 2005).

Description of the surface of DPC16 microcapsules made from RSM and spray-dried at 160 ℃***/80*** ℃

As is shown in Figure 4.4, particles were irregular or spherical in shape with variable sizes. The results were in agreement with the study by Desmond et al. (2002). The particles had a wrinkled surface as they were covered by protein (Xu et al., 2012). According to previous studies, the dense surfaces containing high protein content are formed during drying because of the migration of surface-active protein (Fyfe et al., 2011; Shrestha et al., 2007).



a b

Figure 4. 5 Scanned electron micrograph of DPC16 microcapsules made from 10% skim milk (w/w) and spray-dried at inlet/outlet temperatures of 180 ℃/100 ℃

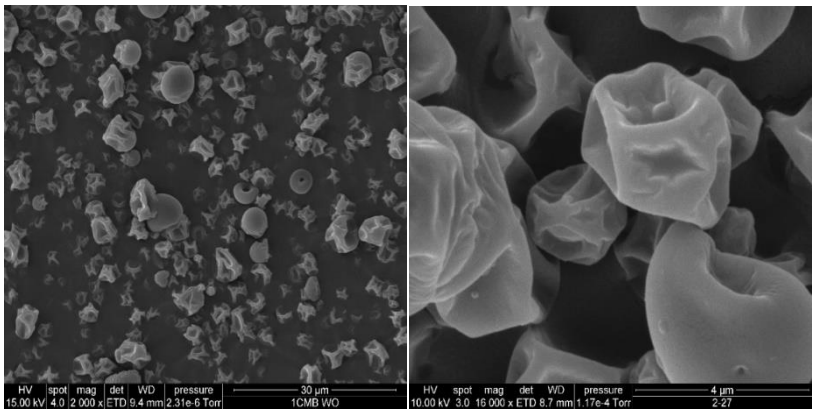
Notes: (a) at ×2,000 magnification, (b) at ×16,000 magnification

Description of the particle size of DPC16 microcapsules made from RSM and spray-dried at 180 ℃/100 ℃

Figure 4.5 is a typical image of *L. reuteri* DPC16 microcapsules made from 10% RSM (w/w) and spray-dried at inlet/outlet temperatures of 180 ℃/100 ℃. The sizes were evenly distributed (3.06±0.11 μm) but smaller compared to the microcapsules spray-dried at 160 ℃/80 ℃ (3.59±0.21 μm) ($P<0.05$). This was in contrast to previous research which reported that the particle size did not change or increased as the inlet/outlet temperatures increased (Maas et al., 2011; Park et al., 2016). The smaller particle size of RSM microcapsules spray-dried at higher inlet/outlet temperatures was probably attributed to a slower, although fluctuating, flow rate of our spray dryer when inlet/outlet temperatures were fixed at higher levels. More water was removed from droplets at the inlet of the dryer while the water content left in powder particles might affect particle size of RSM, the sugar-rich powder (Barbosa-Cánovas et al., 2005).

Description of the surface of DPC16 microcapsules made from RSM and spray-dried at 180 ℃/100 ℃

The particle surface of powders made from RSM and spray-dried at the inlet/outlet temperatures of 180 ℃/100 ℃ was similar as the one produced at 160 ℃/80 ℃. This was in contrast to previous research by Maa et al. (1997), who reported increase of dents and cavities on particle surface and decrease of spherical particles as the outlet temperature increased.



a b

Figure 4. 6 Scanned electron micrograph of DPC16 microcapsules made from the MWM and spray-dried at inlet/outlet temperatures of 160 ℃/80 ℃

Notes: (a) at ×2,000 magnification, (b) at ×16,000 magnification

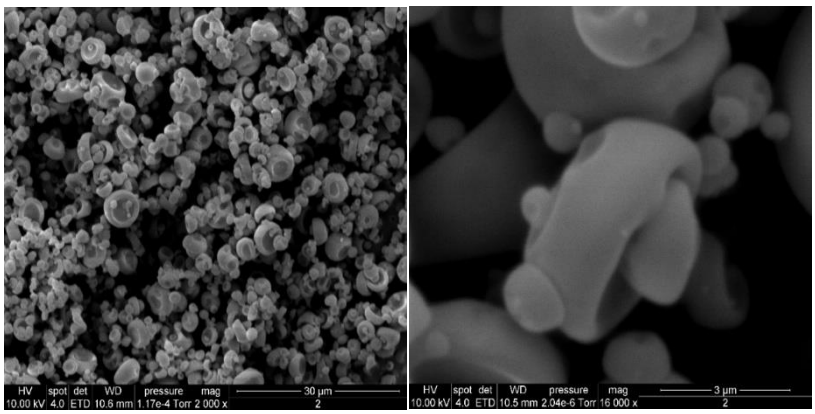
Description of the particle size of DPC16 microcapsules made from the MWM and spray-dried at 160 ℃***/80*** ℃

Figure 4.6 shows the typical morphology of *L. reuteri* DPC16 microcapsules made from the MWM (2.5% whey protein isolate/ 2.5% gum Arabic/ 2.5% inulin/ 2.5% sucrose). The particle sizes were not evenly distributed, and the average particle size was 3.62 ±0.23 μm. However, Arslan et al. (2015) reported that the encapsulated probiotic powder made from 20 % (w/v) whey protein concentrate had a mean surface diameter of 3.31 μm after spray-drying at an inlet temperature of 125 ℃. The addition of sucrose may increase the particle size according to Lay Ma et al. (2008).

Description of the surface of DPC16 microcapsules made from the MWM and spray-dried at 160 ℃***/80*** ℃

Most microcapsules made from the MWM (Figure 4.6) were wrinkled with dents or concavities on the surfaces, which was similar as the surface of powders made from RSM (Figure 4.4). This was probably due to the existence of whey protein isolate in the MWM (Maciel et al., 2014). The morphology of probiotic microcapsules made from skim milk was similar to those made from sweet whey (Maciel et al., 2014). In the MWM, there were also gum Arabic, inulin and sucrose. Adding inulin into the feed solution did not appear to affect the morphology of spray-dried powders (Fritzen-Freire et al., 2012). However, results showed that the morphology of spray-dried powders was affected by the addition of gum Arabic (Fazilah et al., 2019). Among the microcapsules made from the MWM, there were also doughnut-like particles with smooth surface and few dents or roughness, which were presumed to be particles consisting of gum Arabic as reported by (Fazilah et al., 2019). The study reported that a combination of wall material involving gum Arabic contributed to smoother particle surface compared to using gum Arabic alone, which was also observed in the current study (Fazilah et al., 2019; Lian et al., 2002).

The powder made from the MWM had fewer large particles (Figure 4.6) than the powder made from RSM (Figure 4.4), probably due to their difference in the amount of protein. The diameter of microcapsules made from milk protein concentrate increased as the protein content increased during storage at 25 ℃ and 40 ℃ (Babu et al., 2018).



a b

Figure 4. 7 Scanned electron micrograph of DPC16 microcapsules made from 10 % gum Arabic (w/w) and spray-dried at inlet/outlet temperatures of 160 ℃/80 ℃

Notes: (a) at ×2,000 magnification, (b) at ×16,000 magnification

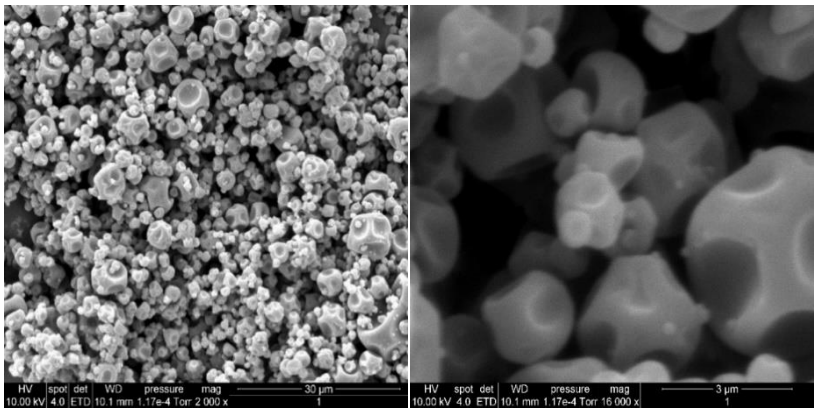
Description of the particle size of DPC16 microcapsules made from gum Arabic and spray-dried at 160 ℃***/80*** ℃

Figure 4.7 shows the morphology of *L. reuteri* DPC16 microcapsules made from 10 % gum Arabic (w/w). The sizes of powder particles were evenly distributed with an average size of 3.79 ±0.30 µm, which was in contrast with previous studies that reported large variable sizes of particles. Microcapsules made from both milk and gum Arabic were spherical with sizes above 5 µm on average (Desmond et al., 2002).

Description of the surface of DPC16 microcapsules made from gum Arabic and spray-dried at 160 ℃***/80*** ℃

The surface of the microcapsules made from skim milk were wrinkled (Figure 4.4), but the surface of the doughnut-shape particles made from gum Arabic was relatively smooth with dents (Figure 4.7). Similar results were also reported by other studies (Bhusari et al., 2014; Ferrari et al., 2012; Rascón et al., 2011). The dents were most likely caused by high water evaporation rate during spray-drying resulting in the shrinkage of particles (Kuck & Noreña, 2016).

Compared to the doughnut-like particles made from the MWM (Figure 4.6), the DPC16 microcapsules made from gum Arabic alone as wall material had more dents on the surface. Similar phenomenon was also reported by previous authors. Fazilah et al. (2019) reported that a combination of gum Arabic and other ingredients as wall material can produce smooth-surface particles. Similar results were also reported by other authors (Bhusari et al., 2014; Ferrari et al., 2012; Rascón et al., 2011).



a b

Figure 4. 8 Scanned electron micrograph of DPC16 microcapsules made from 10 % maltodextrin (w/w) and spray-dried at inlet/outlet temperatures of 160 ℃/80 ℃

Notes. (a) at ×2,000 magnification, (b) at ×16,000 magnification

Description of the particle size of DPC16 microcapsules made from maltodextrin and spray-dried at 160 ℃***/80*** ℃

Figure 4.8 shows the typical morphology of spray-dried DPC16 microcapsules made from 10% maltodextrin (w/w). The particle sizes were not evenly distributed, and the average particle size was 3.59±0.21 µm. Similarly, according to Arslan et al. (2015), encapsulated *Saccharomyces cerevisiae* var. *boulardii* powder made from 20 % maltodextrin (w/v) and spray-dried at the inlet temperatures of 125 ℃ had a mean surface diameter of 3.47 µm.

Description of the surface of DPC16 microcapsules made from maltodextrin and spray-dried at 160 ℃***/80*** ℃

The surface of DPC16 microcapsules made from maltodextrin was similar to results reported by Rodríguez-Huezo et al. (2007). Numerous dents were observed on the surface of most particles and even small particles had wrinkled surfaces. Powder particles with dents were reported to be difficult to dissolve (Reyes et al., 2018), thus microcapsules with smooth surfaces are desirable.

4.7 Summary

In this chapter, the EE, water activity and morphology of DPC16 microcapsules in different treatments were investigated. *L. reuteri* DPC16 cells were encapsulated using four wall materials (RSM, gum Arabic, maltodextrin, MWM comprising whey protein isolate/gum Arabic/inulin/sucrose at the ratio of 1:1:1:1) by spray-drying at the inlet/outlet temperatures of 160 ℃/80 ℃. The performance of skim milk powder alone was also investigated at higher inlet/out temperatures (180 ℃/100 ℃).

The encapsulation of *L. reuteri* DPC16 using 10% RSM (w/w) by spray-drying at elevated inlet/outlet temperatures (180 ℃/100 ℃) resulted in lower EE, water activity and smaller particle size of the microcapsules compared to RSM microcapsules spray-dried at 160 ℃/80 ℃ ($P<0.05$). At lower inlet/outlet temperatures (160 ℃/80 ℃), RSM had the highest EE, and highest water activity of the spray-dried microcapsules which was not desirable. Gum Arabic microcapsules had the lowest results for both EE and water activity ($P<0.05$). No significant differences ($P<0.05$) in particle sizes were found among DPC16 microcapsules made from all wall materials and most of the powder particles had various sizes with some concavities. However, the MWM comprising gum Arabic, whey protein isolate, inulin and sucrose produced microcapsules with relatively high EE, low water activity and some microcapsules with smoother surfaces which could improve powder solubility. Thus, RSM and the MWM were selected as wall materials for the stability tests during storage.

Chapter 5 Storage stability of spray-dried DPC16 microcapsule powders

5.1 Overview

The two selected encapsulation wall materials (RSM and MWM), two vacuum-packaging materials (gas-impermeable film and aluminium foil bags), and two storage temperatures (25 ℃ and 55 ℃) were investigated for their ability to maintain the viability of spray-dried encapsulated DPC16 cells during storage for four weeks. The results of the storage trials are discussed in this chapter.

5.2 Survival of spray-dried encapsulated *L. reuteri* DPC16 during storage at 25 ℃ and 55 ℃

Table 5.1a shows viable cell counts of DPC16 in microcapsule powders during storage for four weeks at 25 ℃. Powders made from RSM had nearly similar initial cell counts (8.71±0.15 log CFU/g) than powders made from MWM (8.24±0.07 log CFU/g). The cell counts in powders of all treatments decreased gradually during storage for four weeks (25 ℃). For the packaging material, spray-dried encapsulated cells packed in aluminium foil bags were relatively stable compared to cell survival in powders packed in gas-impermeable film during storage at 25 ℃. Regarding the encapsulation wall materials, survival of cells preserved in RSM was always higher but the degree of the decrease in cell number was comparable to cells encapsulated in MWM. These two encapsulation wall materials were both made up of protein and small sugar with the latter containing inulin and gum Arabic. According to previous studies, inulin and gum Arabic enhanced cell viability during storage (Corcoran et al., 2004; Desmond et al., 2002; Maciel et al., 2014). Results showed that storage time, type of encapsulation wall materials and packaging materials all had significant effects on cell survival during storage at 25 ℃ ($P<0.05$).

Table 5.1a Viable cell counts (log CFU/g) of spray-dried encapsulated *L. reuteri* DPC16 during storage for four weeks at 25 ℃

Treatment	Storage period (weeks)				
	0	1	2	3	4
RSM film	8.71±0.15	8.62±0.07	8.54±0.14	8.40±0.14	8.13±0.11
MWM film	8.21±0.07	8.38±0.36	7.90±0.14	7.57±0.24	7.18±0.29
RSM alu	8.71±0.15	8.56±0.15	8.64±0.14	8.47±0.10	8.47±0.10
MWM alu	8.21±0.07	7.97±0.18	8.10±0.12	7.81±0.18	7.98±0.15

Table 5.1b Viable cell counts (log CFU/g) of spray-dried encapsulated *L. reuteri* DPC16 during storage for four weeks at 55 ℃

Treatment	Storage period (weeks)				
	0	1	2	3	4
RSM film	8.71±0.15	4.86±0.61	3.68±0.37	3.26±0.07	3.05±0.17
MWM film	8.21±0.07	2.85±0.61	2.55±0.54	1.93±0.79	1.81±0.78
RSM alu	8.71±0.15	3.34±0.29	3.06±0.20	2.96±0.19	1.78±0.34
MWM alu	8.21±0.07	1.86±0.42	1.31±0.76	1.31±0.76	1.24±0.75

Notes.
n = 2 replications with duplicate analysis
± = standard error of mean
RSM film = To encapsulate DPC16 cells in reconstituted skim milk by spray-drying at 160 ℃/80 ℃ (inlet/outlet temperatures) and vacuum-pack the powder in gas-impermeable film
MWM film = To encapsulate DPC16 cells in mixed wall material by spray-drying at 160 ℃/80 ℃ (inlet/outlet temperatures) and vacuum-pack the powder in gas-impermeable film
RSM alu = To encapsulate DPC16 cells in reconstituted skim milk by spray-drying at 160 ℃/80 ℃ (inlet/outlet temperatures) and vacuum-pack the powder in the aluminium foil bag
MWM alu = To encapsulate DPC16 cells in mixed wall material by spray-drying at 160 ℃/80 ℃ (inlet/outlet temperatures) and vacuum-pack the powder in the aluminium foil bag

The initial cell counts in the MWM were lower than that in the RSM. However, during storage, the VCC of DPC16 encapsulated in the two wall materials had comparable reductions of the cells. According to previous studies, MWM which consisted of whey protein: gum Arabic: inulin: sucrose was expected to have less decrease in cell numbers during storage (Corcoran et al., 2004; Desmond et al., 2002; Maciel et al., 2014). As an encapsulation wall material, sweet whey was less effective in preserving microencapsulated cells than RSM when stored at 4 ℃ or 25 ℃ (Maciel et al. (2014). The addition of gum Arabic into skim milk as an encapsulation wall material for spray-drying significantly improved cell viability during storage at either 15 ℃ or 30 ℃ (Desmond et al., 2002), especially at outlet temperatures between 95 and 105 ℃, with

powders packed in sealed polythene bags. The cells were probably protected from oxidative stress due to the formation of a semipermeable wall with gum Arabic (Desmond et al., 2002). The addition of inulin was also expected to have positive effect on cell preservation especially at high temperature storage (37 ℃) in terms of Corcoran et al. (2004). Inulin protects plants from harsh environments such as droughts and frost and can directly interact with membrane lipids (Hincha et al., 2000; Hincha et al., 2002). The addition of sucrose to the wall-material formulation did not show obvious advantage in protecting cells at both storage temperatures in the current study. According to Agudelo et al. (2017), the addition of sucrose to WPI as a wall material for probiotic encapsulation had no influence on cell viability at water activity levels higher than 0.11.

Irrespective of the encapsulation wall material, vacuum-packaging of the DPC16 microcapsule powders in aluminium foil bags provided better protection to DPC16 cells than gas-impermeable film during storage at 25 ℃ ($P<0.05$). Cells encapsulated in both wall materials decreased by < 0.5 logs by the end of storage (Table 5.1a). For powders packed in gas-impermeable film, the decrease in cell numbers was lower when cells were encapsulated in RSM (0.5 log) than in MWM (nearly 1 log) after four weeks at 25 ℃. Higher survival of DPC16 in RSM microcapsules was probably attributed to the presence of large amounts of lactose in RSM which maintained the cell structure (Ying, 2012).

Table 5.1b shows viable cells of DPC16 in microcapsule powders during storage for four weeks at 55 ℃. Storage temperature, encapsulation wall material, packaging material and storage time all had significant effects on cell viability ($P<0.05$). Cells encapsulated in RSM and vacuum-packed in gas-impermeable film maintained higher VCC during storage at 55 ℃ compared to the other treatments. For this treatment, cell counts decreased from 8.71±0.15 to 3.05±0.17 log CFU/g. The results for RSM as the encapsulation wall material with aluminium-bag packaging and MWM with gas-impermeable-film packaging were comparable (Table 5.1b). In these conditions, cells decreased sharply by 5 logs during the first week, then gradually decreased by more than 1 log during elevated-storage temperature (55 ℃). The recovery of DPC16 cells was the lowest when the cells were encapsulated in MWM then vacuum-packed in an aluminium foil bag. Viable cells of this treatment decreased by more than 6 logs within one week and continued decreasing to the end of the storage trial.

The survival of DPC16 cells was markedly higher at 25 ℃ than 55 ℃. The storage temperature was a significant factor that affected the survival of bacterial cells during long-term storage

(P<0.05). Our finding is also supported by other studies which reported that temperature was an important factor for the stability of microorganisms (Agudelo et al., 2017; Ying et al., 2012). According to Teixeira et al. (1996), cell death during storage can be caused by the oxidation of fatty acids in the cellular membrane lipids. The cellular membrane lipids can react with oxygen, light, and moisture especially at high storage temperatures (Morgan et al., 2006).

For probiotics to function, the VCC should be above 10^6 CFU/mL or g in the products at the time of consumption (FAO/WHO, 2003). Therefore, it is important to reduce the loss of cells during spray-drying and storage (Fritzen-Freire et al., 2012). The decrease in cell viability during spray-drying and storage can be severely affected by the water properties of the wall-material solution in which the cells are dried, as well as the temperature (Gardiner et al., 2000; Selmer-Olsen et al., 1999). In this study, during storage at 25 ℃, cell viability was above 10^6 CFU/g in all treatments after four weeks.

5.3 Water activity of spray-dried *L. reuteri* DPC16 microcapsule powders during storage at 25 ℃ and 55 ℃

The water activity of powders stored at 25 ℃ (Table 5.2a) were relatively stable compared to powders stored at 55 ℃ (Table 5.2b), despite some differences in the packaging materials. For vacuum-packed powders in the gas-impermeable film, the water activity levels slightly increased during storage while powders vacuum-packed in aluminium foil bags were stable. The increased water activity was undesirable because it could trigger the oxidation of cellular lipids during storage, leading to the decrease in VCC of DPC16 microcapsules packed in gas-impermeable film during storage at 25 ℃ (Table 5.1a) (Castro et al., 1996).

Table 5.2a Water activity of *L. reuteri* DPC16 microcapsule powders during storage for four weeks at 25 ℃

	Storage period (weeks)				
Treatment	0	1	2	3	4
RSM film	0.268±0.005	0.297±0.008	0.329±0.011	0.299±0.013	0.346±0.010
MWM film	0.239±0.003	0.295±0.008	0.311±0.006	0.274±0.006	0.352±0.011
RSM alu	0.268±0.005	0.236±0.007	0.211±0.013	0.214±0.007	0.222±0.006
MWM alu	0.239±0.003	0.251±0.008	0.226±0.020	0.207±0.007	0.222±0.006

Table 5.2b Water activity of *L. reuteri* DPC16 microcapsule powders during storage for four weeks at 55 ℃

	Storage period (weeks)				
Treatment	0	1	2	3	4
RSM film	0.268±0.005	0.145±0.010	0.139±0.012	0.083±0.007	0.102±0.007
MWM film	0.239±0.003	0.099±0.001	0.091±0.002	0.066±0.002	0.087±0.08
RSM alu	0.268±0.005	0.209±0.006	0.199±0.016	0.193±0.015	0.197±0.014
MWM alu	0.239±0.003	0.203±0.004	0.198±0.006	0.208±0.012	0.219±0.019

Notes.
n = 2 replications with duplicate analysis
± = standard error of mean
RSM film = To encapsulate DPC16 cells in reconstituted skim milk by spray-drying at 160 ℃/80 ℃ (inlet/outlet temperatures) and vacuum-pack the powder in gas-impermeable film
MWM film = To encapsulate DPC16 cells in mixed wall material by spray-drying at 160 ℃/80 ℃ (inlet/outlet temperatures) and vacuum-pack the powder in gas-impermeable film
RSM alu = To encapsulate DPC16 cells in reconstituted skim milk by spray-drying at 160 ℃/80 ℃ (inlet/outlet temperatures) and vacuum-pack the powder in the aluminium foil bag
MWM alu = To encapsulate DPC16 cells in mixed wall material by spray-drying at 160 ℃/80 ℃ (inlet/outlet temperatures) and vacuum-pack the powder in the aluminium foil bag

During storage at 55 ℃, the water activity of powders in all treatments decreased during the first week, then remained stable (Table 5.2b). For powders packed in gas-impermeable film, the water activity decreased sharply during the first week from about 0.250 to nearly 0.100 irrespective of the encapsulation wall material used. However, the decrease of water activity of powders vacuum-packed in aluminium foil bags was low (0.250 to 0.200) during the first week.

Packaging materials and storage temperatures had significant impact on water activity of powders at both storage temperatures ($P<0.05$). The effect of storage temperature on water activity agreed with the study by Teixeira et al. (1995) who reported reduction in water activity with increased storage temperature.

The aluminium foil bag used in this study contributed to the maintenance of the water activity of milk-based probiotic powder at about 0.20, which was desirable because as stated in the last chapter, water activity of 0.25 significantly increase the mortality rate of bacteria (Albadran et al., 2015). However, during storage at 55 ℃, the survival of encapsulated cells packed in aluminium foil bags decreased markedly (Table 5.1b) although the water activity remained low (Table 5.2b). This was initially suspected to be caused by the collapse of the powder. According to Ghandi et al. (2013), water activity, glass transition temperature of the powder and storage

temperature all affect the survival of encapsulated cells. These three factors affect the physical state of the powder and glassy state is preferred by the encapsulated cells. The water activity of RSM microcapsules should be below 0.2 to mitigate the effect of 40 ℃ (Ghandi et al., 2013). Thus, at higher storage temperature (55 ℃) used in this study, the water activity should be lower to maintain the glassy state of powders (Kurtmann, Carlsen, Skibsted, et al., 2009). Even though they did not collapse, they might still have the tendency to collapse (Thomsen et al., 2003). According to the results of the morphology (Figure 11, 12, 15, 16, section 5.5), the powder particles stored at high temperature did not collapse probably due to the low water activity. Thus, the death of the cells encapsulated in RSM and MWM was more likely to be caused by Maillard reactions (discussed in the next section).

The survival of cells encapsulated in RSM and packed in gas-impermeable film decreased slightly compared to cells encapsulated in RSM and packed in the aluminium foil bag (Table 5.1b). This could be attributed to the low water activity of the powders of this treatment which was likely to have maintained a better glassy state of the powder (Table 5.2b). The higher cell survival of encapsulated DPC16 in RSM than in MWM was probably related to the presence of higher amounts of small sugars in RSM (≈ 50%) than in MWM (25%) ("Chapter 3 Lactose content of milk and milk products," 1988; Ying et al., 2012). The presence of small sugars is critical to maintaining the cell structure except for maintaining glassy state during dehydration because it can replace water molecules in the cell membrane (Ying et al., 2012).

5.4 The colour of spray-dried *L. reuteri* DPC16 microcapsule powders during storage at 25 ℃ and 55 ℃

The colour of powders was presented using CIE *L*a*b** system with *L** (whiteness) representing lightness from black (0) to white (100), *a** (redness), from green (-) to red (+), and *b** (yellowness) from blue (-) to yellow (+) (International Colour Consortium, 2004). There was a significant decrease in the lightness and stable redness of powders in all treatments. However, the yellowness of the powders made from MWM and stored at 55 ℃ increased, especially for the samples packed in aluminium foil bags.

The lightness for all the spray-dried powders was high at the beginning of the storage period (Figure 5.1a, Figure 5.1b). The lightness of MWM was about 96 while that of the RSM was

about 95. The results were comparable to the study by Fritzen-Freire et al. (2012) on bifidobacteria microcapsule powder made from RSM and skim milk with inulin.

The lightness of the powders decreased for all samples during storage at the two temperatures (Figure 5.1a, Figure 5.1b). This suggests Maillard reactions taking place in the samples during storage as both encapsulation wall materials contained protein and reducing sugar (Barbosa-Cánovas et al., 2005).

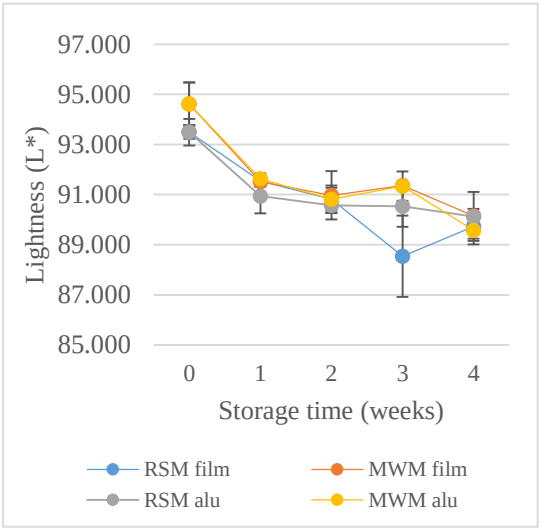


Figure 5.1a Lightness (L*) of DPC16 microcapsule powders during storage for four weeks at 25 ℃

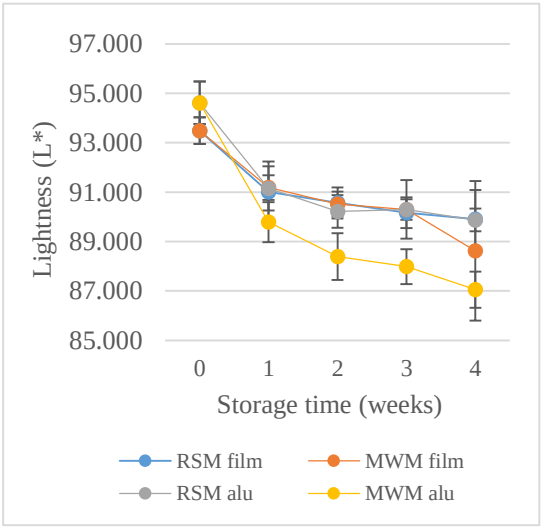


Figure 5.1b Lightness (L*) of DPC16 microcapsule powders during storage for four weeks at 55 ℃

Notes.
n = 2 replications with duplicate analysis
± = standard error bars
RSM film = Lightness of DPC16 microcapsule powder made from reconstituted skim milk and vacuum-packed in gas-impermeable film
MWM film = Lightness of DPC16 microcapsule powder made from mixed wall material and vacuum-packed in gas-impermeable film
RSM alu = Lightness of DPC16 microcapsule powder made from reconstituted skim milk and vacuum-packed in an aluminium foil bag
MWM alu = Lightness of DPC16 microcapsule powder made from mixed wall material and vacuum-packed in an aluminium foil bag

Figure 5.1a shows a change in the lightness of powders during storage at 25 ℃. Encapsulation wall material and time both had significant effects on the lightness of powders ($P<0.05$). Generally, despite differences in the initial lightness, the trend of darkening phenomenon in each powder was similar. The lightness in the powders made from both RSM and MWM decreased continuously during the four-week storage time. The decrease in the lightness was slightly higher during the first week than the last three weeks of storage period. This trend was slightly different from previous work. According to Neves et al. (2019), the lightness of skim milk powder remained stable at 94 during storage at 25 ℃ for 4 weeks. The differences might be caused by the different packaging materials used. In the study by Neves et al. (2019), powders were sealed without a vacuum in polypropylene bags, which left more space between powder particles thereby minimising Maillard reactions.

Figure 5.1b shows changes in lightness of powders during storage at 55 ℃. Results showed that storage temperature, encapsulation wall materials, packaging materials, and storage time all had significant effect on the lightness of powders ($P<0.05$). In all the treatments ($n=4$) stored at 55 ℃, the powders darkened within the first week. However, except the powder made from MWM (vacuum-packed in the aluminium foil bag), the lightness of all other powders decreased slightly during the last three weeks of storage. The decrease of lightness of the powder suggested the occurrence of Maillard reactions (Tan et al., 2012). At elevated storage temperature (55 ℃), the lightness of MWM microcapsules packed in aluminium foil bags decreased more than the MWM microcapsules packed in gas-impermeable film and RSM microcapsules irrespective of the type of packaging. This was possibly attributed to the higher thermal conductivity of aluminium foil bags which accumulated heat inside the package resulting in the Maillard reactions of MWM. Although an increase in temperature may lead to the increase in the thermal conductivity of the plastic materials, plastics are poor heat conductors (Young et al., 1996). The presence of whey protein and gum Arabic in MWM and high water activity of powder in aluminium foil bags could all have contributed to the Maillard reactions and the decrease in lightness of powders (Oliveira et al., 2016). Maillard reactions were possible in MWM microcapsules due to the presence of free reducing oligosaccharides in gum Arabic (Tischer et al., 2000). Previous studies reported that Maillard reactions were used to induce conjugation of proteins with polysaccharides (Pirestani et al., 2016; Zha et al., 2019).

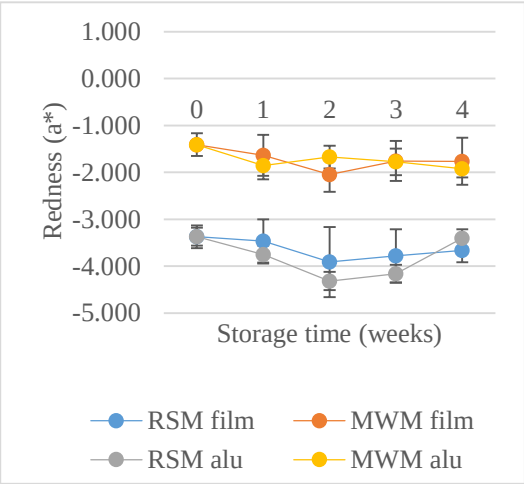


Figure 5.2a Redness (a*) of DPC16 microcapsule powders during storage for four weeks at 25 ℃

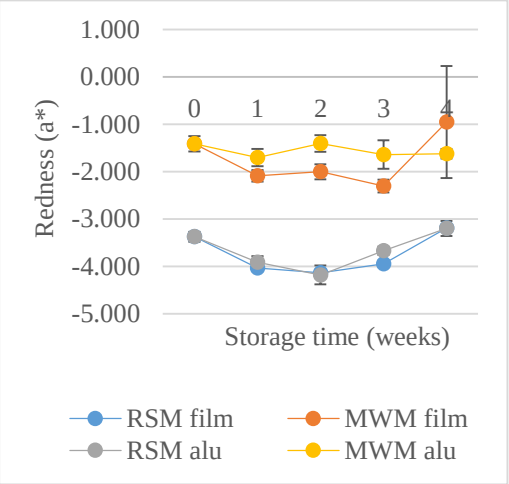


Figure 5.2b Redness (a*) of DPC16 microcapsule powders during storage for four weeks at 55 ℃

Notes.
n = 2 replications with duplicate analysis
± = standard error bars
RSM film = a* of DPC16 microcapsule powder made from reconstituted skim milk and vacuum-packed in gas-impermeable film
MWM film = a* of DPC16 microcapsule powder made from mixed wall material and vacuum-packed in gas-impermeable film
RSM alu = a* of DPC16 microcapsule powder made from reconstituted skim milk and vacuum-packed in an aluminium foil bag
MWM alu = a* of DPC16 microcapsule powder made from mixed wall material and vacuum-packed in an aluminium foil bag

The initial redness (a*) of the powders was all below zero which indicated the colour was greenish (International Colour Consortium, 2004). MWM powders had higher redness than the RSM powders during storage at both 25 ℃ (Figure 5.2a) and 55 ℃ (Figure 5.2b). This indicated that the powder made from MWM was closer to neutral greyness than powder made from RSM. During storage, redness of powders in all treatments were stable. Results showed that only the encapsulation wall material had significant influence on the redness of the powders during storage ($P<0.05$).

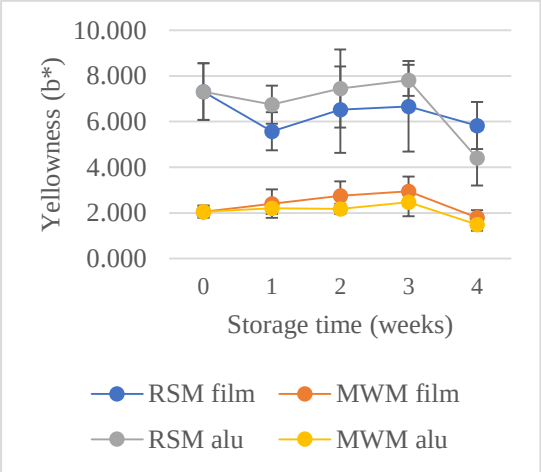


Figure 5.3a Yellowness (b*) of DPC16 microcapsule powders during storage for four weeks at 25 ℃

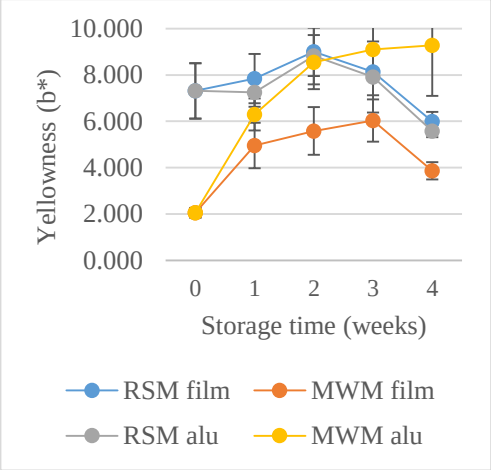


Figure 5.3b Yellowness (b*) of DPC16 microcapsule powders during storage for four weeks at 55 ℃

Notes.
n = 2 replications with duplicate analysis
± = standard error bars
RSM film = b* of DPC16 microcapsule powder made from reconstituted skim milk and vacuum-packed in gas-impermeable film
MWM film = b* of DPC16 microcapsule powder made from mixed wall material and vacuum-packed in gas-impermeable film
RSM alu = b* of DPC16 microcapsule powder made from reconstituted skim milk and vacuum-packed in an aluminium foil bag
MWM alu = b* of DPC16 microcapsule powder made from mixed wall material and vacuum-packed in an aluminium foil bag

The yellowness (b*) of all powders was above zero indicating that the powders were yellowish. The initial yellowness of powders made from MWM was about 2 which was closer to 0 (close to neutral greyness), whereas the yellowness of the RSM powders was about 7. Except for the powders made from MWM and stored at 55 ℃ (Figure 5.8), the yellowness of all the powders was stable during storage at 25 ℃ (Figure 5.3a) and 55 ℃ (Figure 5.3b). The yellowness of the powder made from MWM and packed in gas-impermeable film increased from 2 to 6 during four weeks at 55 ℃ (Figure 5.3b). This indicated that the colour became more yellowish during storage. The yellowness of the powder packed in aluminium foil bags increased more sharply from 2 to 10 within 4 weeks at 55 ℃ (Figure 5.3b). This was probably attributed to the Maillard

reactions in the MWM powders in aluminium-bag packaging stored at high temperature (55 °C). In addition, gum Arabic was originally yellow but the spray-dried MWM microcapsules were initially covered by whey proteins which were white (Xu et al., 2012). After whey proteins were destructed by Maillard reactions, more gum Arabic might be exposed, contributing to the change in colour of the powder from white to yellow. The storage temperature, types of encapsulation wall materials and packaging materials, as well as storage time all had significant effects on yellowness of the powders ($P<0.05$).

The occurrence of Maillard reactions causes cell death (Bhandari et al., 2013), which is suggested by the results in section 5.2 (Table 5.1) where the VCC of DPC16 decreased in all treatments. According to Hedegaard & Skibsted (2013), the oxidation of cellular lipids, destructions of cellular proteins and transformations in DNA and cell wall can all cause cell death during spray-drying and storage (Hedegaard & Skibsted, 2013). Maillard reactions can destroy proteins, causing the collapse of the glassy state of sugar matrix, and also indirectly leading to lipid oxidation. The lipid oxidation is caused by the production of water and increase of water activity during Maillard reactions (Hodge, 1953).

5.5 The moisture content and bulk density of spray-dried *L. reuteri* DPC16 microcapsule powders during storage at 25 °C and 55 °C

Figure 5.4 shows the moisture content and the bulk density of DPC16 microcapsule powders during storage for four weeks at 25 °C. Results showed that the types of encapsulation wall materials, packaging materials, and storage time all had significant influence on moisture content of probiotic powders ($P<0.05$).

Overall, the moisture content of powders made from RSM (0.045-0.060) was higher than that made from MWM (0.035~0.055) during storage at 25 °C. According to Pisecky (1997), in dried milk products, at $0.200 < a_w < 0.600$, the moisture content is dominated by the physical state of lactose. For all powders stored under 25 °C, the a_w was between 0.200 and 0.350 (Table 5.2).

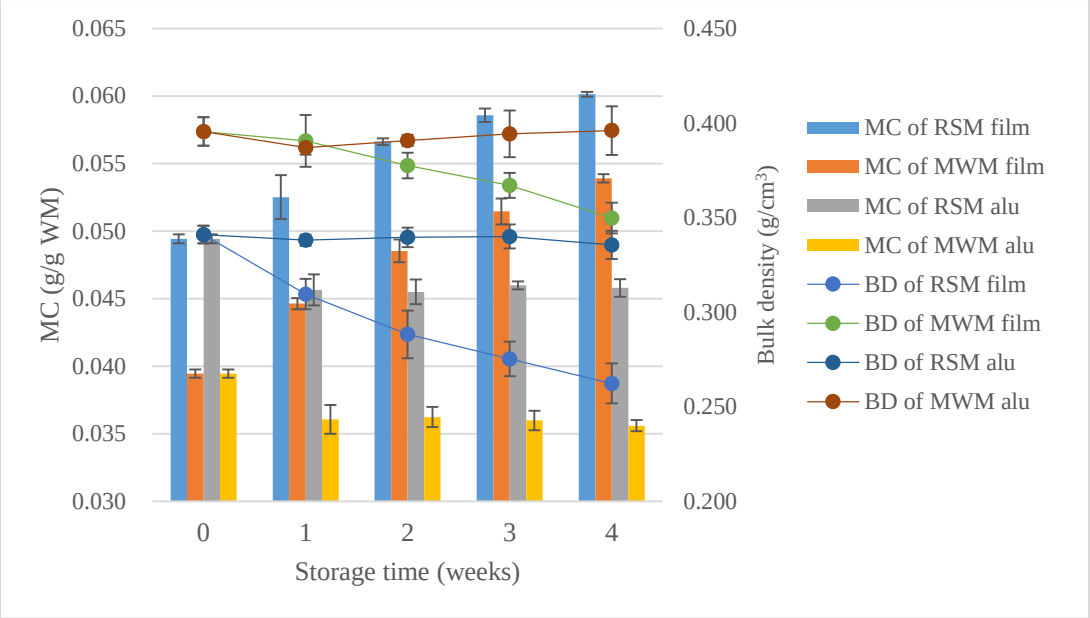


Figure 5. 4 The moisture content (g/g WM) and the bulk density of DPC16 microcapsule powders during storage for four weeks at 25 °C

Notes.
n = 2 replications with duplicate analysis
± = standard error bars
MC = moisture content
WM = wet matter
MC of RSM film = the moisture content of DPC16 microcapsule powder made from reconstituted skim milk and vacuum-packed in gas-impermeable film
MC of MWM film = the moisture content of DPC16 microcapsule powder made from mixed wall material and vacuum-packed in gas-impermeable film
MC of RSM alu = the moisture content of DPC16 microcapsule powder made from reconstituted skim milk and vacuum-packed in an aluminium foil bag
MC of MWM alu = the moisture content of DPC16 microcapsule powder made from mixed wall material and vacuum-packed in an aluminium foil bag
BD of RSM film = the bulk density of DPC16 microcapsule powder made from reconstituted skim milk and vacuum-packed in gas-impermeable film
BD of MWM film = the bulk density of DPC16 microcapsule powder made from mixed wall material and vacuum-packed in gas-impermeable film
BD of RSM alu = the bulk density of DPC16 microcapsule powder made from reconstituted skim milk and vacuum-packed in an aluminium foil bag
BD of MWM alu = the bulk density of DPC16 microcapsule powder made from mixed wall material and vacuum-packed in an aluminium foil bag

It seems that aluminium foil bags preserved the moisture content of powders better than the gas-impermeable film. The moisture content of DPC16 microcapsules vacuum-packed in gas-impermeable film increased during storage. However, the moisture content of the sample packed in aluminium foil bags decreased in week one and then stabilised during the rest of the storage time. This might be caused by the low permeability of the aluminium foil bag than the film. The moisture content of powders (0.049 to 0.060) made from RSM packed in gas-

impermeable film was not in the acceptable range (0.028 – 0.056) during storage at 25 ℃, but the RSM microcapsules packed in aluminium foil bags had desirable moisture content (0.046 – 0.049). Dehydrated milk powder is recommended to be stored at or below a moisture content of 0.06, 40% relative humidity, to prevent the crystallization of lactose (Warburton & Pixton, 1978). However, the moisture content of probiotic powders , ranging from 0.028 to 0.056 was reported to prolong their shelf life (Khem et al., 2016) and 0.040 was regarded optimal (Masters, 1985). Thus, the moisture content of RSM microcapsules packed in gas-impermeable film was not in the optimal range (Figure 5.4). Packing the RSM microcapsules in aluminium foil bags obtained more desirable moisture content of powder during storage at 25 ℃.

The initial moisture content of powders made from RSM (0.049±0.000) was similar to a previous study by Liao et al. (2017). Liao et al. used an outlet temperature of 70 ℃ during spray-drying and the moisture content was 0.042. Meanwhile, Maciel et al. (2014) spray-dried a feed solution of 30% skim milk (w/w) at inlet/outlet temperatures of 180 ℃/85 ℃ and the powder contained 0.043±0.002 moisture content.

The initial moisture content (0.040±0.000) of powders made from MWM was lower than the moisture content of powders made from RSM which agreed with previous reports. The addition of gum Arabic, inulin and sucrose can lower the moisture content of spray-dried powder made from milk protein (Desmond et al., 2002; Fritzen-Freire et al., 2012; Liao et al., 2002), although whey protein isolate powders may have higher moisture content than RSM following the same spray-drying conditions (Maciel et al., 2014). During storage at 25 ℃, the increase in moisture content of powders made from MWM was slightly higher than RSM. As the water activities of the powders were in the range $0.2 < a_w < 0.4$, the moisture adsorption might be caused by whey protein (Foster et al., 2005; Hardy et al., 2002), gum Arabic (Rodríguez-Bernal et al., 2015) and inulin (Zimeri & Kokini, 2002).

In contrast to the powders packed in the gas-impermeable film, the moisture content of powders packed in aluminium foil bags were stable for four weeks, mostly likely due to the low permeability of the packaging.

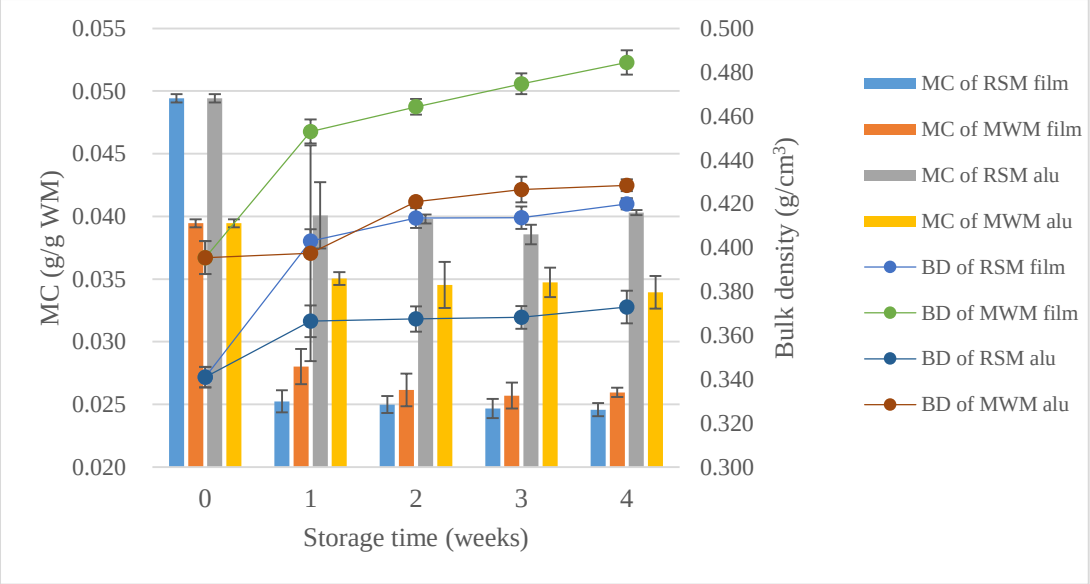


Figure 5. 5 The moisture content (g/g WM) and the bulk density of encapsulated DPC16 microcapsule powders during the four-week storage time at 55 °C.

Notes.
n = 2 replications with duplicate analysis
± = standard error bars
MC = moisture content
WM = wet matter
MC of RSM film = the moisture content of DPC16 microcapsule powder made from reconstituted skim milk and vacuum-packed in gas-impermeable film
MC of MWM film = the moisture content of DPC16 microcapsule powder made from mixed wall material and vacuum-packed in gas-impermeable film
MC of RSM alu = the moisture content of DPC16 microcapsule powder made from reconstituted skim milk and vacuum-packed in an aluminium foil bag
MC of MWM alu = the moisture content of DPC16 microcapsule powder made from mixed wall material and vacuum-packed in an aluminium foil bag
BD of RSM film = the bulk density of DPC16 microcapsule powder made from reconstituted skim milk and vacuum-packed in gas-impermeable film
BD of MWM film = the bulk density of DPC16 microcapsule powder made from mixed wall material and vacuum-packed in gas-impermeable film
BD of RSM alu = the bulk density of DPC16 microcapsule powder made from reconstituted skim milk and vacuum-packed in an aluminium foil bag
BD of MWM alu = the bulk density of DPC16 microcapsule powder made from mixed wall material and vacuum-packed in an aluminium foil bag

Figure 5.5 shows the moisture content and bulk density of DPC16 microcapsule powders during storage for four weeks at 55 °C. Results showed that types of encapsulation wall materials, packaging materials and storage time all had significant effects on the moisture content of powders during storage at 55 °C ($P<0.05$). Powders in all treatments were dehydrated during the storage period. However, powders packed in the gas-impermeable film had a higher decrease in the moisture content compared to that packed in the aluminium foil bags during the first week of storage. Thereafter, for all treatments, the moisture content was stable during the

last three weeks of storage. The smaller changes in moisture content of powders in aluminium foil bags was presumably attributed to the low permeability of aluminium foil bags.

The powders made from skim milk had a higher decrease in moisture content than powders made from MWM. This agreed with the water sorption behaviour of the components of the powders comprising whey protein isolate, gum Arabic, inulin and sucrose. As the water activity decreased from 0.240 to 0.060, the moisture content of whey protein decreased from nearly 0.050 to 0.020 at the storage temperature of 50 ℃ (Foster et al., 2005), indicating a higher moisture content of whey protein at a lower storage temperature. However, the moisture content of soluble sodium caseinate was less than 0.020 at the same water activity level ($0.060 < a_w < 0.240$) at the storage temperature of 30 ℃ (Bajpai & Tiwari, 2014). Casein is the major milk protein in milk which accounts for 80% of the total milk protein (Silva & Malcata, 2005). In addition, inulin and gum Arabic have higher hygroscopicity than sucrose or lactose (Bronlund & Paterson, 2004; Rodríguez-Bernal et al., 2015; Yu et al., 2008; Zimeri & Kokini, 2002).

5.6 The bulk density of spray-dried *L. reuteri* DPC16 microcapsule powders during storage at 25 ℃ and 55 ℃

Figure 5.4 shows the changes in bulk density of powders of different treatments during storage at 25 ℃ for four weeks. The bulk density of powders decreased gradually when packed in gas-impermeable film but were stable when packed in aluminium foil bags. At both storage temperatures (25 ℃ and 55 ℃), the bulk density of powders made from the skim milk was always lower than that made from MWM. A possible reason is that, some MWM microcapsules had smoother particle surfaces than the RSM microcapsules (Figure 4.4, Figure 4.6). Smooth surface of particles are reported to increase the bulk density of the powder (Binsi et al., 2017). During storage, the bulk density of powders containing sugars such as sucrose and lactose can decrease after absorbing moisture because of the hygroscopic property of sugar (Barbosa-Cánovas et al., 2005; Maidannyk et al., 2020) and both RSM and MWM had large amounts of sugars. Therefore, the bulk density of both microcapsules decreased as moisture content increased in the film packaging caused by the higher gas permeability (Sebranek & Houser, 2006). Results also showed that the bulk density of MWM microcapsules packaged in gas-impermeable film decreased more slightly compared to the RSM microcapsules although MWM contained larger amounts of sugar (Aalaei et al., 2016). This was probably caused by

the gum Arabic in MWM that caused stickiness (Figure 5.10), which in turn increased the bulk density of the powder (Barbosa-Cánovas et al., 2005; Werner et al., 2007). The encapsulation wall materials, packaging materials and storage time all affected the bulk density of powders during storage at 25 ℃ ($P<0.05$).

The graphs in Figure 5.5 show the changes in bulk density of powders during storage for four weeks at 55 ℃. Bulk density of powders in all the treatments increased gradually, which was presumably caused by the dehydration of powders especially sugar as smaller particles are denser (Bahram et al., 2014; Elversson & Millqvist-Fureby, 2005; Maidannyk et al., 2020). A higher increase in the bulk density of powders packed in gas-impermeable film was observed compared to the powders packed in aluminium foil bags, probably due to a higher loss of moisture content (Bahram et al., 2014; Maidannyk et al., 2020). The types of encapsulation wall materials, packaging materials and storage time all had significant effects on the bulk density of powders during storage at 55 ℃ ($P<0.05$).

Powders with high bulk density of powders can be stored in large amounts in small containers, which facilitate easier handling of the products (Carneiro et al., 2013). Further, such powders would have a lower amount of air between the particles, which could lead to better protection of cells in the powder (Carneiro et al., 2013). Therefore, powders with high bulk density are more desirable for powder preservation.

5.7 The particle size of spray-dried *L. reuteri* DPC16 microcapsule powders during storage at 25 ℃ and 55 ℃

Figure 5.6a shows the average particle size of DPC16 microcapsule powders during storage at 25 ℃. The particle size during storage was relatively stable (3 - 4.5 μm). According to our results, the type of packaging material had significant effect on the particle size of DPC16 microcapsule powders during storage at this temperature ($P<0.05$). The effect of packaging was probably caused by the particle size being affected by moisture sorption of the powder (Viswanathan et al., 2000). However, the moisture sorption of powders could have been affected by the packaging used due to the different gas permeabilities (Sebranek & Houser, 2006; Tatipata, 2009).

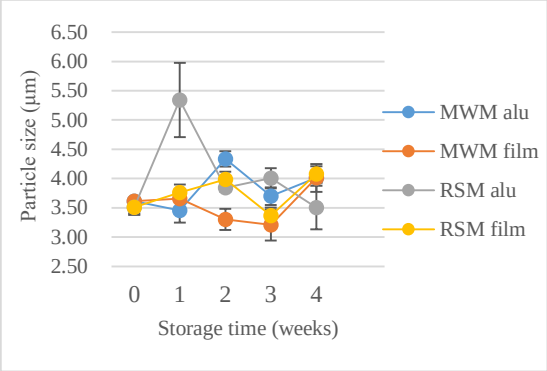


Figure 5.6a Particle size (µm) of DPC16 microcapsule powders during storage for four weeks at 25 ℃

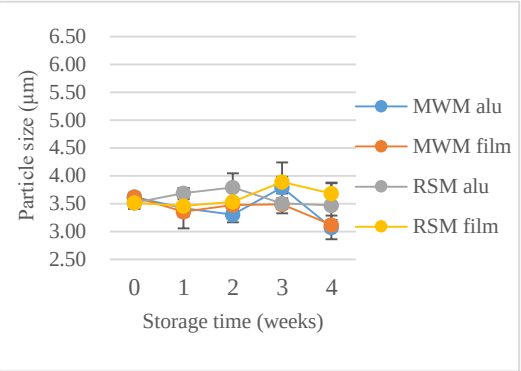


Figure 5.6b Particle size (µm) of DPC16 microcapsule powders during storage for four weeks at 55 ℃

Notes.
n = 2 replications with duplicate analysis
± = standard error bars
MWM alu = the particle size of encapsulated DPC16 microcapsule powder particles in mixed wall material and vacuum-packed in the aluminium foil bag
MWM film = the particle size of encapsulated DPC16 microcapsule powder particles in mixed wall material and vacuum-packed in gas-impermeable film
RSM alu = the particle size of encapsulated DPC16 microcapsule powder particles in reconstituted skim milk and vacuum-packed in the aluminium foil bag
RSM film = the particle size of encapsulated DPC16 microcapsule powder particles in reconstituted skim milk and vacuum-packed in gas-impermeable film

The particle size of DPC16 microcapsule powders during storage at 55 ℃ is shown in Figure 5.6b. The particle size was stable at 3 to 4 µm during storage for four weeks and their sizes among different formulations were not variable ($P<0.05$). Data do not show any obvious factors that may have affected the particle size of powders during storage at 55 ℃ ($P<0.05$). Similar results were reported in previous studies. According to Babu et al. (2018), no changes in the size of powder particles made from 70% (w/w) milk protein concentrate were observed after storage for 12 weeks at 40 ℃.

According to the results of the particle size of microcapsules stored at both temperatures, the storage temperature and packaging material were both significant factors that possibly influenced the particle size of powders during storage ($P<0.05$). Powders stored at a higher temperature (55 ℃) had lower average particle sizes (Appendix 4, Table 4.29) ($P<0.05$). The initial particle sizes of RSM and MWM microcapsules have been discussed in the section on morphology (4.6.2) in Chapter 4. The smaller particle sizes during storage at 55 ℃ might be

related to the shrinkage of powder particles due to loss of moisture and changes in the shapes of the particles (Barbosa-Cánovas et al., 2005; Maidannyk et al., 2020). More regular-shaped particles of milk protein concentrate powder were reported at high temperature of storage (Babu et al. (2018).

5.8 The surface appearance of spray-dried *L. reuteri* DPC16 microcapsule powders during storage at 25 ℃ and 55 ℃

According to Walton (2000), morphology affects bulk density, rehydration properties and flowability of powders. Compared with the broken whey protein microcapsules in which bacterial cells were embedded, our results showed that in all conditions, no bacteria cells were on the surface of powder particles, indicating the efficiency of the encapsulating wall materials (Khem et al., 2016). The morphology of spray-dried RSM powders packed in different packaging materials and stored at different temperatures did not change significantly during storage period. However, for powders made from MWM, apparent changes in morphology (Figure 5.8 to Figure 5.12) were observed during storage of the samples at elevated temperature (55 ℃), probably due to moisture sorption especially for the surface of particles composed of gum Arabic in MWM (Silva et al., 2013). Aggregation of particles was observed (week 4) in the powder made from MWM, packed in gas-impermeable film and stored at 25 ℃. This phenomenon was probably caused by the moisture adsorption of the sugar-rich powder. Gum Arabic in MWM very likely caused stickiness (Werner et al., 2007). In addition, Lay Ma et al. (2008) reported large agglomerates in the spay-dried powder made from concentrated milk with 7.5% and 10% sucrose. In their study, the aggregation was formed due to the stickiness of sucrose after spray-drying at inlet/outlet temperatures of 179 ℃/72 ℃. In our study, 160 ℃/ 80 ℃ (inlet/outlet temperatures) were used. The higher outlet temperature was more likely to have caused the stickiness of particles at the surface (Adhikari et al., 2005). However, for the other treatments (MWM microcapsules packed in aluminium foil bags stored at 25 ℃, MWM microcapsules packed in either aluminium foil bags or gas-impermeable film stored at 55 ℃) as well as RSM microcapsules, no aggregation of powders was observed during storage. The absence of aggregation of particles might be attributed to presence of high concentration of proteins with high molecular weight in RSM which can increase the glass transition temperature of powders and low gas permeability of aluminium foil bags (Aalaei et al., 2016; Rajam & Anandharamakrishnan, 2015; Tatipata, 2009). In addition, the water activity of RSM

microcapsules (peak 0.346, Table 4.2), which was below 0.4, was also probably not high enough to cause the transformation of powder morphology of the RSM microcapsules. Due to the high levels of the lactose in RSM (50% - 53%), the disaccharide probably characterised the physical state of the wall material (Hedegaard & Skibsted, 2013). According to Jouppila & Roos (1994), the water activity of 0.4 is a threshold value that separates the glassy and non-glassy state as the main form of lactose at ambient temperature. At about 20 ℃, the collapse of milk powder is likely to happen at a_w of 0.4 (Bhandari et al., 2013).

Figure 5.7 and Figure 5.8 show the SEMs of DPC16 microcapsule powders made from RSM and MWM at week 0, respectively. The surface of the particles has been discussed in section 4.6.2. Generally, particles had spherical or irregular shapes with various sizes. Most of the particles made from the two encapsulation wall materials (RSM and MWM) were wrinkled on the surface with visible dents or concavities. However, there were also doughnut-like particles with smooth surface and few dents or roughness among particles made from MWM.

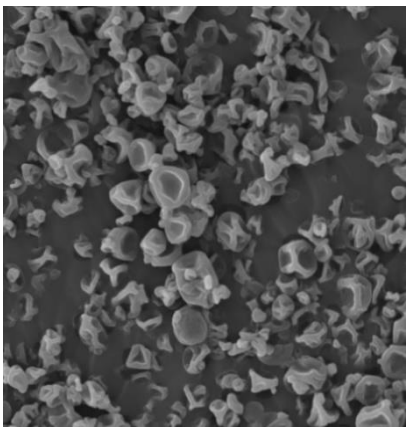


Figure 5.7a. SEM of DPC16 microcapsule powder (week 0) made from reconstituted skim milk (2,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

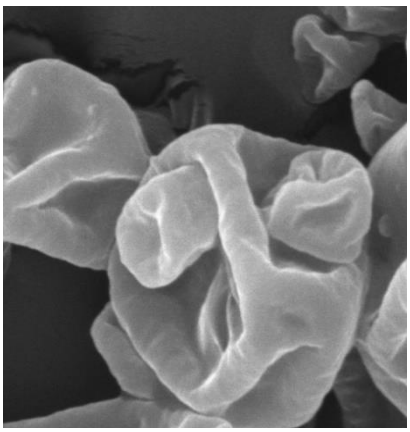


Figure 5.7b. SEM of DPC16 microcapsule powder (week 0) made from reconstituted skim milk (16,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

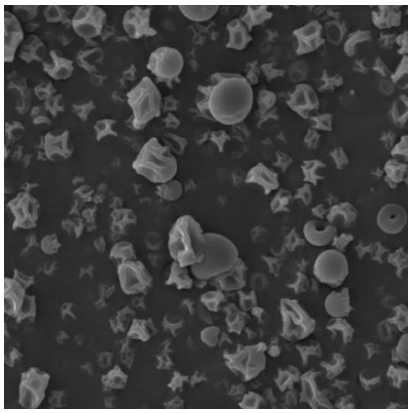


Figure 5.8a. SEM of DPC16 microcapsule powder (week 0) made from the mixed wall material (2,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

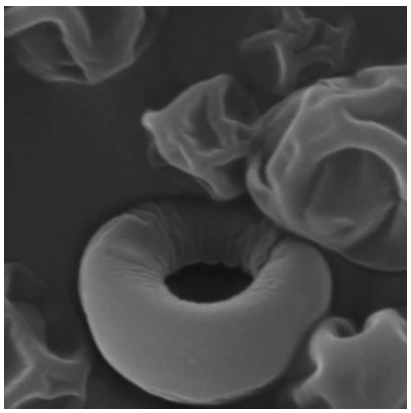


Figure 5.8b. SEM of DPC16 microcapsule powder (week 0) made from the mixed wall material (16,000 ×magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

Figure 5.9 to Figure 5.12 show the SEMs of DPC16 microcapsule powders made from MWM during storage for four weeks (25 ℃). Figure 5.9a, b shows the SEMs of DPC16 microcapsule powder made from MWM and vacuum-packed in aluminium foil bag during storage (25 ℃) in week 1 (Figure 5.9a, b) and week 4 (Figure 5.9c, d), respectively. Compared to Figure 8, which shows the same powder in week 0, the distribution, size and surface of MWM microcapsules had no apparent changes except the doughnut-like particle. According to Figure 5.9b which is the micrograph of the powder with 16,000 × magnification in week 1, the doughnut-like particle appeared more wrinkled, which might be caused by the loss of moisture.

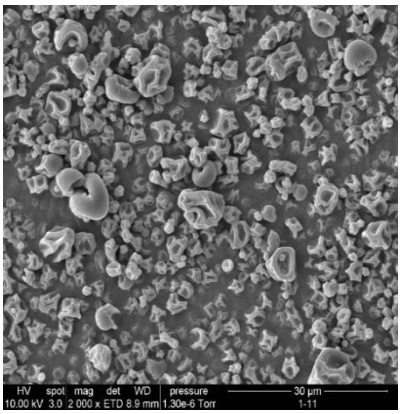


Figure 5.9a. SEM of DPC16 microcapsule powder (week 1) made from the mixed wall material, vacuum-packed in the aluminium foil bag, and stored at 25 ℃ (2,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

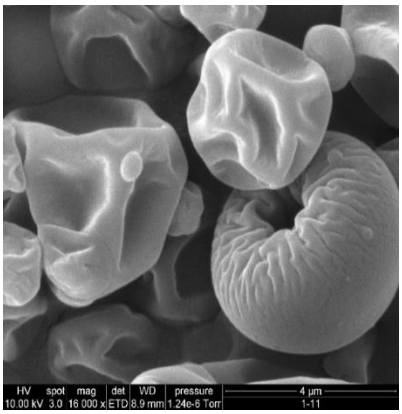


Figure 5.9b. SEM of DPC16 microcapsule powder (week 1) made from the mixed wall material, vacuum-packed in the aluminium foil bag, and stored at 25 ℃ (16,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

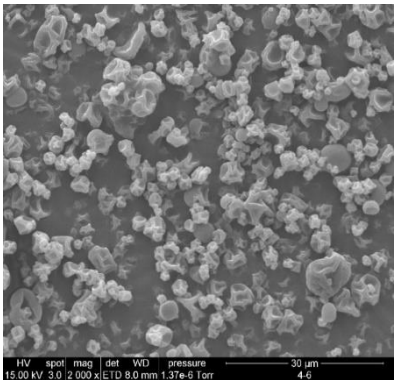


Figure 5.9c. SEM of DPC16 microcapsule powder (week 4) made from the mixed wall material, vacuum-packed in the aluminium foil bag, and stored at 25 ℃ (2,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

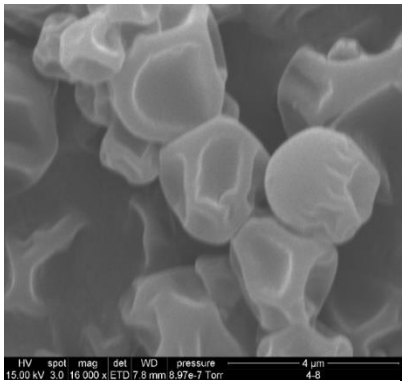


Figure 5.9 d. SEM of DPC16 microcapsule powder (week 4) made from the mixed wall material, vacuum-packed in the aluminium foil bag, and stored at 25 ℃ (16,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

Figure 5.10 is the SEMs of DPC16 microcapsule powder made from MWM and vacuum-packed in gas-impermeable film during storage in week 1 and week 4, respectively at 25 ℃. Compared to the same powder in week 0 (refer to Figure 5.8), the powder during storage in week 1 did not show any change (Figure 5.10a, b), however, aggregation was observed in week 4 (Figure 5.10 d). In addition, more spherical particles with smooth surface were found probably due to the adsorption of moisture in week 4 (Figure 5.10c). Since aggregation is an unexpected phenomenon in powder products (Bhandari et al., 2013), the occurrence of aggregation of the powders in the film packaging suggested the use of non-ideal packaging (i.e. film) and encapsulation wall material (i.e. MWM) for the storage (25 ℃) of DPC16 microcapsule powder.

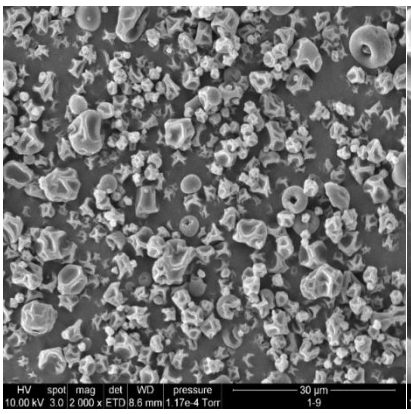


Figure 5.10a. SEM of DPC16 microcapsule powder (week 1) made from the mixed wall material, vacuum-packed in gas-impermeable film, and stored at 25 ℃ (2,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

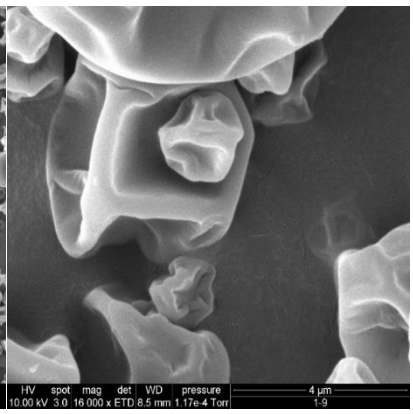


Figure 5.10b. SEM of DPC16 microcapsule powder (week 1) made from the mixed wall material, vacuum-packed in gas-impermeable film, and stored at 25 ℃ (16,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

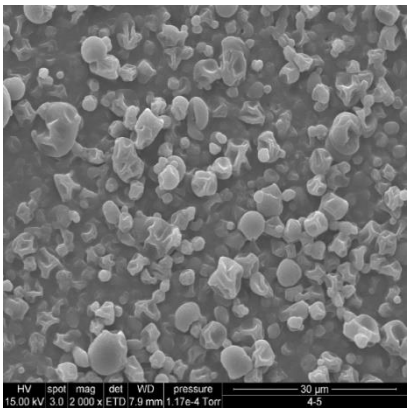


Figure 5.10c. SEM of DPC16 microcapsule powder (week 4) made from the mixed wall material, vacuum-packed in gas-impermeable film, and stored at 25 ℃ (2,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

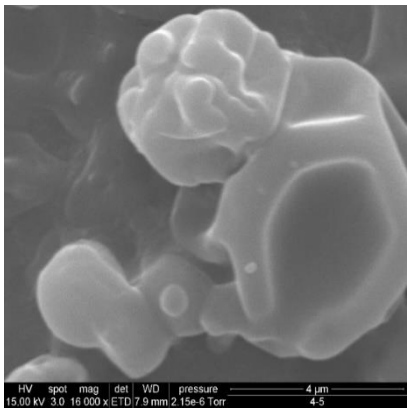


Figure 5.10d. SEM of DPC16 microcapsule powder (week 4) made from the mixed wall material, vacuum-packed in gas-impermeable film, and stored at 25 ℃ (16,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

Figure 5.11 shows the SEMs of DPC16 microcapsule powder made from MWM, vacuum-packed in aluminium foil bag during storage (55 ℃) in week 1 (Figure 5.11a, b) and week 4 (Figure 5.11c, d), respectively. Compared to the same powder in the same packaging material stored at 25 ℃ (Figure 5.9) and to the powder in week 0 (Figure 5.8), the powder stored at 55 ℃ appeared more wrinkled. Not only did the doughnut-like particles (Figure 5.11a) became more wrinkled which was also observed during storage at 25 ℃ (Figure 5.9b), but also to the particles with irregular or spherical shapes (Figure 5.11b, d). The phenomenon of forming wrinkles might be caused by desorption of moisture during storage at 55 ℃.

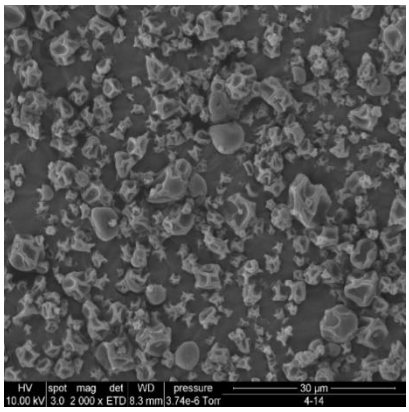


Figure 5.11a. SEM of DPC16 microcapsule powder (week 1) made from the mixed wall material, vacuum-packed in the aluminium foil bag, and stored at 55 ℃ (2,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

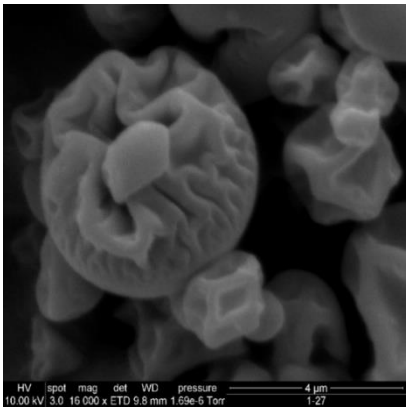


Figure 5.11b. SEM of DPC16 microcapsule powder (week 1) made from the mixed wall material, vacuum-packed in the aluminium foil bag, and stored at 55 ℃ (16,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

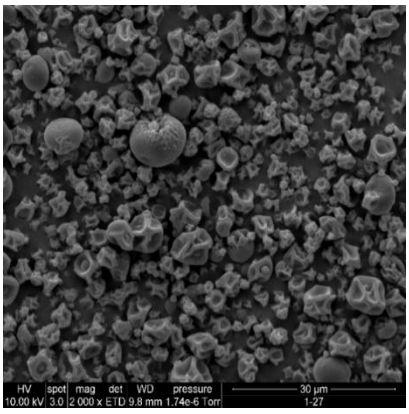


Figure 5.11c. SEM of DPC16 microcapsule powder (week 4) made from the mixed wall material, vacuum-packed in the aluminium foil bag, and stored at 55 ℃ (2,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

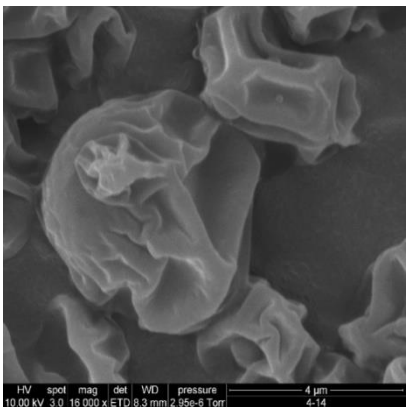


Figure 5.11d. SEM of DPC16 microcapsule powder (week 4) made from the mixed wall material, vacuum-packed in the aluminium foil bag, and stored at 55 ℃ (16,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

Figure 5.12 shows the DPC16 microcapsule powder made from MWM, vacuum-packed in gas-impermeable film followed by storage at 55 ℃. Shrinkage of the doughnut-like particles was be observed in week 1 (Figure 5.12b) presumably due to the loss of moisture. This was also noted in the same (MWM) microcapsules packed in aluminium foil bags and stored at the same temperature (55 ℃) (Figure 5.11). In week 4, some cracks were observed on the surface of the doughnut-like particles (Figure 5.12d). According to Buma and Henstra (1970), such cracks suggest the mechanical stresses caused by uneven heating at different parts of particles and shrinkage of some wall substances. The doughnut-like particles with smooth surface caused by the addition of gum Arabic as the wall may be not strong enough against the mechanical stresses compared to particles with dented surfaces, although they (smooth-surface particles) contribute to better solubility, fluidity and higher bulk density (Binsi et al., 2017; Fernandes et al., 2014; Reyes et al., 2018). The cracks on microcapsules can expose the microcapsules as reported by Li et al. (2015). Thus, the MWM containing gum Arabic may not be suitable as an encapsulation wall material intended for microencapsulation by spray-drying technology if the (spray-dried) powder may be subjected to storage at high temperature such as 55 ℃.

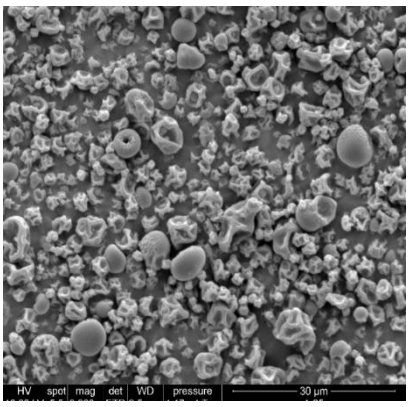


Figure 5.12a. SEM of DPC16 microcapsule powder (week 1) made from the mixed wall material, vacuum-packed in gas-impermeable film, and stored at 55 ℃ (2,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

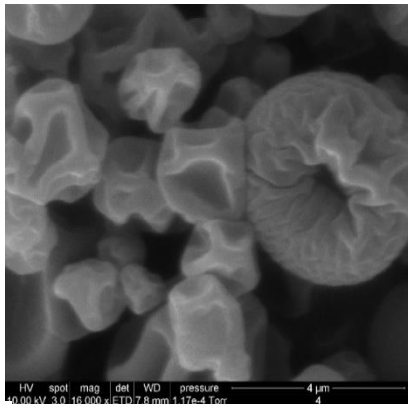


Figure 5.12b. SEM of DPC16 microcapsule powder (week 1) made from the mixed wall material, vacuum-packed in gas-impermeable film, and stored at 55 ℃ (16,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

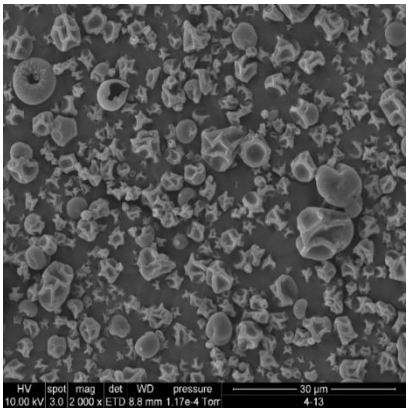


Figure 5.12c. SEM of DPC16 microcapsule powder (week 4) made from the mixed wall material, vacuum-packed in gas-impermeable film, and stored at 55 ℃ (2,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

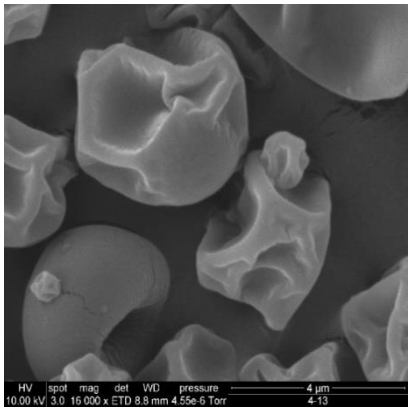


Figure 5.12d. SEM of DPC16 microcapsule powder (week 4) made from the mixed wall material, vacuum-packed in gas-impermeable film, and stored at 55 ℃ (16,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

Figure 5.13 to Figure 5.16 show the SEMs of DPC16 microcapsule powders made from RSM during storage. Overall, no distinctive particles such as the doughnut-like ones were found among RSM microcapsules. Particles had various sizes and irregular shapes with dents on surfaces during storage for four weeks. Figure 5.13 shows the microscopic images of the DPC16 microcapsule powder made from RSM, vacuum-packed in aluminium foil bags during storage (25 ℃) for one week (Figure 5.13a, b) and four weeks (Figure 5.13c, d). Comparing the powder during storage in week 0 (Figure 5.7), the powder packed in aluminium foil bags and stored at 25 ℃ did not show changes in particle sizes, shapes and surface appearance in week 4 (Figure 5.13c, d).

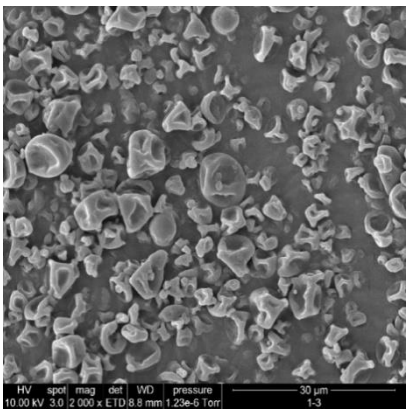


Figure 5.13a. SEM of DPC16 microcapsule powder (week 1) made from reconstituted skim milk, vacuum-packed in the aluminium foil bag, and stored at 25 ℃ (2,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

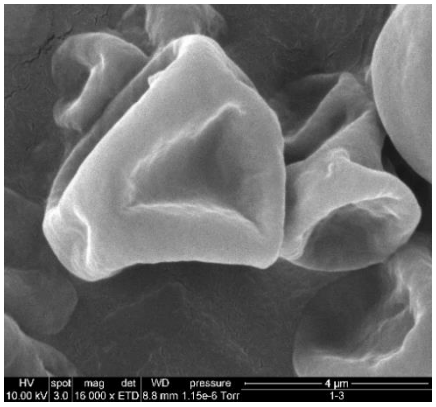


Figure 5.13b. SEM of DPC16 microcapsule powder (week 1) made from reconstituted skim milk, vacuum-packed in the aluminium foil bag, and stored at 25 ℃ (16,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

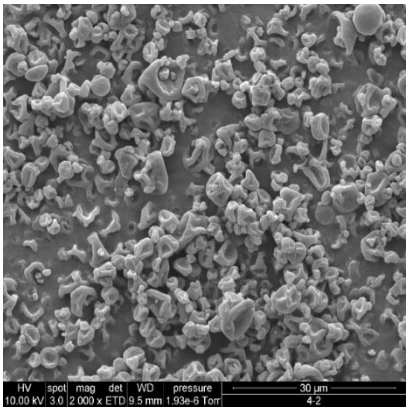


Figure 5.13c. SEM of DPC16 microcapsule powder (week 4) made from reconstituted skim milk, vacuum-packed in the aluminum foil bag, and stored at 25 ℃ (2,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

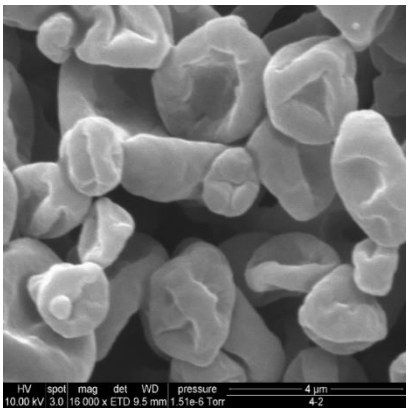


Figure 5.13d. SEM of DPC16 microcapsule powder (week 4) made from reconstituted skim milk, vacuum-packed in the aluminum foil bag, and stored at 25 ℃ (16,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

Figure 5.14 shows the SEMs of DPC16 microcapsule powder made from RSM, vacuum-packed in gas-impermeable film and stored at 25 ℃. No significant changes were observed compared to the powders stored for zero and four weeks (Figure 5.7). The particles were still various in size with dented surface during storage time.

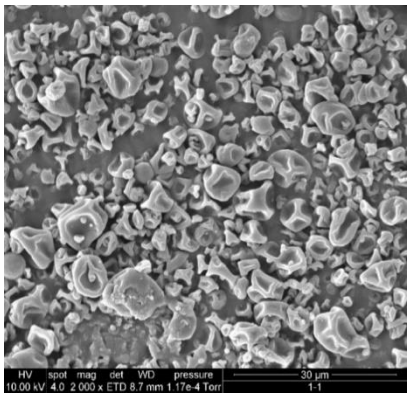


Figure 5.14a. SEM of DPC16 microcapsule powder (week 1) made from reconstituted skim milk, vacuum-packed in gas-impermeable film, and stored at 25 ℃ (2,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

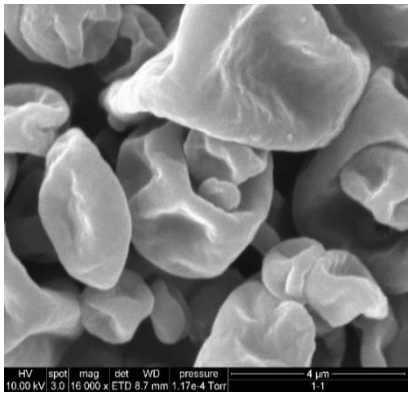


Figure 5.14b. SEM of DPC16 microcapsule powder (week 1) made from reconstituted skim milk, vacuum-packed in gas-impermeable film, and stored at 25 ℃ (16,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

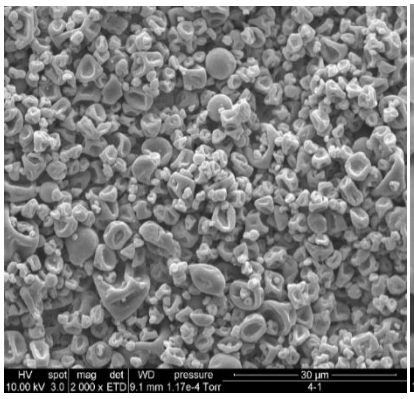


Figure 5.14c. SEM of DPC16 microcapsule powder (week 4) made from reconstituted skim milk, vacuum-packed in gas-impermeable film, and stored at 25 ℃ (2,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

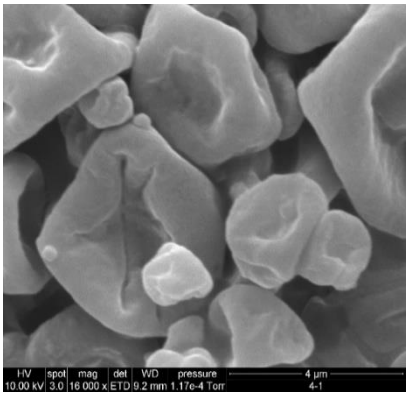


Figure 5.14d. SEM of DPC16 microcapsule powder (week 4) made from reconstituted skim milk, vacuum-packed in gas-impermeable film, and stored at 25 ℃ (16,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

Figure 5.15 is the microscopic image of DPC16 microcapsule powder made from RSM, vacuum-packed in aluminium foil bags, then stored at 55 ℃. No changes (particle sizes, surface, shapes) were observed during storage compared to the powder in week 0 (Figure 5.7). However, Babu et al. (2018) reported that the milk protein powders containing 70 % to 90% proteins became more regular in shape at high storage temperature. The RSM microcapsules in this study were not found to have more regular shape during storage at 55 ℃ probably because of the relatively lower percentage of protein content used in RSM.

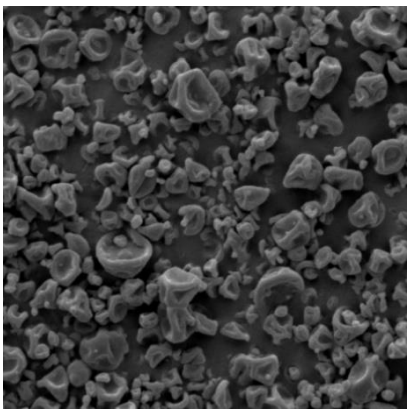


Figure 5.15a. SEM of DPC16 microcapsule powder (week 1) made from reconstituted skim milk, vacuum-packed in the aluminum foil bag, and stored at 55 ℃ (2,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

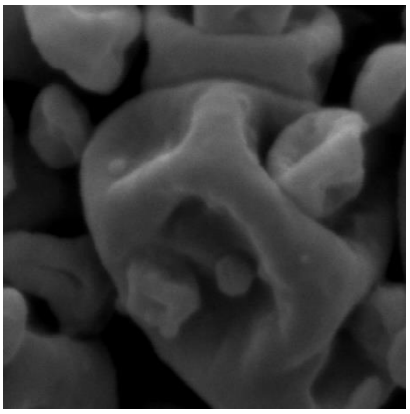


Figure 5.15b. SEM of DPC16 microcapsule powder (week 1) made from reconstituted skim milk, vacuum-packed in the aluminum foil bag, and stored at 55 ℃ (16,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

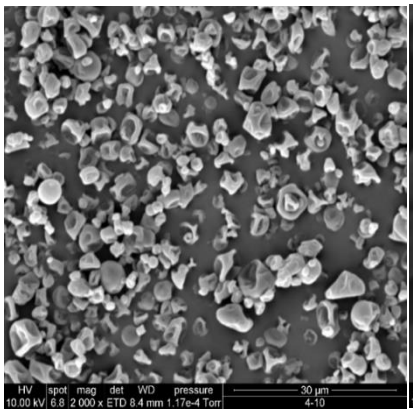


Figure 5.15c. SEM of DPC16 microcapsule powder (week 4) made from reconstituted skim milk, vacuum-packed in the aluminum foil bag, and stored at 55 ℃ (2,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

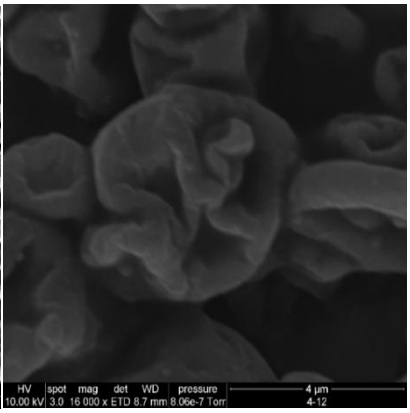


Figure 5.15d. SEM of DPC16 microcapsule powder (week 4) made from reconstituted skim milk, vacuum-packed in the aluminum foil bag, and stored at 55 ℃ (16,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

Figure 5.16 shows the SEMs of DPC16 microcapsule powder made from RSM, vacuum-packed in gas-impermeable film and stored at 55 ℃. No changes were observed compared to the same powder in week 0 (Figure 5.9).

According to Figure 5.13 to Figure 5.16, no needle-like shapes were observed on the surface of particles during storage, suggesting that crystallization did not take place in the lactose of RSM. Therefore, the storage conditions for the powders were suitable for the RSM powders containing lactose. According to Maidannyk et al. (2020), lactose crystals are observed as needle-like “tomahawk” on the surface of particles. They may be found on the surface of humidified milk protein concentrates containing 40% proteins at 54.5% relative humidity. To prevent the crystallization of lactose, moisture content below 6% and water activity below 0.4 were recommended for the preservation of milk-based powders (Bhandari et al., 2013; Warburton & Pixton, 1978). In the current study, the moisture content and water activity of the RSM microcapsules were much less than the moisture content and water activity mentioned here.

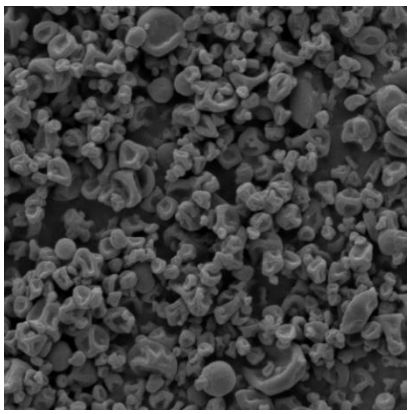


Figure 5.16a. SEM of DPC16 microcapsule powder (week 1) made from skim milk, vacuum-packed in gas- impermeable film, and stored at 55 ℃ (2,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

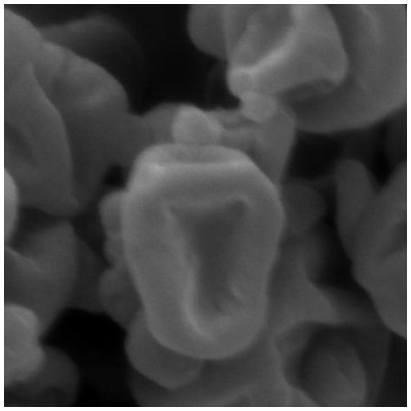


Figure 5.16b. SEM of DPC16 microcapsule powder (week 1) made from skim milk, vacuum-packed in gas-impermeable film, and stored at 55 ℃ (16,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

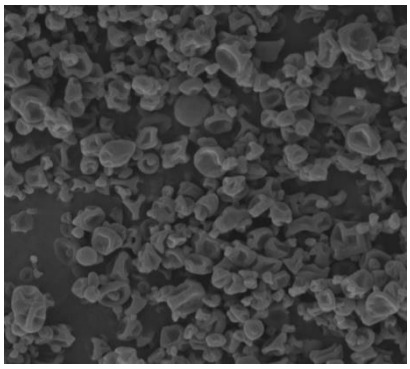


Figure 5.16c. SEM of DPC16 microcapsule powder (week 4) made from reconstituted skim milk, vacuum-packed in gas-impermeable film, and stored at 55 ℃ (2,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

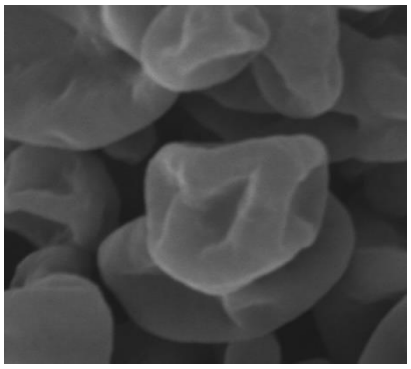


Figure 5.16d. SEM of DPC16 microcapsule powder (week 4) made from reconstituted skim milk, vacuum-packed in gas-impermeable film, and stored at 55 ℃ (16,000 × magnification), captured by the FEI Electron Optics, Quanta 200 (The Netherlands).

5.9 Summary

In this chapter, a four-week storage trial for spray-dried DPC16 microcapsules was carried out to evaluate the potential of two selected encapsulation wall materials (RSM and MWM), two packaging materials (gas-impermeable film and aluminium foil bags) and two storage temperatures (25 ℃; 55 ℃). During storage at 25 ℃, both RSM and MWM maintained high cell survival of DPC16 (> 7 log CFU/g) and the colour was stable irrespective of the type of packaging used. However, due to the relatively high gas permeability of gas-impermeable film, the microcapsules increased in moisture content and water activity in the film packaging, which might be harmful for cells during longer storage and led to swelling and aggregation of some MWM microcapsules. Therefore, the gas-impermeable film and MWM may be unsuitable for packaging wall-material-encapsulated DPC16 microcapsules for storage at ambient temperature.

During storage at 55 ℃, the reduction of DPC16 cells encapsulated in RSM and vacuum-packed in gas-impermeable film were lower than cells encapsulated in RSM packaged in aluminium foil bags and cells encapsulated in MWM irrespective of the type of packaging. Meanwhile, MWM microcapsules packed in aluminium foil bags had the lowest cell survival and highest colour change and morphology of the powder. Thus, MWM and aluminium foil bags would be unsuitable encapsulation wall material and the packaging material for DPC16 cell preservation at high storage temperature (55 ℃).

Therefore, the results showed that RSM is an encapsulation wall material with high potential for the preservation of DPC16 at both ambient and higher storage temperatures compared to MWM. Results also showed that vacuum-packing the RSM microcapsules in aluminium foil bags could maintain high quality of the powders during storage at ambient temperature, however, the DPC16 cells might be more susceptible to high storage temperature in this treatment compared to packaging RSM microcapsules in gas-impermeable film.

Chapter 6 Conclusion

The study investigated the long-term preservation of spray-dried *L. reuteri* DPC16 cells encapsulated in selected wall materials. The results of the study may be concluded as below:
The DPC16 cells encapsulated in 10% RSM (w/w), spray-dried at 160°C /80°C and vacuum-packed in aluminium foil bags had relatively stable and highest cell survival (>8.47 log CFU/g) during storage at 25°C for 4 weeks, but during storage at 55 °C, the cell survival decreased from 8.71±0.15 to 1.78±0.34 within one week, which was not the best performance among all treatments. There was a slight decrease in viable cells when the spray-dried DPC16 cells were encapsulated in RSM, vacuum-packed in gas-impermeable film and stored at 25°C (from 8.71±0.15 to 8.13±0.11). However, during storage at 55°C, the decrease of cells was slowest in this treatment, which was desired. Cells decreased from 8.71±0.15 to 4.86±0.61 within one week. Even at the fourth week, there was still 3.05±0.17 left while in all other treatments only less than 2 logs of cells were left.

Mixed wall material didn't have as good performance as RSM regarding EE during drying, it didn't perform well in storage trial as expected either. The decrease of cells encapsulated in MWM was larger than in RSM in all treatments when they were packed and stored in the same condition.

Chapter 7. Recommendations

Cell viability when passing through the GIT

Probiotics function at colon and the cells must be alive. Therefore, they must also survive the harsh conditions in the GIT. Since reconstituted skim milk has been selected as wall material for cell encapsulation, the next step should be to investigate its protective effect on cells in GIT and its ability to target-release the cells to the colon. If the performance is not ideal, an approach needs to be found to achieve that.

Addition to other food products

The encapsulated DPC16 powder can be added to traditional food products such as bread, sausages, yogurt, ice-cream and jelly to enhance their value as functional foods. Still, the cell-target-release property of the food need to be evaluated.

Rehydration of the powder

Cells could be released by the rehydration of the spray-dried microcapsules. Thus, powder-rehydration properties are important characteristics of encapsulated DPC16 powder products. Rehydration properties includes wettability, sinkability, dispersibility and solubility, etc.

Particle size measurement

The particle size measurement in this project was limited by access to instrument so it may not be precise. Mastersizer is preferred for particle size measurement rather than manual measurement on scanned electron microscopy.

Vacuum degree for packaging

During packaging, the vacuum degree was set to 2 torr which was the lowest degree that the equipment can achieve. Further research can be carried out to find the proper higher value which can save both time and energy during packaging process.

Packaging material

Due to the high cost of aluminium bags, other economical packaging materials such as other gas-impermeable film can be tested for their protective effect on encapsulated probiotic powder.

Mechanism of protective effect of skim milk as the wall material for encapsulation

The mechanism of the protective effect of skim milk on DPC16 cells can be studied further. For instance, to investigate whether it is related to the ratio of protein to sugar or it is because of the existence of other components, which can provide more information to encapsulate other probiotic strains.

Safety concern of DPC16

L. reuteri DPC16 was first isolated in New Zealand in recent decades. More research needs to be done on the safety of this strain as probiotic for human.

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Chapter 8. References

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Appendix

Appendix 1 Material and Methods

Preparation of microbiological media for analysis of encapsulated DPC16 powder

A. 0.1% peptone water (w/w) (Merck, Germany)

Direction: completely dissolve 1g universal peptone in 1000 mL distilled water. Small portions for series dilution were made by dispensing 9 mL 0.1% peptone water into dilution bottles using a dispenser. Sterilize the media by autoclave at 121 ℃ for 15 min then cool down to below 50 ℃ before use.

B. de Mann Rogosa Shape (MRS) agar, (Supplier: Oxoid, Australia)

Direction: dissolve 62 g MRS agar in 1000 mL distilled water. Dispense into bottles and sterilize the media by autoclave at 121 ℃ for 15 min then cool down to below 50 ℃ before use.

C. MRS broth

Direction: dissolve 52 g MRS broth in 1000 mL distilled water. Dispense into bottles and sterilize the media by autoclave at 121 ℃ for 15 min then cool down to below 50 ℃ before use.

Appendix 2 Raw data at the screening stage

Table 2.1 Log viable cells (log CFU/mL) of DPC16 during incubation for 18 h

	Log viable cell (log CFU/g)					
Time (h)	Rep1	Rep2	Rep3	Rep4	Average	Std
0	5.72	5.68	5.83	5.57	5.70	0.02
1	5.65	5.68	5.80	5.54	5.67	0.02
2	5.69	5.69	5.82	5.56	5.69	0.01
3	5.79	5.60	5.82	5.57	5.70	0.13
4	5.79	5.89	5.97	5.71	5.84	0.07
5	6.02	6.12	6.20	5.94	6.07	0.08
6	6.63	6.69	6.79	6.53	6.66	0.04
7	7.01	7.23	7.25	6.99	7.12	0.16
8	7.68	7.48	7.71	7.45	7.58	0.14
9	8.02	7.94	8.11	7.85	7.98	0.05
10	8.38	8.27	8.46	8.20	8.33	0.07
11	8.60	8.62	8.74	8.48	8.61	0.01
12	8.88	8.81	8.97	8.71	8.84	0.05
13	8.89	8.89	9.02	8.76	8.89	0.01
14	8.90	8.89	9.02	8.77	8.90	0.01
15	8.92	8.91	9.04	8.79	8.92	0.01
16	8.95	8.94	9.08	8.82	8.95	0.01
17	8.98	8.96	9.10	8.84	8.97	0.01
18	8.98	8.98	9.11	8.85	8.98	0.00

Table 2.2 Optical density$_{595nm}$ during incubation for 18 h

	Replication					
Time (h)	1	Rep 2	Rep3	Rep4	Average	Std
0	0.097	0.014	0.068	0.044	0.056	0.042
1	0.031	0.029	0.042	0.018	0.030	0.001
2	0.037	0.035	0.048	0.024	0.036	0.001
3	0.034	0.032	0.045	0.021	0.033	0.001
4	0.050	0.036	0.055	0.031	0.043	0.007
5	0.060	0.049	0.067	0.043	0.055	0.005
6	0.075	0.074	0.087	0.063	0.075	0.001
7	0.149	0.147	0.160	0.136	0.148	0.001
8	0.289	0.272	0.293	0.269	0.281	0.009
9	0.532	0.488	0.522	0.498	0.510	0.022
10	0.929	0.891	0.922	0.898	0.910	0.019
11	1.708	1.051	1.392	1.368	1.380	0.329

12	1.962	1.890	1.938	1.914	1.926	0.036
13	2.060	2.012	2.048	2.024	2.036	0.024
14	2.133	2.089	2.123	2.099	2.111	0.022
15	2.201	2.163	2.194	2.170	2.182	0.019
16	2.612	1.954	2.295	2.271	2.283	0.329
17	2.430	2.358	2.406	2.382	2.394	0.036
18	2.524	2.476	2.512	2.488	2.500	0.024

Table 2.3. Encapsulation efficiency (%) of wall materials

Wall material	Inlet/outlet temperatures(℃/℃)	Replication	log CFU/g before drying	log CFU/g after drying	%EE	Average EE (%)	SEM of EE (%)
maltodextrin	160/80	1	8.602	8.045	93.53	92.50	0.37
		2	8.740	8.025	91.82		
		3	8.556	7.892	92.24		
		4	8.681	8.021	92.40		
gum Arabic	160/80	1	9.111	8.049	88.35	90.63	3.08
		2	9.076	8.954	98.66		
		3	8.643	7.903	91.43		
		4	8.602	7.230	84.05		
RSM	160/80	1	9.068	9.086	100.20	98.06	0.86
		2	9.320	9.021	96.79		
		3	9.188	8.869	96.54		
		4	9.079	8.964	98.73		
MWM	160/80	1	9.258	8.336	90.05	93.97	1.49
		2	8.949	8.253	92.22		
		3	8.806	8.465	96.13		
		4	8.845	8.623	97.49		
RSM	180/100	1	8.954	7.748	86.53	83.85	1.50
		2	9.064	7.826	86.34		
		3	8.672	7.086	81.71		
		4	8.708	7.037	80.82		

Table 2.4 Water activity of DPC16 microcapsules made from different wall materials and spray-dried at different inlet/outlet temperatures

Inlet/outlet temperatures (℃/℃)	Wall material	a_w					
		Replication 1	Replication 2	Replication 3	Replication 4	Mean	SEM
160/80	Maltodextrin	0.259	0.218	0.223	0.246	0.237	0.010
	Gum Arabic	0.173	0.158	0.166	0.181	0.170	0.005
	RSM	0.270	0.284	0.286	0.294	0.284	0.005
	MWM	0.232	0.183	0.187	0.182	0.196	0.010
180/100	RSM	0.212	0.189	0.203	0.196	0.200	0.004

Table 2.5a. Particle size of DPC16 microcapsule powders made from different wall materials (μm) just after drying

Temperature(℃/℃)	Batch#	Replication	RSM	gum Arabic	maltodextrin	MWM
160/80	1	1	5.594	3.765	5.749	7.039
160/80	1	2	1.921	5.604	3.345	3.421

160/80	1	3	5.447	2.642	2.096	2.450
160/80	1	4	3.916	3.265	2.319	2.710
160/80	1	5	3.608	3.566	2.626	2.559
160/80	1	6	3.284	4.565	2.575	5.793
160/80	1	7	3.265	2.871	3.604	2.580
160/80	1	8	4.031	5.429	5.293	3.045
160/80	1	9	1.652	5.309	4.284	3.503
160/80	1	10	2.089	2.062	2.517	2.668
160/80	1	11	2.155	4.173	3.986	5.818
160/80	1	12	2.213	7.275	3.939	2.778
160/80	1	13	2.838	2.680	4.771	3.514
160/80	1	14	4.642	2.556	4.007	2.571
160/80	1	15	3.806	3.176	3.162	2.882
160/80	2	1	3.827	3.531	5.499	2.205
160/80	2	2	4.163	5.207	2.690	3.571
160/80	2	3	5.618	2.284	3.191	3.453
160/80	2	4	4.408	2.531	2.639	3.452
160/80	2	5	3.564	3.131	2.252	2.333
160/80	2	6	3.137	5.130	2.151	4.319
160/80	2	7	1.490	2.741	3.207	6.375
160/80	2	8	5.060	5.858	5.586	3.203
160/80	2	9	3.029	6.618	4.569	3.646
160/80	2	10	4.994	2.124	2.034	2.705
160/80	2	11	3.280	4.345	3.971	3.193
160/80	2	12	3.363	6.550	3.877	3.762
160/80	2	13	4.354	1.359	5.542	4.496
160/80	2	14	2.984	1.112	4.014	5.252
160/80	2	15	3.827	2.352	2.325	3.190

Notes.

	RSM	gum Arabic	maltodextrin	MWM
Mean particle size (μm)	3.585	3.794	3.594	3.616
SEM (μm)	0.208	0.298	0.214	0.226

Table 2.5b. Particle size (μm) of DPC16 microcapsule powders made from 10% reconstituted skim milk (w/w) spray-dried at different inlet/outlet temperatures

Batch #	Replication	160 ℃/80 ℃	180 ℃/100 ℃
1	1	5.594	3.772
1	2	1.921	2.618
1	3	5.447	2.237
1	4	3.916	3.329
1	5	3.608	2.474
1	6	3.284	3.236
1	7	3.265	2.271
1	8	4.031	2.219
1	9	1.652	2.697
1	10	2.089	3.296

1	11	2.155	3.089
1	12	2.213	2.886
1	13	2.838	3.234
1	14	4.642	4.289
1	15	3.806	3.606
2	1	3.827	3.543
2	2	4.163	2.237
2	3	5.618	2.474
2	4	4.408	3.659
2	5	3.564	2.948
2	6	3.137	3.473
2	7	1.490	2.542
2	8	5.060	2.438
2	9	3.029	2.394
2	10	4.994	3.593
2	11	3.280	3.177
2	12	3.363	2.771
2	13	4.354	3.468
2	14	2.984	3.579
2	15	3.827	4.212

Notes.

	160 ℃/80 ℃	180 ℃/100 ℃
Mean particle size (μm)	3.585	3.059
SEM	0.208	0.109

Appendix 3. Raw data at the storage stage

Table 3.1a. Cell survival (log CFU/g) of DPC16 microcapsule powders in different treatments during storage at 25 ℃

				Cell survival (log CFU/g) during storage (weeks)				
Temperature (℃)	Wall	Package	Replication	0	1	2	3	4
25	1	1	1	8.455	8.471	8.450	8.152	7.964
25	1	1	2	8.455	8.519	8.207	8.146	8.004
25	1	1	3	8.959	8.699	8.845	8.613	8.458
25	1	1	4	8.959	8.778	8.653	8.672	8.107
25	2	1	1	8.081	7.968	7.571	7.083	6.716
25	2	1	2	8.081	8.079	7.799	7.230	6.690
25	2	1	3	8.328	8.000	8.064	8.013	7.869
25	2	1	4	8.328	9.461	8.170	7.968	7.462
25	1	2	1	8.455	8.299	8.408	8.270	8.417
25	1	2	2	8.455	8.312	8.401	8.320	8.272
25	1	2	3	8.959	8.724	8.914	8.613	8.446
25	1	2	4	8.959	8.886	8.851	8.663	8.732
25	2	2	1	8.081	7.792	7.934	7.342	7.613
25	2	2	2	8.081	7.560	7.851	7.748	7.869
25	2	2	3	8.328	8.173	8.270	8.033	8.204
25	2	2	4	8.328	8.340	8.352	8.130	8.246

Table 3.1b. Cell survival of DPC16 microcapsule powders in different treatments during storage at 55 ℃

				Cell survival (log CFU/g) during storage (weeks)				
Temperature (℃)	Wall	Package	Replication	0	1	2	3	4
55	1	1	1	8.455	3.763	2.903	3.090	2.724
55	1	1	2	8.455	3.851	3.204	3.217	3.491
55	1	1	3	8.959	6.064	4.352	3.378	2.898
55	1	1	4	8.959	5.771	4.241	3.340	3.100
55	2	1	1	8.081	4.083	3.519	3.188	3.117
55	2	1	2	8.081	3.681	3.439	3.241	3.121
55	2	1	3	8.328	1.602	1.477	1.301	1.000
55	2	1	4	8.328	2.041	1.778	0.000	0.000
55	1	2	1	8.455	2.690	2.477	3.470	1.477
55	1	2	2	8.455	3.037	3.303	3.013	1.000
55	1	2	3	8.959	3.949	3.248	2.568	2.114
55	1	2	4	8.959	3.663	3.225	2.778	2.544
55	2	2	1	8.081	2.544	2.556	2.580	1.954
55	2	2	2	8.081	2.602	2.699	2.653	3.000
55	2	2	3	8.328	1.301	0.000	0.000	0.000
55	2	2	4	8.328	1.000	0.000	0.000	0.000

Notes.
Temperature = storage temperature.
Wall = wall material.
In the column of Wall, 1 = reconstituted skim milk, 2 = mixed wall material.
Package = packaging material.
In the column of Package, 1 = gas-impermeable film, 2 = aluminium foil bag.

Table 3.2a. Water activity of DPC16 microcapsule powders in different treatments during storage at 25 ℃

				Water activity of powder during storage (weeks)				
Temperature (℃)	Wall	Package	Replication	0	1	2	3	4
25	1	1	1	0.277	0.299	0.329	0.332	0.374
25	1	1	2	0.277	0.308	0.356	0.306	0.331
25	1	1	3	0.259	0.309	0.331	0.281	0.333
25	1	1	4	0.259	0.273	0.302	0.276	0.348
25	2	1	1	0.233	0.282	0.307	0.266	0.329
25	2	1	2	0.233	0.306	0.325	0.262	0.35
25	2	1	3	0.244	0.281	0.295	0.286	0.347
25	2	1	4	0.244	0.313	0.315	0.282	0.382
25	1	2	1	0.277	0.23	0.225	0.227	0.212
25	1	2	2	0.277	0.22	0.205	0.207	0.227
25	1	2	3	0.259	0.25	0.236	0.226	0.236
25	1	2	4	0.259	0.243	0.178	0.196	0.212
25	2	2	1	0.233	0.226	0.2	0.197	0.219
25	2	2	2	0.233	0.255	0.186	0.194	0.206
25	2	2	3	0.244	0.264	0.259	0.22	0.231
25	2	2	4	0.244	0.259	0.261	0.216	0.234

Table 3.2b. Water activity of DPC16 microcapsule powders during storage at 55 ℃

				Water activity of powder during storage (weeks)				
Temperature (℃)	Wall	Package	Replication	0	1	2	3	4
55	1	1	1	0.277	0.165	0.152	0.083	0.123
55	1	1	2	0.277	0.161	0.163	0.063	0.091
55	1	1	3	0.259	0.125	0.130	0.097	0.099
55	1	1	4	0.259	0.129	0.110	0.088	0.094
55	2	1	1	0.233	0.102	0.096	0.064	0.073
55	2	1	2	0.233	0.100	0.085	0.069	0.086
55	2	1	3	0.244	0.097	0.090	0.068	0.077
55	2	1	4	0.244	0.097	0.095	0.063	0.110
55	1	2	1	0.277	0.228	0.233	0.173	0.210
55	1	2	2	0.277	0.200	0.207	0.197	0.157
55	1	2	3	0.259	0.205	0.198	0.234	0.199
55	1	2	4	0.259	0.205	0.158	0.169	0.221
55	2	2	1	0.233	0.191	0.182	0.188	0.198
55	2	2	2	0.233	0.204	0.202	0.195	0.182
55	2	2	3	0.244	0.209	0.209	0.209	0.269
55	2	2	4	0.244	0.208	0.201	0.241	0.228

Notes.
Temperature = storage temperature.
Wall = wall material.
In the column of Wall, 1 = reconstituted skim milk, 2 = mixed wall material.
Package = packaging material.
In the column of Package, 1 = gas-impermeable film, 2 = aluminium foil bag.

Table 3.3a. Lightness of DPC16 microcapsule powders in different treatments during storage at 25 ℃

					Lightness of powders during storage (weeks)				
Temperature (℃)	Wall	Package	Batch#	Replication	0	1	2	3	4
25	1	1	1	1	94.550	93.710	91.700	87.170	90.770
25	1	1	1	2	94.550	93.010	92.970	91.180	92.370
25	1	1	2	1	95.275	93.450	93.090	92.930	92.170
25	1	1	2	2	95.275	93.310	93.370	92.500	92.330
25	2	1	1	1	96.380	93.320	93.680	93.120	92.520
25	2	1	1	2	96.380	93.560	92.860	93.210	92.470
25	2	1	2	1	95.210	93.300	91.860	93.320	91.960
25	2	1	2	2	95.210	93.170	93.220	93.220	92.400
25	1	2	1	1	94.550	92.420	92.640	92.320	93.370
25	1	2	1	2	94.550	92.400	92.910	91.810	91.860
25	1	2	2	1	95.275	93.370	92.480	92.740	92.040
25	1	2	2	2	95.275	93.360	92.330	93.360	91.650
25	2	2	1	1	96.380	93.430	92.780	92.610	91.660
25	2	2	1	2	96.380	93.580	93.280	93.560	92.370
25	2	2	2	1	95.210	93.540	92.870	93.580	91.290
25	2	2	2	2	95.210	93.190	92.210	93.030	91.830

Notes.
Temperature = storage temperature.
Wall = wall material.
In the column of Wall, 1 = reconstituted skim milk, 2 = mixed wall material.
Package = packaging material.
In the column of Package, 1 = gas-impermeable film, 2 = aluminium foil bag.

Treatments	Mean/SEM	Lightness of powders during storage (weeks)				
		0	1	2	3	4
RSM, gas-impermeable film	Mean	94.913	93.370	92.783	90.945	91.910
	SEM	0.209	0.146	0.370	1.312	0.382
RSM, aluminium foil bag	Mean	94.913	92.888	92.590	92.558	92.230
	SEM	0.209	0.276	0.124	0.328	0.388
MWM, gas-impermeable film	Mean	95.795	93.338	92.905	93.218	92.338
	SEM	0.675	0.163	0.773	0.082	0.256
MWM, aluminium foil bag	Mean	95.795	93.435	92.785	93.195	91.788
	SEM	0.338	0.088	0.220	0.233	0.225

Table 3.3b. Lightness of DPC16 microcapsule powders in different treatments during storage at 55 ℃

Temperature (℃)	Wall	Package	Replication	Lightness of powder during storage (weeks)				
				0	1	2	3	4
55	1	1	1	94.550	91.470	92.020	91.980	90.720
55	1	1	2	94.550	93.330	92.550	93.010	92.860
55	1	1	3	95.275	93.480	93.240	91.940	92.570
55	1	1	4	95.275	93.490	92.510	92.130	92.060
55	2	1	1	96.380	93.750	92.530	92.260	92.190
55	2	1	2	96.380	93.550	91.600	92.440	92.200
55	2	1	3	95.210	92.280	92.850	92.000	92.250
55	2	1	4	95.210	92.620	92.240	92.780	91.470
55	1	2	1	94.550	93.150	92.880	93.170	91.660
55	1	2	2	94.550	92.500	92.650	91.930	92.380
55	1	2	3	95.275	93.400	91.960	91.250	87.630
55	1	2	4	95.275	93.260	92.690	93.140	92.410
55	2	2	1	96.380	92.650	89.820	90.760	90.310
55	2	2	2	96.380	91.400	90.920	91.160	90.170
55	2	2	3	95.210	92.370	90.970	90.290	89.530
55	2	2	4	95.210	91.410	91.670	89.850	89.010

Notes.
Temperature = storage temperature.
Wall = wall material.
In the column of Wall, 1 = reconstituted skim milk, 2 = mixed wall material.
Package = packaging material.
In the column of Pack, 1 = gas-impermeable film, 2 = aluminium foil bag.

Treatments	Mean/SEM	Lightness of powders during storage (weeks)				
		0	1	2	3	4
RSM, gas-impermeable film	Mean	94.913	92.943	92.580	92.265	92.053
	SEM	0.209	0.492	0.251	0.252	0.474
RSM, aluminium foil bag	Mean	94.913	93.078	92.545	92.373	91.020
	SEM	0.419	0.398	0.403	0.945	2.286
MWM, gas-impermeable film	Mean	95.795	93.050	92.305	92.370	92.028
	SEM	0.676	0.711	0.532	0.328	0.373
MWM, aluminium foil bag	Mean	95.795	91.958	90.845	90.515	89.755
	SEM	0.338	0.324	0.382	0.284	0.301

Table 3.4a. Redness of DPC16 microcapsule powders in different treatments during storage at 25 ℃

				Redness of powder during storage (weeks)				
Temperature (℃)	Wall	Package	Replication	0	1	2	3	4
25	1	1	1	-3.580	-2.900	-3.030	-3.040	-3.400
25	1	1	2	-3.520	-3.870	-3.580	-3.670	-3.480
25	1	1	3	-3.040	-3.270	-4.350	-4.050	-3.870
25	1	1	4	-3.340	-3.810	-4.680	-4.350	-3.890
25	2	1	1	-1.460	-1.600	-2.620	-1.500	-1.930
25	2	1	2	-1.380	-2.020	-2.120	-1.980	-1.490
25	2	1	3	-1.400	-1.490	-1.950	-2.030	-2.100
25	2	1	4	-1.400	-1.440	-1.500	-1.520	-1.530
25	1	2	1	-3.580	-3.140	-4.140	-4.000	-3.980
25	1	2	2	-3.520	-4.180	-3.880	-4.470	-2.890
25	1	2	3	-3.040	-3.820	-4.580	-4.540	-3.090
25	1	2	4	-3.340	-3.870	-4.660	-3.630	-3.660
25	2	2	1	-1.460	-1.570	-1.840	-2.050	-1.960
25	2	2	2	-1.380	-2.090	-1.440	-1.550	-2.040
25	2	2	3	-1.400	-1.630	-1.920	-1.510	-2.040
25	2	2	4	-1.400	-2.120	-1.490	-1.990	-1.660

Notes.
Temperature = storage temperature.
Wall = wall material.
In the column of Wall, 1 = reconstituted skim milk, 2 = mixed wall material.
Package = packaging material.
In the column of Package, 1 = gas-impermeable film, 2 = aluminium foil bag.

		Redness of powders during storage (weeks)				
Treatments	Mean/SEM	0	1	2	3	4
RSM, gas-impermeable film	Mean	-3.370	-3.463	-3.910	-3.778	-3.660
	SEM	0.121	0.231	0.373	0.282	0.128
RSM, aluminium foil bag	Mean	-3.370	-3.753	-4.315	-4.160	-3.405
	SEM	0.121	0.219	0.185	0.214	0.252
MWM, gas-impermeable film	Mean	-1.410	-1.638	-2.048	-1.758	-1.763
	SEM	0.017	0.132	0.231	0.143	0.150
MWM, aluminium foil bag	Mean	-1.410	-1.853	-1.673	-1.775	-1.925
	SEM	0.017	0.146	0.121	0.142	0.090

Table 3.4b. Redness of DPC16 microcapsule powders in different treatments during storage at 55 ℃

				Redness of powder during storage (weeks)				
Temperature (℃)	Wall	Package	Replication	0	1	2	3	4
55	1	1	1	-3.550	-4.010	-4.530	-3.980	-3.070
55	1	1	2	-3.550	-4.100	-3.960	-3.910	-3.450
55	1	1	3	-3.190	-3.830	-3.640	-4.160	-3.150
55	1	1	4	-3.190	-4.180	-4.390	-3.710	-3.070
55	2	1	1	-1.420	-1.920	-2.190	-1.990	-1.940
55	2	1	2	-1.420	-2.370	-1.720	-2.450	-2.400
55	2	1	3	-1.400	-1.850	-2.350	-2.580	2.590
55	2	1	4	-1.400	-2.210	-1.740	-2.180	-2.040
55	1	2	1	-3.550	-3.580	-4.230	-3.530	-2.900
55	1	2	2	-3.550	-4.160	-4.720	-3.590	-3.390
55	1	2	3	-3.190	-3.860	-3.940	-3.610	-3.540
55	1	2	4	-3.190	-4.030	-3.820	-3.930	-2.950

55	2	2	1	-1.420	-1.410	-1.870	-1.340	-2.000
55	2	2	2	-1.420	-1.980	-1.050	-1.990	-1.510
55	2	2	3	-1.400	-1.430	-1.500	-1.880	-2.150
55	2	2	4	-1.400	-1.980	-1.190	-1.340	-0.810

Notes.
Temperature = storage temperature.
Wall = wall material.
In the column of Wall, 1 = reconstituted skim milk, 2 = mixed wall material.
Package = packaging material.
In the column of Package, 1 = gas-impermeable film, 2 = aluminium foil bag.

		Redness of powders during storage (weeks)				
Treatments	Mean/SEM	0	1	2	3	4
RSM, gas-impermeable film	Mean	-3.370	-4.030	-4.130	-3.940	-3.185
	SEM	0.104	0.075	0.203	0.093	0.090
RSM, aluminium foil bag	Mean	-3.370	-3.908	-4.178	-3.665	-3.195
	SEM	0.104	0.125	0.200	0.090	0.159
MWM, gas-impermeable film	Mean	-3.370	-4.030	-4.130	-3.940	-3.185
	SEM	0.104	0.075	0.203	0.093	0.090
MWM, aluminium foil bag	Mean	-1.410	-1.700	-1.403	-1.638	-1.618
	SEM	0.006	0.162	0.182	0.173	0.302

Table 3.5a. Yellowness of DPC16 microcapsule powders in different treatments during storage at 25 ℃

				Yellowness of powder during storage (weeks)				
Temperature (℃)	Wall	Package	Replication	0	1	2	3	4
25	1	1	1	8.480	4.340	5.020	5.290	4.830
25	1	1	2	8.210	5.820	4.810	5.360	6.510
25	1	1	3	5.870	6.060	8.600	6.490	5.060
25	1	1	4	6.690	6.080	7.640	9.540	6.900
25	2	1	1	2.090	3.110	2.850	3.670	1.540
25	2	1	2	2.370	2.210	2.540	2.210	1.750
25	2	1	3	1.740	2.670	2.030	3.240	2.270
25	2	1	4	2.010	1.640	3.560	2.660	1.610
25	1	2	1	8.480	6.090	8.590	8.460	6.220
25	1	2	2	8.210	7.320	5.320	7.810	3.630
25	1	2	3	5.870	7.600	9.040	8.100	3.800
25	1	2	4	6.690	5.970	6.850	6.870	3.990
25	2	2	1	2.090	2.380	2.360	2.280	1.330
25	2	2	2	2.370	2.400	2.210	3.300	1.890
25	2	2	3	1.740	2.200	1.860	2.470	1.300
25	2	2	4	2.010	1.840	2.270	1.830	1.450

Notes.
Temperature = storage temperature.
Wall = wall material.
In the column of Wall, 1 = reconstituted skim milk, 2 = mixed wall material.
Package = packaging material.
In the column of Package, 1 = gas-impermeable film, 2 = aluminium foil bag.

		Yellowness of powders during storage (weeks)				
Treatments	Mean/SEM	0	1	2	3	4
RSM, gas-impermeable film	Mean	7.313	5.575	6.518	6.670	5.825
	SEM	0.622	0.416	0.947	0.995	0.516
RSM, aluminium foil bag	Mean	7.313	6.745	7.450	7.810	4.410
	SEM	0.622	0.417	0.853	0.340	0.608

MWM, gas-impermeable film	Mean	2.053	2.408	2.745	2.945	1.793
	SEM	0.130	0.315	0.330	0.321	0.165
MWM, aluminium foil bag	Mean	2.053	2.205	2.175	2.470	1.493
	SEM	0.130	0.130	0.109	0.307	0.136

Table 3.5b. Yellowness of DPC16 microcapsule powders in different treatments during storage at 55 ℃

				Yellowness of powder during storage (weeks)				
Temperature (℃)	Wall	Package	Replication	0	1	2	3	4
55	1	1	1	8.345	9.360	9.690	7.370	6.380
55	1	1	2	8.345	7.140	9.920	9.610	5.840
55	1	1	3	6.280	7.780	7.630	7.740	6.280
55	1	1	4	6.280	7.090	8.750	7.800	5.480
55	2	1	1	2.230	4.400	4.150	7.260	3.990
55	2	1	2	2.230	3.960	5.850	5.580	4.240
55	2	1	3	1.875	6.180	5.740	6.140	3.880
55	2	1	4	1.875	5.290	6.590	5.150	3.360
55	1	2	1	8.345	6.970	8.920	5.820	5.720
55	1	2	2	8.345	7.250	9.720	9.420	5.540
55	1	2	3	6.280	8.100	7.050	8.560	5.830
55	1	2	4	6.280	6.670	9.610	7.860	5.230
55	2	2	1	2.230	6.200	6.950	9.530	7.450
55	2	2	2	2.230	5.360	9.620	7.290	8.660
55	2	2	3	1.875	6.900	9.180	9.490	8.560
55	2	2	4	1.875	6.720	8.460	10.070	12.470

Notes.
Temperature = storage temperature.
Wall = wall material.
In the column of Wall, 1 = reconstituted skim milk, 2 = mixed wall material.
Package = packaging material.
In the column of Package, 1 = gas-impermeable film, 2 = aluminium foil bag.

		Yellowness of powders during storage (weeks)				
Treatments	Mean/SEM	0	1	2	3	4
RSM, gas-impermeable film	Mean	7.313	7.843	8.998	8.130	5.995
	SEM	0.596	0.530	0.521	0.502	0.208
RSM, aluminium foil bag	Mean	7.313	7.248	8.825	7.915	5.580
	SEM	0.596	0.308	0.618	0.768	0.131
MWM, gas-impermeable film	Mean	2.053	4.958	5.583	6.033	3.868
	SEM	0.102	0.493	0.513	0.457	0.185
MWM, aluminium foil bag	Mean	2.053	6.295	8.553	9.095	9.285
	SEM	0.102	0.345	0.585	0.616	1.096

Table 3.6a. Moisture content of DPC16 microcapsule powders in different treatments during storage at 25 ℃

				Moisture content of powder during storage (weeks)				
Temperature (℃)	Wall	Package	Replication	0	1	2	3	4
25	1	1	1	0.0491	0.053	0.0565	0.0587	0.0603
25	1	1	2	0.0496	0.0536	0.057	0.0586	0.0601
25	1	1	3	0.0492	0.0501	0.0565	0.0579	0.0599
25	1	1	4	0.0498	0.0534	0.0565	0.0591	0.0602
25	2	1	1	0.0396	0.0442	0.0473	0.0511	0.0535
25	2	1	2	0.0398	0.0445	0.0487	0.0519	0.0541
25	2	1	3	0.0393	0.0446	0.0492	0.0525	0.0538

25	2	1	4	0.0391	0.0452	0.0489	0.0503	0.0542
25	1	2	1	0.0491	0.0445	0.0442	0.0459	0.0455
25	1	2	2	0.0496	0.0465	0.0461	0.0463	0.0459
25	1	2	3	0.0492	0.0467	0.0462	0.0456	0.0451
25	1	2	4	0.0498	0.0448	0.0455	0.0461	0.0466
25	2	2	1	0.0396	0.0374	0.0373	0.0365	0.036
25	2	2	2	0.0398	0.0348	0.0356	0.0349	0.0351
25	2	2	3	0.0393	0.0359	0.0361	0.0362	0.0358
25	2	2	4	0.0391	0.0361	0.0359	0.0363	0.0354

Notes.
Temperature = storage temperature.
Wall = wall material.
In the column of Wall, 1 = reconstituted skim milk, 2 = mixed wall material.
Package = packaging material.
In the column of Package, 1 = gas-impermeable film, 2 = aluminium foil bag.

Treatments	Mean/SEM	Moisture content of powders during storage (weeks)				
		0	1	2	3	4
RSM, gas-impermeable film	Mean	0.0494	0.0525	0.0566	0.0586	0.0601
	SEM	0.0002	0.0008	0.0001	0.0002	0.0001
MWM, gas-impermeable film	Mean	0.0395	0.0446	0.0485	0.0515	0.0539
	SEM	0.0002	0.0002	0.0004	0.0005	0.0002
RSM, aluminium foil bag	Mean	0.0494	0.0456	0.0455	0.0460	0.0458
	SEM	0.0002	0.0006	0.0005	0.0001	0.0003
MWM, aluminium foil bag	Mean	0.0395	0.0361	0.0362	0.0360	0.0356
	SEM	0.0002	0.0005	0.0004	0.0004	0.0002

Table 3.6b. Moisture content of DPC16 microcapsule powders in different treatments during storage at 55 ℃

Temperature (℃)	Wall	Package	Replication	Moisture content of powder during storage (weeks)				
				0	1	2	3	4
55	1	1	1	0.0491	0.0245	0.0250	0.0238	0.0250
55	1	1	2	0.0496	0.0265	0.0257	0.0251	0.0238
55	1	1	3	0.0492	0.0251	0.0241	0.0243	0.0248
55	1	1	4	0.0498	0.0249	0.0252	0.0255	0.0247
55	2	1	1	0.0396	0.0269	0.0257	0.0254	0.0264
55	2	1	2	0.0398	0.0280	0.0267	0.0271	0.0257
55	2	1	3	0.0393	0.0281	0.0247	0.0237	0.0261
55	2	1	4	0.0391	0.0291	0.0275	0.0266	0.0256
55	1	2	1	0.0491	0.0400	0.0403	0.0378	0.0402
55	1	2	2	0.0496	0.0435	0.0398	0.0393	0.0401
55	1	2	3	0.0492	0.0397	0.0396	0.0392	0.0403
55	1	2	4	0.0498	0.0371	0.0395	0.0380	0.0406
55	2	2	1	0.0396	0.0354	0.0368	0.0355	0.0345
55	2	2	2	0.0398	0.0352	0.0345	0.0359	0.0351
55	2	2	3	0.0393	0.0353	0.0345	0.0343	0.0341
55	2	2	4	0.0391	0.0343	0.0323	0.0333	0.0321

Notes.
Temperature = storage temperature.
Wall = wall material.

In the column of Wall, 1 = reconstituted skim milk, 2 = mixed wall material.
Package = packaging material.
In the column of Package, 1 = gas-impermeable film, 2 = aluminium foil bag.

		Moisture content of powders during storage (weeks)				
Treatments	Mean/SEM	0	1	2	3	4
MC of RSM film	Mean	0.0494	0.0252	0.0250	0.0247	0.0246
	SEM	0.0002	0.0004	0.0003	0.0004	0.0003
MC of MWM film	Mean	0.0395	0.0280	0.0262	0.0257	0.0260
	SEM	0.0002	0.0007	0.0006	0.0005	0.0002
MC of RSM alu	Mean	0.0494	0.0401	0.0398	0.0386	0.0403
	SEM	0.0002	0.0013	0.0002	0.0004	0.0001
MC of MWM alu	Mean	0.0395	0.0351	0.0345	0.0347	0.0339
	SEM	0.0002	0.0003	0.0009	0.0006	0.0007

Table 3.7a. Bulk density (g/cm3) of DPC16 microcapsule powders in different treatments during storage at 25 ℃

				Bulk density content of powder during storage (weeks)				
Temperature (℃)	Wall	Package	Replication	0	1	2	3	4
25	1	1	1	0.345	0.310	0.290	0.271	0.264
25	1	1	2	0.345	0.315	0.295	0.279	0.267
25	1	1	3	0.337	0.298	0.270	0.265	0.247
25	1	1	4	0.337	0.315	0.298	0.286	0.271
25	2	1	1	0.402	0.410	0.385	0.376	0.351
25	2	1	2	0.402	0.390	0.381	0.366	0.338
25	2	1	3	0.389	0.382	0.374	0.366	0.356
25	2	1	4	0.389	0.380	0.370	0.360	0.354
25	1	2	1	0.345	0.341	0.340	0.345	0.345
25	1	2	2	0.345	0.340	0.343	0.345	0.329
25	1	2	3	0.337	0.336	0.332	0.332	0.338
25	1	2	4	0.337	0.335	0.343	0.338	0.330
25	2	2	1	0.402	0.390	0.395	0.383	0.386
25	2	2	2	0.402	0.390	0.390	0.384	0.386
25	2	2	3	0.389	0.382	0.388	0.405	0.413
25	2	2	4	0.389	0.386	0.390	0.405	0.399

Notes.
Temperature = storage temperature.
Wall = wall material.
In the column of Wall, 1 = reconstituted skim milk, 2 = mixed wall material.
Package = packaging material.
In the column of Package, 1 = gas-impermeable film, 2 = aluminium foil bag.

		Bulk density of powders during storage (weeks)				
	Mean/SEM	0	1	2	3	4
RSM, gas-impermeable film	Mean	0.341	0.310	0.288	0.275	0.262
	SEM	0.002	0.004	0.006	0.005	0.005
MWM, gas-impermeable film	Mean	0.396	0.391	0.378	0.367	0.350
	SEM	0.004	0.007	0.003	0.003	0.004
RSM, aluminium foil bag	Mean	0.341	0.338	0.340	0.340	0.336
	SEM	0.002	0.001	0.003	0.003	0.004
MWM, aluminium foil bag	Mean	0.396	0.387	0.391	0.394	0.396
	SEM	0.004	0.002	0.001	0.006	0.006

Table 3.7b. Bulk density (g/cm3) of DPC16 microcapsule powders in different treatments during storage at 55 ℃

				Bulk density of powder during storage (weeks)				
Temperature (℃)	Wall	Package	Replication	0	1	2	3	4
55	1	1	1	0.345	0.409	0.413	0.419	0.423
55	1	1	2	0.345	0.397	0.410	0.407	0.417
55	1	1	3	0.337	0.400	0.411	0.416	0.421
55	1	1	4	0.337	0.406	0.420	0.413	0.419
55	2	1	1	0.402	0.459	0.466	0.479	0.487
55	2	1	2	0.402	0.456	0.467	0.475	0.479
55	2	1	3	0.389	0.450	0.459	0.468	0.481
55	2	1	4	0.389	0.447	0.465	0.477	0.491
55	1	2	1	0.345	0.375	0.374	0.376	0.384
55	1	2	2	0.345	0.370	0.368	0.365	0.371
55	1	2	3	0.337	0.361	0.368	0.366	0.369
55	1	2	4	0.337	0.360	0.360	0.366	0.368
55	2	2	1	0.402	0.426	0.422	0.418	0.427
55	2	2	2	0.402	0.324	0.421	0.431	0.429
55	2	2	3	0.389	0.417	0.422	0.429	0.426
55	2	2	4	0.389	0.423	0.419	0.428	0.432

Notes.
Temperature = storage temperature.
Wall = wall material.
In the column of Wall, 1 = reconstituted skim milk, 2 = mixed wall material.
Package = packaging material.
In the column of Package, 1 = gas-impermeable film, 2 = aluminium foil bag.

		Bulk density of powders during storage (weeks)				
Treatments	Mean/SEM	0	1	2	3	4
RSM, gas-impermeable film	Mean	0.341	0.403	0.414	0.414	0.420
	SEM	0.002	0.003	0.002	0.003	0.001
MWM, gas-impermeable film	Mean	0.396	0.453	0.464	0.475	0.485
	SEM	0.004	0.003	0.002	0.002	0.003
RSM, aluminium foil bag	Mean	0.341	0.367	0.368	0.368	0.373
	SEM	0.002	0.004	0.003	0.003	0.004
MWM, aluminium foil bag	Mean	0.396	0.398	0.421	0.427	0.429
	SEM	0.004	0.025	0.001	0.003	0.001

Table 3.8a. Particle size (μm) of DPC16 microcapsule powders in different treatments during storage at 25 ℃

					Particle size of powders during storage (weeks)				
Temperature (℃)	Wall	Package	Batch#	Replication	0	1	2	3	4
25	1	1	1	1	5.594	2.507	2.973	3.773	2.909
25	1	1	1	2	1.921	4.364	3.561	5.440	7.139
25	1	1	1	3	5.447	2.834	3.809	2.397	3.051
25	1	1	1	4	3.916	5.577	5.323	4.156	4.227
25	1	1	1	5	3.608	4.280	2.417	1.848	1.919
25	1	1	1	6	3.284	4.882	6.682	3.105	1.841
25	1	1	1	7	3.265	2.889	3.068	5.945	4.996
25	1	1	1	8	4.031	6.700	3.509	2.614	3.861
25	1	1	1	9	1.652	3.071	5.377	5.009	3.772
25	1	1	1	10	2.089	6.646	2.508	4.515	3.996
25	1	1	1	11	2.155	3.712	4.807	6.234	3.930
25	1	1	1	12	2.213	1.352	10.018	2.083	2.148

25	1	1	1	13	2.838	5.762	2.022	2.654	3.069
25	1	1	1	14	4.642	5.280	5.376	3.141	4.734
25	1	1	1	15	3.806	3.097	3.187	2.440	3.082
25	1	1	2	1	3.827	2.252	2.222	1.636	3.468
25	1	1	2	2	4.163	2.044	6.284	1.780	6.809
25	1	1	2	3	5.618	3.141	3.883	3.579	3.653
25	1	1	2	4	4.408	2.203	1.634	2.504	4.720
25	1	1	2	5	3.564	6.940	2.568	4.386	3.062
25	1	1	2	6	3.137	2.433	5.282	1.715	2.073
25	1	1	2	7	1.490	1.946	2.282	6.518	1.767
25	1	1	2	8	5.060	5.536	3.331	2.486	1.940
25	1	1	2	9	3.029	4.591	3.985	2.066	7.055
25	1	1	2	10	4.994	2.074	1.967	1.547	7.342
25	1	1	2	11	3.280	4.772	7.243	4.842	3.831
25	1	1	2	12	3.363	3.712	6.574	2.476	5.255
25	1	1	2	13	4.354	1.891	1.878	5.982	6.218
25	1	1	2	14	2.984	3.993	3.641	1.886	4.113
25	1	1	2	15	3.827	2.424	2.075	2.311	6.310
25	1	2	1	1	5.594	4.358	3.660	3.313	2.432
25	1	2	1	2	1.921	4.941	2.976	2.598	3.298
25	1	2	1	3	5.447	8.024	3.843	5.597	3.590
25	1	2	1	4	3.916	2.485	2.764	4.973	3.360
25	1	2	1	5	3.608	3.051	3.319	1.899	2.188
25	1	2	1	6	3.284	7.910	3.819	8.585	2.588
25	1	2	1	7	3.265	4.462	4.182	1.169	4.794
25	1	2	1	8	4.031	4.158	2.234	2.204	3.922
25	1	2	1	9	1.652	4.837	6.870	2.687	2.640
25	1	2	1	10	2.089	3.840	2.286	5.909	1.690
25	1	2	1	11	2.155	5.356	2.917	2.437	2.850
25	1	2	1	12	2.213	2.895	3.280	3.432	2.938
25	1	2	1	13	2.838	2.805	7.841	6.017	2.270
25	1	2	1	14	4.642	4.384	5.467	2.450	3.915
25	1	2	1	15	3.806	3.182	3.698	3.145	2.244
25	1	2	2	1	3.827	3.437	4.501	4.943	3.196
25	1	2	2	2	4.163	6.293	5.011	2.576	3.996
25	1	2	2	3	5.618	12.662	7.271	6.676	2.861
25	1	2	2	4	4.408	9.876	2.920	3.145	2.091
25	1	2	2	5	3.564	6.243	2.752	3.756	4.266
25	1	2	2	6	3.137	4.884	6.804	4.546	5.329
25	1	2	2	7	1.490	3.495	2.925	2.562	5.112
25	1	2	2	8	5.060	7.283	2.517	2.563	9.001
25	1	2	2	9	3.029	3.927	3.092	8.082	2.733
25	1	2	2	10	4.994	5.382	2.810	2.437	3.363
25	1	2	2	11	3.280	3.965	3.328	7.234	1.699
25	1	2	2	12	3.363	4.084	2.428	4.886	5.564
25	1	2	2	13	4.354	12.765	2.064	6.538	3.873
25	1	2	2	14	2.984	4.878	4.775	1.146	4.274
25	1	2	2	15	3.827	4.377	2.993	2.648	3.069
25	2	1	1	1	7.039	2.316	4.325	2.548	4.526

25	2	1	1	2	3.421	2.769	1.392	3.051	2.964
25	2	1	1	3	2.450	1.667	2.368	1.849	4.145
25	2	1	1	4	2.710	2.518	1.952	2.819	5.054
25	2	1	1	5	2.559	2.204	2.695	3.276	6.785
25	2	1	1	6	5.793	3.720	3.973	2.491	5.976
25	2	1	1	7	2.580	2.585	1.605	3.480	4.878
25	2	1	1	8	3.045	10.728	1.729	3.781	3.772
25	2	1	1	9	3.503	2.960	1.759	3.482	4.510
25	2	1	1	10	2.668	2.965	6.705	3.051	3.219
25	2	1	1	11	5.818	3.712	2.692	3.602	3.864
25	2	1	1	12	2.778	1.352	5.494	6.354	2.637
25	2	1	1	13	3.514	5.762	2.835	3.338	3.587
25	2	1	1	14	2.571	5.280	2.825	6.200	5.417
25	2	1	1	15	2.882	3.097	3.362	4.408	3.866
25	2	1	2	1	2.205	1.678	4.690	1.581	3.755
25	2	1	2	2	3.571	3.284	3.885	4.044	2.293
25	2	1	2	3	3.453	1.788	2.066	2.956	6.798
25	2	1	2	4	3.452	3.404	1.399	2.887	1.908
25	2	1	2	5	2.333	4.843	2.334	2.315	4.113
25	2	1	2	6	4.319	2.679	5.525	2.220	2.970
25	2	1	2	7	6.375	3.616	3.791	2.692	5.212
25	2	1	2	8	3.203	7.354	3.051	4.450	2.556
25	2	1	2	9	3.646	5.014	1.862	2.246	4.080
25	2	1	2	10	2.705	4.780	2.754	3.811	3.695
25	2	1	2	11	3.193	4.461	4.876	2.083	2.701
25	2	1	2	12	3.762	2.423	3.501	2.912	6.793
25	2	1	2	13	4.496	2.844	7.975	2.138	2.761
25	2	1	2	14	5.252	4.501	2.516	4.208	3.582
25	2	1	2	15	3.190	5.410	3.167	1.938	1.913
25	2	2	1	1	7.039	2.583	3.024	4.872	3.367
25	2	2	1	2	3.421	4.469	4.315	2.720	2.953
25	2	2	1	3	2.450	3.239	3.697	1.699	2.124
25	2	2	1	4	2.710	2.238	5.390	4.005	3.014
25	2	2	1	5	2.559	2.980	4.693	5.215	6.037
25	2	2	1	6	5.793	3.055	6.130	3.477	5.953
25	2	2	1	7	2.580	3.351	3.285	3.003	2.707
25	2	2	1	8	3.045	2.623	2.395	4.814	9.873
25	2	2	1	9	3.503	2.486	5.587	4.096	2.661
25	2	2	1	10	2.668	7.905	3.281	4.311	4.259
25	2	2	1	11	5.818	3.093	5.840	2.620	5.504
25	2	2	1	12	2.778	4.612	2.658	2.013	2.948
25	2	2	1	13	3.514	4.099	4.370	2.202	2.911
25	2	2	1	14	2.571	6.296	2.785	3.004	3.531
25	2	2	1	15	2.882	3.257	10.405	4.288	4.401
25	2	2	2	1	2.205	3.816	3.367	3.626	5.213
25	2	2	2	2	3.571	7.330	2.953	7.168	2.506
25	2	2	2	3	3.453	2.128	2.124	2.313	4.270
25	2	2	2	4	3.452	2.516	3.014	2.581	4.739
25	2	2	2	5	2.333	2.722	6.037	3.286	4.365

25	2	2	2	6	4.319	2.284	5.953	2.827	2.649
25	2	2	2	7	6.375	1.617	2.707	4.359	2.346
25	2	2	2	8	3.203	7.182	9.873	2.464	12.509
25	2	2	2	9	3.646	3.018	2.661	7.379	3.174
25	2	2	2	10	2.705	2.132	4.259	3.938	2.817
25	2	2	2	11	3.193	2.431	5.504	3.067	2.766
25	2	2	2	12	3.762	2.701	2.948	2.740	2.394
25	2	2	2	13	4.496	4.408	2.911	3.694	2.635
25	2	2	2	14	5.252	1.961	3.531	3.378	3.371
25	2	2	2	15	3.190	1.164	4.401	5.806	2.404

Notes.
Temperature = storage temperature.
Wall = wall material.
In the column of Wall, 1 = reconstituted skim milk, 2 = mixed wall material.
Package = packaging material.
In the column of Package, 1 = gas-impermeable film, 2 = aluminium foil bag.

		Bulk density of powders during storage (weeks)				
Treatments	Mean/SEM	0	1	2	3	4
RSM, gas-impermeable film	Mean	3.585	3.763	3.983	3.369	4.076
	SEM	0.208	0.293	0.359	0.283	0.308
MWM, gas-impermeable film	Mean	3.616	3.724	3.303	3.207	4.011
	SEM	0.230	0.352	0.292	0.207	0.252
RSM, aluminium foil bag	Mean	3.585	5.341	3.845	4.005	3.505
	SEM	0.208	0.479	0.288	0.371	0.267
MWM, aluminium foil bag	Mean	3.616	3.456	4.337	3.699	4.013
	SEM	0.230	0.311	0.364	0.253	0.414

Table 3.8b. Particle size (μm) of DPC16 microcapsule powders in different treatments during storage at 55 ℃

					Particle size of powders during storage (weeks)				
Temperature (℃)	Wall	Package	Batch#	Replication	0	1	2	3	4
55	1	1	1	1	5.594	2.486	2.619	5.850	4.987
55	1	1	1	2	1.921	2.182	4.262	3.379	4.426
55	1	1	1	3	5.447	3.238	4.535	6.753	6.064
55	1	1	1	4	3.916	5.144	9.122	2.431	1.531
55	1	1	1	5	3.608	3.526	3.474	4.491	3.780
55	1	1	1	6	3.284	3.187	2.429	2.913	4.101
55	1	1	1	7	3.265	2.239	2.936	3.756	3.683
55	1	1	1	8	4.031	3.765	3.057	3.155	2.515
55	1	1	1	9	1.652	2.467	4.369	5.438	3.485
55	1	1	1	10	2.089	5.609	3.560	3.842	4.264
55	1	1	1	11	2.155	3.264	2.827	6.155	4.383
55	1	1	1	12	2.213	2.029	2.085	5.666	4.977
55	1	1	1	13	2.838	3.559	4.488	3.644	5.593
55	1	1	1	14	4.642	2.138	2.109	1.933	1.866
55	1	1	1	15	3.806	4.469	1.732	6.357	3.414
55	1	1	2	1	3.827	4.369	4.383	4.786	3.702
55	1	1	2	2	4.163	3.659	4.624	3.143	2.109
55	1	1	2	3	5.618	2.564	1.630	1.964	2.363

55	1	1	2	4	4.408	4.613	1.837	2.938	4.329
55	1	1	2	5	3.564	4.114	2.662	3.325	6.262
55	1	1	2	6	3.137	3.337	2.362	4.137	2.088
55	1	1	2	7	1.490	4.479	2.410	2.589	3.637
55	1	1	2	8	5.060	3.753	2.766	3.202	3.800
55	1	1	2	9	3.029	2.082	2.607	5.120	2.401
55	1	1	2	10	4.994	2.855	5.416	3.247	4.811
55	1	1	2	11	3.280	3.917	3.249	5.836	2.901
55	1	1	2	12	3.363	4.215	4.413	2.415	2.762
55	1	1	2	13	4.354	2.180	4.085	2.460	2.197
55	1	1	2	14	2.984	3.547	6.508	2.594	5.686
55	1	1	2	15	3.827	4.767	3.368	3.135	2.363
55	1	2	1	1	5.594	3.307	2.914	3.329	4.834
55	1	2	1	2	1.921	1.402	4.824	3.933	3.892
55	1	2	1	3	5.447	3.870	2.369	1.983	2.896
55	1	2	1	4	3.916	2.777	2.840	3.863	6.419
55	1	2	1	5	3.608	2.219	4.644	2.615	2.250
55	1	2	1	6	3.284	2.263	2.228	7.252	2.193
55	1	2	1	7	3.265	6.032	7.202	2.658	5.556
55	1	2	1	8	4.031	4.086	4.629	7.085	6.217
55	1	2	1	9	1.652	3.616	3.316	3.808	5.419
55	1	2	1	10	2.089	5.849	5.277	4.716	2.595
55	1	2	1	11	2.155	1.611	4.800	3.708	2.574
55	1	2	1	12	2.213	4.514	3.261	2.698	2.877
55	1	2	1	13	2.838	5.227	6.350	4.426	2.994
55	1	2	1	14	4.642	5.042	2.425	2.088	3.978
55	1	2	1	15	3.806	1.589	5.118	2.184	6.027
55	1	2	2	1	3.827	3.643	2.134	5.709	3.823
55	1	2	2	2	4.163	6.175	6.008	3.848	2.484
55	1	2	2	3	5.618	3.842	2.589	4.533	2.361
55	1	2	2	4	4.408	2.661	4.971	2.189	2.329
55	1	2	2	5	3.564	2.175	4.771	2.684	2.392
55	1	2	2	6	3.137	5.789	5.558	3.375	2.401
55	1	2	2	7	1.490	5.892	2.134	3.021	2.579
55	1	2	2	8	5.060	3.629	2.073	4.151	5.879
55	1	2	2	9	3.029	2.257	3.325	2.644	2.221
55	1	2	2	10	4.994	3.007	2.443	2.221	2.684
55	1	2	2	11	3.280	2.641	5.211	3.807	5.222
55	1	2	2	12	3.363	2.910	2.511	2.684	1.502
55	1	2	2	13	4.354	2.626	2.245	3.374	2.445
55	1	2	2	14	2.984	7.324	3.267	2.857	2.894
55	1	2	2	15	3.827	2.658	2.325	1.656	2.191
55	2	1	1	1	7.039	4.997	4.929	2.342	2.218
55	2	1	1	2	3.421	2.501	2.625	3.354	1.941
55	2	1	1	3	2.450	2.794	3.178	3.049	2.525
55	2	1	1	4	2.710	2.067	1.855	2.307	4.253
55	2	1	1	5	2.559	4.689	4.367	2.382	2.675
55	2	1	1	6	5.793	2.111	1.817	3.820	5.693
55	2	1	1	7	2.580	2.030	1.832	4.099	2.715

55	2	1	1	8	3.045	2.418	1.826	4.375	3.042
55	2	1	1	9	3.503	4.608	4.298	2.274	5.593
55	2	1	1	10	2.668	3.501	3.002	3.548	2.599
55	2	1	1	11	5.818	3.046	3.180	5.739	3.714
55	2	1	1	12	2.778	3.126	3.355	6.718	3.065
55	2	1	1	13	3.514	2.493	4.473	3.161	3.240
55	2	1	1	14	2.571	4.302	4.435	2.435	3.843
55	2	1	1	15	2.882	4.456	2.684	2.634	4.720
55	2	1	2	1	2.205	3.188	5.003	4.928	2.348
55	2	1	2	2	3.571	3.346	3.120	2.646	1.868
55	2	1	2	3	3.453	2.936	2.103	5.974	3.852
55	2	1	2	4	3.452	2.590	2.608	3.793	1.943
55	2	1	2	5	2.333	2.544	1.838	1.718	2.482
55	2	1	2	6	4.319	3.978	2.227	1.736	5.060
55	2	1	2	7	6.375	2.573	4.489	2.556	1.420
55	2	1	2	8	3.203	2.879	3.914	4.594	2.829
55	2	1	2	9	3.646	5.360	3.878	3.461	4.078
55	2	1	2	10	2.705	2.042	10.141	3.827	2.371
55	2	1	2	11	3.193	2.707	4.108	2.946	1.487
55	2	1	2	12	3.762	2.256	2.890	4.978	2.658
55	2	1	2	13	4.496	4.577	5.586	3.060	3.598
55	2	1	2	14	5.252	6.384	1.450	2.145	4.246
55	2	1	2	15	3.190	4.072	3.047	4.033	1.499
55	2	2	1	1	7.039	2.583	3.024	4.872	3.367
55	2	2	1	2	3.421	4.469	4.315	2.720	2.953
55	2	2	1	3	2.450	3.239	3.697	1.699	2.124
55	2	2	1	4	2.710	2.238	5.390	4.005	3.014
55	2	2	1	5	2.559	2.980	4.693	5.215	6.037
55	2	2	1	6	5.793	3.055	6.130	3.477	5.953
55	2	2	1	7	2.580	3.351	3.285	3.003	2.707
55	2	2	1	8	3.045	2.623	2.395	4.814	9.873
55	2	2	1	9	3.503	2.486	5.587	4.096	2.661
55	2	2	1	10	2.668	7.905	3.281	4.311	4.259
55	2	2	1	11	5.818	3.093	5.840	2.620	5.504
55	2	2	1	12	2.778	4.612	2.658	2.013	2.948
55	2	2	1	13	3.514	4.099	4.370	2.202	2.911
55	2	2	1	14	2.571	6.296	2.785	3.004	3.531
55	2	2	1	15	2.882	3.257	10.405	4.288	4.401
55	2	2	2	1	2.205	3.816	3.367	3.626	5.213
55	2	2	2	2	3.571	7.330	2.953	7.168	2.506
55	2	2	2	3	3.453	2.128	2.124	2.313	4.270
55	2	2	2	4	3.452	2.516	3.014	2.581	4.739
55	2	2	2	5	2.333	2.722	6.037	3.286	4.365
55	2	2	2	6	4.319	2.284	5.953	2.827	2.649
55	2	2	2	7	6.375	1.617	2.707	4.359	2.346
55	2	2	2	8	3.203	7.182	9.873	2.464	12.509
55	2	2	2	9	3.646	3.018	2.661	7.379	3.174
55	2	2	2	10	2.705	2.132	4.259	3.938	2.817
55	2	2	2	11	3.193	2.431	5.504	3.067	2.766

55	2	2	2	12	3.762	2.701	2.948	2.740	2.394
55	2	2	2	13	4.496	4.408	2.911	3.694	2.635
55	2	2	2	14	5.252	1.961	3.531	3.378	3.371
55	2	2	2	15	3.190	1.164	4.401	5.806	2.404

Notes.
Temperature = storage temperature.
Wall = wall material.
In the column of Wall, 1 = reconstituted skim milk, 2 = mixed wall material.
Package = packaging material.
In the column of Package, 1 = gas-impermeable film, 2 = aluminium foil bag.

		Particle size of powders during storage (weeks)				
Treatments	Mean/SEM	0	1	2	3	4
RSM, gas-impermeable film	Mean	3.585	3.458	3.531	3.888	3.683
	SEM	0.208	0.181	0.287	0.257	0.239
MWM, gas-impermeable film	Mean	3.616	3.352	3.475	3.488	3.119
	SEM	0.230	0.207	0.308	0.231	0.215
RSM, aluminium foil bag	Mean	3.585	3.688	3.792	3.503	3.471
	SEM	0.208	0.289	0.273	0.249	0.270
MWM, aluminium foil bag	Mean	3.616	3.456	4.337	3.699	4.013
	SEM	0.230	0.311	0.364	0.253	0.414

Appendix 4. Statistical outputs

A. Data analysis for results at the screening stage

a. Viable cell counts of DPC16 in feed colloids

Table 4.1a. One-way ANOVA: Viable cell counts of DPC16 in reconstituted skim milk before and after spray-drying

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time	1	1.80500E+14	1.80500E+14	0.08	0.792
Error	6	1.43075E+16	2.38458E+15		
Total	7	1.44880E+16			

Notes.
Method: Null hypothesis: All means are equal
Alternative hypothesis: At least one mean is different
Significance level: $\alpha = 0.05$
Equal variances were assumed for the analysis.
Factor information: Factor: Time
Levels: 2
Values: 1, 2

Table 4.1b. Grouping information using the Tukey method and 95% confidence: Viable cell counts of DPC16 in reconstituted skim milk before and after spray-drying

Time	N	Mean	Grouping
1	4	95750000	A
2	4	86250000	A

Notes. Means that do not share a letter are significantly different.

Table 4.2a. One-way ANOVA: Viable cell counts of DPC16 in gum Arabic before and after spray-drying

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time	1	6.12500E+12	6.12500E+12	0.00	0.954
Error	6	1.02958E+16	1.71596E+15		
Total	7	1.03019E+16			

Notes.
Method: Null hypothesis: All means are equal
Alternative hypothesis: At least one mean is different
Significance level: $\alpha = 0.05$
Equal variances were assumed for the analysis.
Factor information: Factor: Time
Levels: 2

Values: 1, 2

Table 4.2b. Grouping information using the Tukey method and 95% confidence: Viable cell counts of DPC16 in gum Arabic before and after spray-drying

Time	N	Mean	Grouping
1	4	86500000	A
2	4	84750000	A

Notes. Means that do not share a letter are significantly different.

Table 4.3a. One-way ANOVA: Viable cell counts of DPC16 in maltodextrin before and after spray-drying

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time	1	2.45000E+13	2.45000E+13	0.49	0.510
Error	6	2.99500E+14	4.99167E+13		
Total	7	3.24000E+14			

Notes.
Method: Null hypothesis: All means are equal
Alternative hypothesis: At least one mean is different
Significance level $\alpha = 0.05$
Equal variances were assumed for the analysis.
Factor information: Factor: Time
Levels: 2
Values: 1, 2

Table 4.3b. Grouping information using the Tukey method and 95% confidence: Viable cell counts of DPC16 in maltodextrin before and after spray-drying

Time	N	Mean	Grouping
2	4	45750000	A
1	4	42250000	A

Notes. Means that do not share a letter are significantly different.

Table 4.4a. One-way ANOVA: Viable cell counts of DPC16 in mixed wall material before and after spray-drying

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time	1	7.02113E+15	7.02113E+15	0.55	0.487
Error	6	7.69998E+16	1.28333E+16		
Total	7	8.40209E+16			

Notes.
Method: Null hypothesis: All means are equal
Alternative hypothesis: At least one mean is different
Significance level: $\alpha = 0.05$
Equal variances were assumed for the analysis.
Factor information: Factor: Time
Levels: 2
Values: 1, 2

Table 4.4b. Grouping information using the Tukey method and 95% confidence: Viable cell counts of DPC16 in mixed wall material before and after spray-drying

Time	N	Mean	Grouping
1	4	156500000	A
2	4	97250000	A

Notes. Means that do not share a letter are significantly different.

b. Data analysis of encapsulation efficiency of wall materials and water activity of microcapsules

Table 4.5a Analysis of variance for the general linear model: encapsulation efficiency versus wall materials, temperature, batch

Source	DF	Adj SS	Adj MS	F-Value	P-Value
wall materials	3	0.011997	0.003999	4.26	0.035
temperature	1	0.040405	0.040405	43.08	0.000
batch	1	0.001228	0.001228	1.31	0.279
wall materials*batch	3	0.006589	0.002196	2.34	0.135
temperature*batch	1	0.000926	0.000926	0.99	0.344
Error	10	0.009379	0.000938		
Total	19	0.062286			

Notes.
Method: Factor coding (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
wall materials	Fixed	4	1, 2, 3, 4
temperature	Fixed	2	1, 2
batch	Fixed	2	1, 2

Model Summary:
S = 0.0306256, R-sq = 84.94%, R-sq (adj) = 71.39%, R-sq (pred) = 39.77%.
Residual plots:

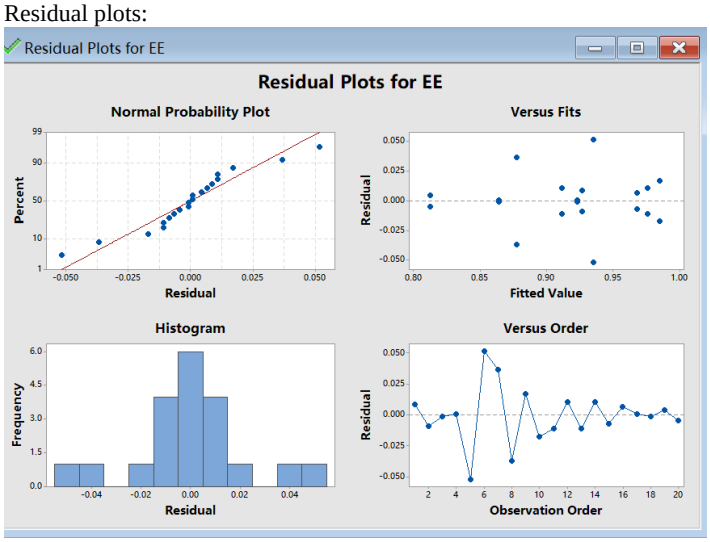


Table 4.5b Grouping information of wall materials regarding their effects on the encapsulation efficiency of DPC16 using Tukey pairwise comparisons and 95% confidence.

Wall materials	N	Mean	Grouping
3	8	0.909576	A
4	4	0.868652	A B
1	4	0.853884	A B
2	4	0.835189	B

Notes. Means that do not share a letter are significantly different.
In the column of Wall materials,
1 = maltodextrin, 2 = gum Arabic, 3 = reconstituted skim milk, 4 = mixed wall material.

Table 4.5c Grouping Information of temperatures regarding their effects on the encapsulation efficiency of DPC16 using the Tukey method and 95% Confidence

Temperature	N	Mean	Grouping
1	16	0.937893	A
2	4	0.795757	B

Notes. Means that do not share a letter are significantly different.
Temperature = inlet/outlet temperatures, in the column of temperature, 1 = 160 ℃/80 ℃, 2 = 180℃/100℃.

Table 4.6a Analysis of variance for the general linear model: a_w versus wall materials, temperature and batch

Source	DF	Adj SS	Adj MS	F-Value	P-Value
wall materials	3	0.029693	0.009898	33.42	0.000
temperature	1	0.013944	0.013944	47.08	0.000
batch	1	0.000144	0.000144	0.49	0.501
wall materials*batch	3	0.000769	0.000256	0.87	0.491
temperature*batch	1	0.000098	0.000098	0.33	0.578
Error	10	0.002962	0.000296		
Total	19	0.034896			

Notes.
Method: Factor coding (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
wall materials	Fixed	4	1, 2, 3, 4
temperature	Fixed	2	1, 2
batch	Fixed	2	1, 2

Model summary:
S = 0.0172105, R-sq = 91.51%, R-sq (adj) = 83.87%, R-sq (pred) = 66.05%.
Residual plots:

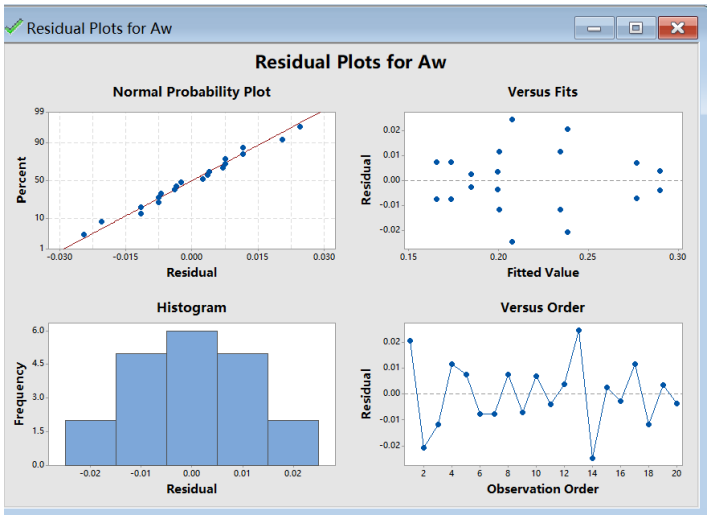


Table 4.6b. Grouping Information of wall materials about their effects on a_w using the Tukey method and 95% confidence

Wall materials	N	Mean	Grouping
3	8	0.24175	A
1	4	0.19475	B
4	4	0.15425	C
2	4	0.12775	C

Notes. Means that do not share a letter are significantly different.
In the column of Wall materials,
1 = maltodextrin, 2 = gum Arabic, 3 = reconstituted skim milk, 4 = mixed wall material.

Table 4.6c. Grouping information of temperatures about their effects on a_w using the Tukey method and 95% confidence

Temperature	N	Mean	Grouping
1	16	0.221375	A
2	4	0.137875	B

Notes. Means that do not share a letter are significantly different.
Temperature = inlet/outlet temperatures, in the column of temperature, 1 = 160 ℃/80 ℃, 2 = 180℃/100℃.

c. Data analysis: particle size of microcapsules at screening stage

Table 4.7a Analysis of variance for the general linear model for the particle size (μm) of DPC16 microcapsule powders versus inlet/outlet temperatures, batch number, replication and wall materials

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	1	4.159	4.1587	5.86	0.019
Batch	1	0.210	0.2096	0.30	0.589
Replication	14	28.047	2.0034	2.83	0.003
Wall	3	0.872	0.2908	0.41	0.746
Temperature*Batch	1	0.483	0.4832	0.68	0.413
Temperature*Replication	14	13.876	0.9911	1.40	0.185
Batch*Replication	14	9.378	0.6698	0.94	0.519
Batch*Wall	3	2.035	0.6785	0.96	0.420
Replication*Wall	42	110.274	2.6256	3.70	0.000
Error	56	39.712	0.7092		
Total	149	220.147			

Notes.
Method: Factor coding (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Temperature	Fixed	2	1, 2
Batch	Fixed	2	1, 2
Replication	Fixed	15	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15
Wall	Fixed	4	1, 2, 3, 4

Model Summary:
S = 0.842110, R-sq = 81.96%, R-sq (adj) = 52.00%, R-sq (pred) = 0.00%.
Residual plots:

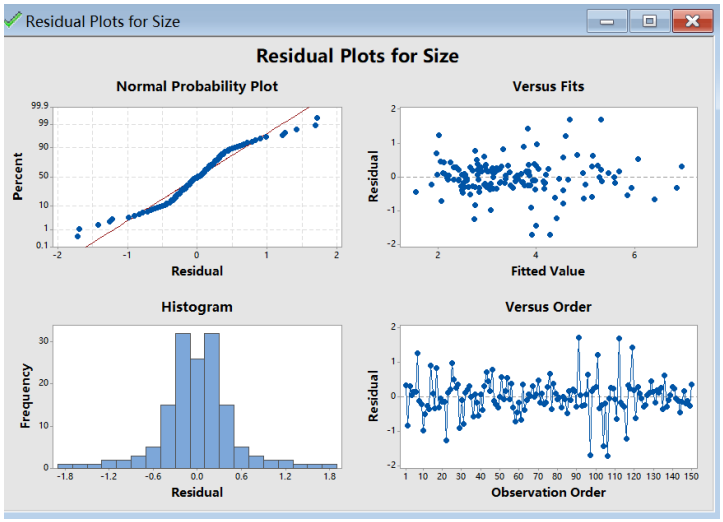


Table 4.7b. Grouping information of particle size (μm) of DPC16 microcapsules spray-dried at different inlet/outlet temperatures using the Tukey pairwise comparisons and 95% Confidence

Inlet/outlet temperatures	N	Mean	Grouping
1	120	3.64732	A
2	30	3.12078	B

Notes. Means that do not share a letter are significantly different.
In the column of Inlet/outlet temperatures, 1 = 160 ℃/80 ℃; 2 = 180 ℃/100 ℃.

Table 4.7c. Grouping information of particle size (μm) of DPC16 microcapsules measured at different replication using the Tukey pairwise comparisons and 95% Confidence

Replication	N	Mean	Grouping
12	10	4.23976	A
9	10	4.17890	A
1	10	4.12455	A
11	10	4.07299	A
6	10	3.94125	A
8	10	3.57979	A B
13	10	3.57187	A B
14	10	3.45274	A B
2	10	3.43316	A B
7	10	3.28104	A B
15	10	3.13636	A B
4	10	2.82111	A B
10	10	2.60053	A B
5	10	2.51738	A B
3	10	1.80932	B

Notes. Means that do not share a letter are significantly different.

Table 4.7d. Grouping information of particle size (μm) of DPC16 microcapsules at different replication*wall material using the Tukey pairwise comparisons and 95% Confidence

Replication*Wall	N	Mean	Grouping
12 2	2	6.93257	A
9 2	2	6.06620	A B
6 4	2	5.12803	A B C
2 2	2	5.09834	A B C
1 3	2	5.09730	A B C
13 3	2	5.03421	A B C
6 2	2	4.91955	A B C
11 4	2	4.71347	A B
8 2	2	4.53530	A B C
9 3	2	4.52908	A B C
7 4	2	4.49179	A B C
11 2	2	4.46675	A B C
8 3	2	4.33118	A B C
11 3	2	4.18654	A B C
1 1	4	4.18415	A B C

1 4	2	4.09537	A B C
14 3	2	4.07109	A B C
14 4	2	3.97171	A B
3 1	4	3.94407	A B C
12 3	2	3.92806	A B C
13 4	2	3.88244	A B C
14 1	4	3.87376	A B C
15 1	4	3.86281	A B C
4 1	4	3.82800	A B C
9 4	2	3.67745	A B C
10 1	4	3.49304	A B C
13 1	4	3.47357	A B C
8 1	4	3.43720	A B C
7 3	2	3.41982	A B C
12 4	2	3.29022	A B C
6 1	4	3.28248	B C
2 4	2	3.18907	A B C
5 1	4	3.14846	B C
1 2	2	3.12138	A B C
15 4	2	3.08219	A B C
11 1	4	2.92520	B C
5 2	2	2.91082	A B C
7 2	2	2.82038	A B C
15 2	2	2.81046	A B C
12 1	4	2.80817	B C
15 3	2	2.78997	A B C
4 4	2	2.74731	A B C
2 1	4	2.73496	B C
2 3	2	2.71028	A B C
10 4	2	2.63776	A B C
4 2	2	2.56400	A B C
9 1	4	2.44287	B C
6 3	2	2.43496	B C
7 1	4	2.39218	C
10 3	2	2.22706	B C
4 3	2	2.14511	B C
10 2	2	2.04427	B C
8 4	2	2.01550	B C
5 4	2	2.00842	B C
5 3	2	2.00182	B C
13 2	2	1.89726	B C
14 2	2	1.89440	B C
3 4	2	1.36309	C
3 3	2	1.05509	C
3 2	2	0.87502	C

Means that do not share a letter are significantly different.
In the column of Replication*Wall, the second number is the type of wall material. For the type of wall material, 1 = RSM, 2 = reconstituted skim milk, 3 = maltodextrin, 4 = mixed wall material.

B. Data analysis for results at the storage stage

a. Cell survival of DPC16 in microcapsules

Table 4.8. Analysis of variance for general linear model: survival versus full factors (storage temperature, wall-material type, packaging-material type and storage time) and interactions during storage

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Storage Temperature	1	1085.06	1085.06	1989.28	0.000
Wall material type	1	35.70	35.70	65.46	0.000
packaging material type	1	3.94	3.94	7.23	0.008
Time	4	359.69	89.92	164.86	0.000
Storage Temperature*Wall material type	1	6.08	6.08	11.16	0.001
Storage Temperature*packaging material type	1	9.25	9.25	16.95	0.000
Storage Temperature*Time	4	279.27	69.82	128.00	0.000
Wall material type*packaging material type	1	1.05	1.05	1.92	0.167
Wall material type*Time	4	3.89	0.97	1.78	0.134
packaging material type*Time	4	4.67	1.17	2.14	0.077
Storage Temperature*Wall material type*packaging material type	1	0.48	0.48	0.88	0.351
Storage Temperature*Wall material type*Time	4	5.88	1.47	2.69	0.032

Storage Temperature*packaging material type*Time	4	2.75	0.69	1.26	0.287
Wall material type*packaging material type*Time	4	0.65	0.16	0.30	0.878
Error	204	111.27	0.55		
Lack-of-Fit	4	0.26	0.07	0.12	0.976
Pure Error	200	111.01	0.56		
Total	239	1909.64			

Notes.
Method: Factor coding (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Storage Temperature	Fixed	2	1, 2
Wall material type	Fixed	2	1, 2
packaging material type	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model Summary:
Mpdel a. S = 0.738547, R-sq = 94.17%, R-sq (adj) = 93.17%, R-sq (pred) = 91.94%;
Residual plots:

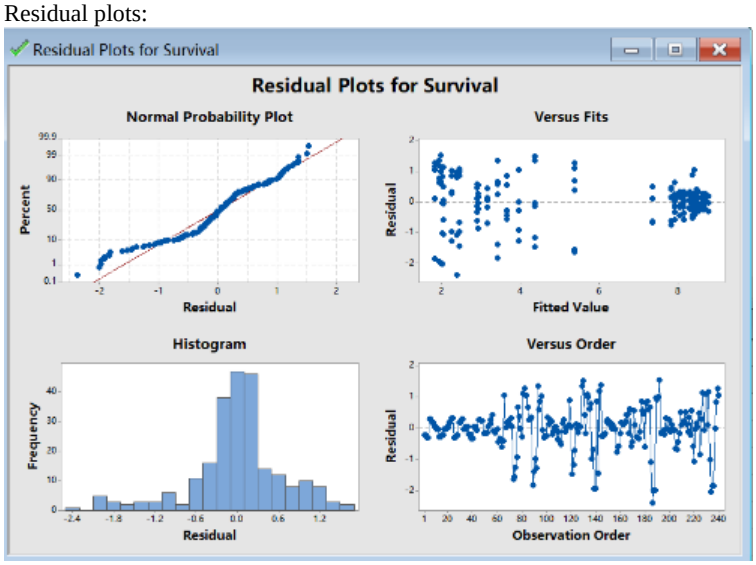


Table 4.9a. Analysis of variance for general linear model: survival versus full factors (wall-material type, packaging-material type and storage time) and their interactions during storage at 25 ℃

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Wall-material type	1	6.1550	6.15503	59.80	0.000
Packaging-material type	1	0.5568	0.55677	5.41	0.022
Time	4	4.3536	1.08839	10.58	0.000
Wall-material type*Packaging-material type	1	0.0556	0.05558	0.54	0.464
Wall-material type*Time	4	0.4343	0.10858	1.06	0.383
Packaging-material type*Time	4	1.8602	0.46505	4.52	0.002
Wall-material type*Packaging-material type*Time	4	0.3725	0.09312	0.90	0.464
Error	100	10.2920	0.10292		
Total	119	24.0799			

Model Summary
Notes.
Method: Factor coding (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Wall material type	Fixed	2	1, 2
packaging material type	Fixed	2	1, 2
Storage Time	Fixed	5	0, 1, 2, 3, 4

Model Summary:
S = 0.320811, R-sq = 57.26%, R-sq (adj) =49.14%, R-sq (pred) = 38.45%.
Residual plots:

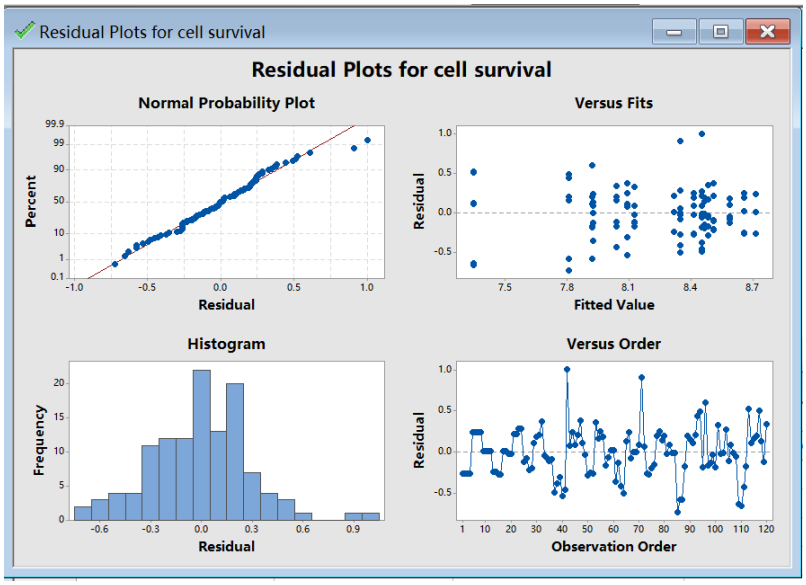


Table 4.9b. Grouping information of DPC16 cell survival encapsulated in different types of wall materials during storage at 25 ℃ using the Tukey pairwise comparisons and 95% Confidence

Wall-material type	N	Mean	Grouping
1	40	8.52306	A
2	40	7.93104	B

Notes.
Means that do not share a letter are significantly different.
In the column of wall-material type, 1 = reconstituted skim milk, 2 = mixed wall material.

Table 4.9c. Grouping information of the survival of encapsulated DPC16 cells packed in different packaging mateirals during storage at 25 ℃ using the Tukey pairwise comparisons and 95% Confidence

Packaging-material type	N	Mean	Grouping
2	40	8.29083	B
1	40	8.16327	A

Notes.
Means that do not share a letter are significantly different.
In the column of packaging-material type, 1 = gas-impermeable film, 2 = aluminium foil bag.

Table 4.9d. Grouping information of the survival of encapsulated DPC16 cells at different time points during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Time	N	Mean	Grouping
week 0	16	8.45575	A
week 1	16	8.37891	A B
week 2	16	8.29642	A B
week 3	16	8.06234	B C
week 4	16	7.94182	C

Notes.
Means that do not share a letter are significantly different.

Table 4.9e. Grouping information of the survival of encapsulated DPC16 cells against different packaging material type*Time during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Packaging-material type*Time	N	Mean	Grouping
1 1	8	8.49690	A
1 0	8	8.45575	A
2 0	8	8.45575	A
2 2	8	8.37277	A
2 1	8	8.26091	A
2 4	8	8.22477	A
1 2	8	8.22007	A
2 3	8	8.13995	A B
1 3	8	7.98473	A B
1 4	8	7.65888	B

Notes.

Means that do not share a letter are significantly different.
In the column of Packaging-material type*Time, the first number is the type of packaging material and the second number is the time point. For example,
1 1 = gas-impermeable film * week 1,
2 1 = aluminium foil bag * week 1.

Table 4.10a. Analysis of variance for general linear model: survival versus wall-material type, packaging-material type and storage time and their interactions during storage at 55 ℃

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Wall-material type	1	35.634	35.634	35.38	0.000
Packaging-material type	1	12.634	12.634	12.54	0.001
Time	4	634.614	158.653	157.52	0.000
Wall material type*packaging material type	1	1.471	1.471	1.46	0.230
Wall material type*Time	4	9.329	2.332	2.32	0.062
Packaging material type*Time	4	5.558	1.390	1.38	0.246
Wall material type*packaging material type*Time	4	0.543	0.136	0.13	0.969
Error	100	100.718	1.007		
Total	119	800.502			

Notes.
Method: Factor coding (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Wall-material type	Fixed	2	1, 2
Packaging-material type	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model summary:
S = 1.00358, R-sq = 87.42%, R-sq (adj) = 85.03%, R-sq (pred) = 81.88%.
Residual plots:

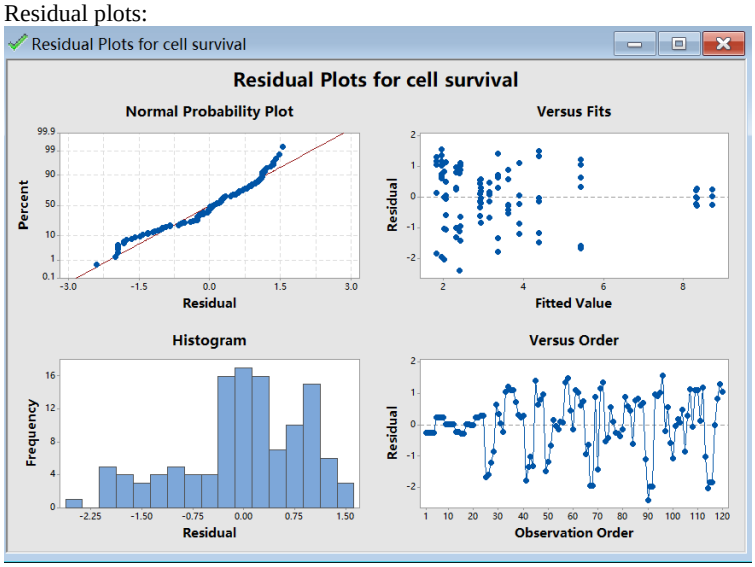


Table 4.10b. Grouping information of DPC16 cell survival encapsulated in different types of wall materials during storage at 55 ℃ using the Tukey pairwise comparisons and 95% Confidence

Wall-material type	N	Mean	Grouping
1	40	4.34008	A
2	40	3.12783	B

Notes.
Means that do not share a letter are significantly different.
In the column of wall-material type, 1 = reconstituted skim milk, 2 = mixed wall material.

Table 4.10c. Grouping information of the survival of encapsulated DPC16 cells packed in different packaging mateirals during storage at 55 ℃ using the Tukey pairwise comparisons and 95% Confidence

Packaging-material type	N	Mean	Grouping
1	40	4.09058	A
2	40	3.37733	B

Notes.
Means that do not share a letter are significantly different.
In the column of packaging-material type, 1 = gas-impermeable film, 2 = aluminium foil bag.

Table 4.10d. Grouping information of the survival of encapsulated DPC16 cells at different time points during storage at 55 °C using the Tukey pairwise comparisons and 95% confidence

Time	N	Mean	Grouping
week 0	16	8.45575	A
week 1	16	3.22778	B
week 2	16	2.65137	B C
week 3	16	2.36358	B C
week 4	16	1.97130	C

Notes.
Means that do not share a letter are significantly different.

b. Water activity of microcapsules

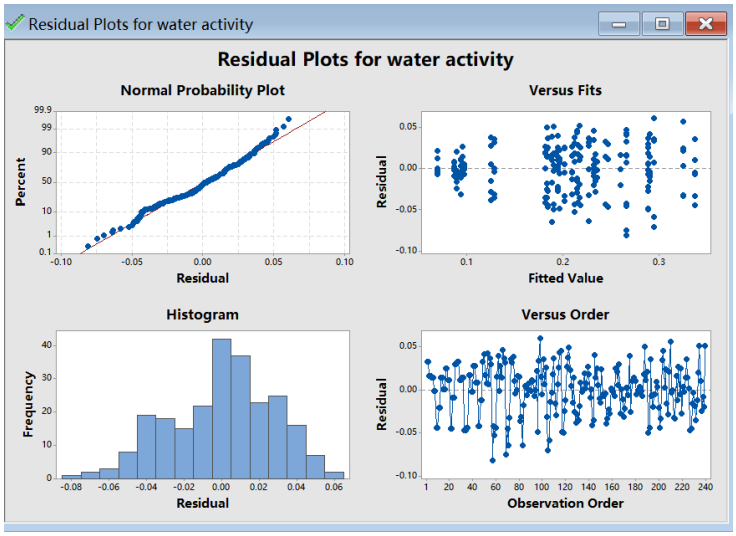
Table 4.11 Analysis of variance for general linear model: survival versus full factors (wall-material type, packaging-material type and storage time) and their interactions during storage

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Storage Temperature	1	0.47200	0.471999	512.74	0.000
Wall-material type	1	0.00406	0.004057	4.41	0.037
Packaging-material type	1	0.00415	0.004154	4.51	0.035
Time	4	0.06756	0.016890	18.35	0.000
Storage Temperature*Wall-material type	1	0.00032	0.000315	0.34	0.559
Storage Temperature*Packaging-material type	1	0.28214	0.282145	306.50	0.000
Storage Temperature*Time	4	0.12240	0.030600	33.24	0.000
Wall-material type*Packaging-material type	1	0.00563	0.005625	6.11	0.014
Wall-material type*Time	4	0.00509	0.001272	1.38	0.241
Packaging-material type*Time	4	0.00230	0.000575	0.62	0.645
Storage Temperature*Wall-material type*Packaging-material type	1	0.00223	0.002234	2.43	0.121
Storage Temperature*Wall-material type*Time	4	0.00329	0.000822	0.89	0.469
Storage Temperature*Packaging-material type*Time	4	0.09082	0.022706	24.67	0.000
Wall-material type*Packaging-material type*Time	4	0.00102	0.000255	0.28	0.892
Error	204	0.18779	0.000921		
Lack-of-Fit	4	0.00312	0.000780	0.84	0.498
Pure Error	200	0.18467	0.000923		
Total	239	1.25080			

Notes.
Method: Factor coding (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Storage Temperature	Fixed	2	1, 2
Wall material type	Fixed	2	1, 2
packaging material type	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model Summary:
S = 0.0303404, R-sq = 84.99%, R-sq (adj) = 82.41%, R-sq (pred) = 79.22%.



Residual plots:

Table 4.12a. Analysis of variance for general linear model: a_w versus packaging-material type, storage time and packaging material*time at 25 ℃

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Wall-material type	1	0.001055	0.001055	0.97	0.328
Packaging-material type	1	0.108914	0.108914	99.72	0.000
Time	4	0.029706	0.007426	6.80	0.000
Wall-material type*Packaging-material type	1	0.000384	0.000384	0.35	0.554
Wall-material type*Time	4	0.003850	0.000962	0.88	0.478
Packaging-material type*Time	4	0.051860	0.012965	11.87	0.000
Wall-material type*Packaging-material type*Time	4	0.000939	0.000235	0.22	0.930
Error	100	0.109217	0.001092		
Total	119	0.305925			

Notes.
Method: Factor coding (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Wall-material type	Fixed	2	1, 2
Packaging material type	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model Summary:
S = 0.0330480, R-sq = 64.30%, R-sq (adj) = 57.52%, R-sq (pred) = 48.59%.
Residual plots:

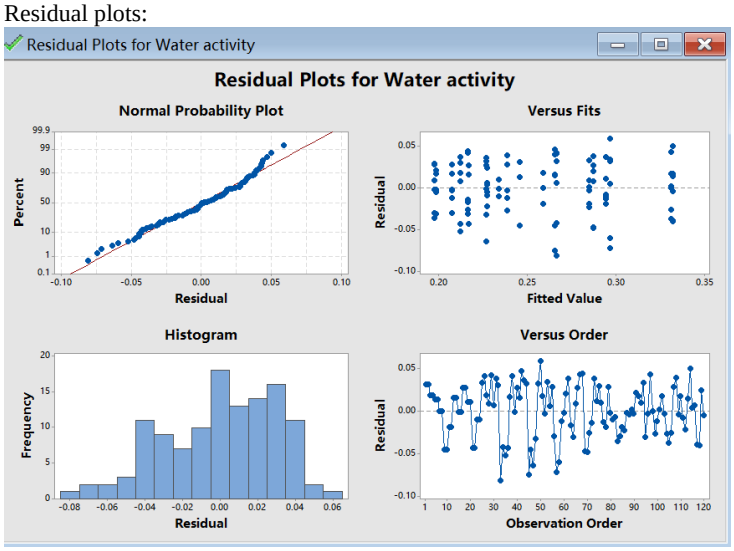


Table 4.12b. Grouping information of the water activity of DPC16 microcapsules packed in different types of packaging materials during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Packaging-material type	N	Mean	Grouping
1	60	0.281965	A
2	60	0.221712	B

Notes.
Means that do not share a letter are significantly different.
In the column of Packaging-material type, 1 = gas-impermeable film, 2 = aluminium foil bag.

Table 4.12c. Grouping information of the water activity of DPC16 microcapsules at different time points during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Time	N	Mean	Grouping
week 4	24	0.282117	A
week 2	24	0.251917	B
week 3	24	0.243733	B
week 1	24	0.242675	B
week 0	24	0.238750	B

Notes.
Means that do not share a letter are significantly different.

Table 4.12d. Grouping information of the water activity of DPC16 microcapsules at different packaging material type*Time during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Packaging material type*Time	N	Mean	Grouping
1 4	12	0.331400	A
1 2	12	0.291775	A B
1 3	12	0.289417	A B
1 1	12	0.265900	B C
2 0	12	0.246167	C D
2 4	12	0.232833	C D E
1 0	12	0.231333	C D E
2 1	12	0.219450	D E
2 2	12	0.212058	D E
2 3	12	0.198050	E

Notes.
Means that do not share a letter are significantly different.
In the column of Packaging-material type*Time, the first number is the type of packaging material and the second number is the time point. For example,
1 1 = gas-impermeable film * week 1,
2 1 = aluminium foil bag * week 1.

Table 4.13a. Analysis of variance for general linear model: water activity versus wall-material type, packaging-material type and storage time at 55 ℃

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Wall-material type	1	0.006491	0.006491	8.70	0.004
Packaging-material type	1	0.114264	0.114264	153.17	0.000
Time	3	0.151979	0.050660	67.91	0.000
Wall-material type*Packaging-material type	1	0.003121	0.003121	4.18	0.044
Wall-material type*Time	3	0.000308	0.000103	0.14	0.937
Packaging-material type*Time	3	0.033785	0.011262	15.10	0.000
Wall-material type*Packaging-material type*Time	3	0.000898	0.000299	0.40	0.753
Error	80	0.059678	0.000746		
Total	95	0.370525			

Notes.
Method: Factor coding: (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Wall material type	Fixed	2	1, 2
Packaging material type	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model Summary:
S = 0.0273125, R-sq = 83.89%, R-sq (adj) = 80.87%, R-sq (pred) = 76.81%.
Residual plots:

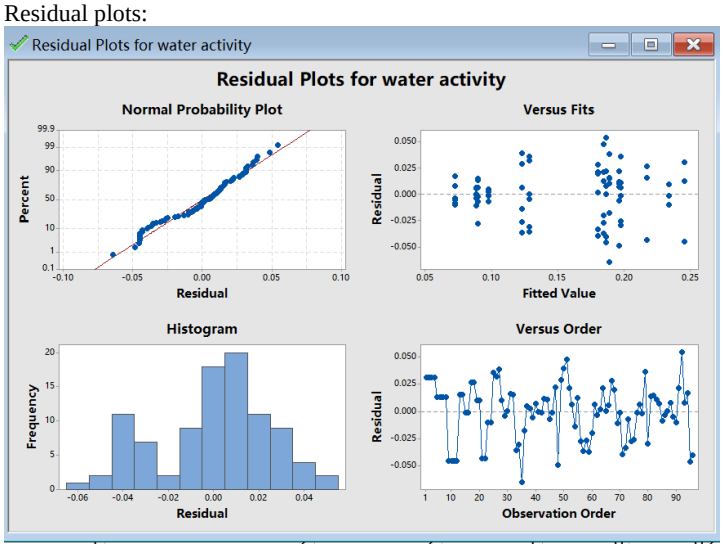


Table 4.13b. Grouping information of the water activity of DPC16 microcapsules made from different types of wall materials during storage at 55 ℃ using the Tukey pairwise comparisons and 95% Confidence

Wall-material type	N	Mean	Grouping
1	60	0.168402	A
2	60	0.157887	B

Notes.
Means that do not share a letter are significantly different.
In the column of wall-material type, 1 = reconstituted skim milk, 2 = mixed wall material.

Table 4.13c. Grouping information of the water activity of DPC16 microcapsules packed in different packaging materials during storage at 55 ℃ using the Tukey pairwise comparisons and 95% Confidence

Packaging-material type	N	Mean	Grouping
2	60	0.201592	A
1	60	0.124697	B

Notes.
Means that do not share a letter are significantly different.
In the column of packaging-material type, 1 = gas-impermeable film, 2 = aluminium foil bag.

Table 4.13d. Grouping information of the water activity of DPC16 microcapsules at different time points during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Time	N	Mean	Grouping
week 0	24	0.235500	A
week 1	24	0.152825	B
week 4	24	0.146537	B
week 2	24	0.144146	B
week 3	24	0.136712	B

Notes.
Means that do not share a letter are significantly different.

Table 4.13e. Grouping information of the water activity of DPC16 microcapsules against different packaging-material type*Time during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type*packaging-material type	N	Mean	Grouping
2 2	30	0.204227	A
1 2	30	0.198957	A
1 1	30	0.137847	B
2 1	30	0.111547	C

Notes.
Means that do not share a letter are significantly different.
In the column of Wall-material type*packaging-material type, the first number is the type of wall materials and the second number is the type of packaging materials. For example,
1 1 = reconstituted skim milk * gas-impermeable film,
2 2 = mixed wall material * aluminium foil bag.

Table 4.13f. Grouping information of the water activity of DPC16 microcapsules against different packaging-material type*Time during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Packaging material type*Time	N	Mean	Grouping
2 0	12	0.239667	A
1 0	12	0.231333	A B
2 4	12	0.200775	B C
2 1	12	0.192767	C
2 3	12	0.192267	C
2 2	12	0.182483	C
1 1	12	0.112883	D
1 2	12	0.105808	D
1 4	12	0.092300	D
1 3	12	0.081158	D

Notes.
Means that do not share a letter are significantly different.

c. Colour of microcapsules

Table 4.14 Analysis of variance for general linear model: lightness versus full factors (storage temperature, wall-material type, packaging-material type and storage time) and their interactions during storage

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Storage Temperature	1	11.220	11.2201	18.30	0.000
Wall-material type	1	0.151	0.1507	0.25	0.621
Packaging-material type	1	4.682	4.6820	7.64	0.007
Time	4	267.841	66.9602	109.23	0.000
Storage Temperature*Wall-material type	1	9.521	9.5209	15.53	0.000
Storage Temperature*Packaging-material type	1	6.671	6.6708	10.88	0.001
Storage Temperature*Time	4	3.346	0.8365	1.36	0.250

Wall-material type*Packaging-material type	1	5.941	5.9406	9.69	0.002
Wall-material type*Time	4	9.099	2.2746	3.71	0.007
Packaging-material type*Time	4	4.113	1.0283	1.68	0.159
Storage Temperature*Wall-material type*Packaging-material type	1	1.602	1.6020	2.61	0.109
Storage Temperature*Wall-material type*Time	4	5.956	1.4889	2.43	0.051
Storage Temperature*Packaging-material type*Time	4	4.489	1.1224	1.83	0.127
Wall-material type*Packaging-material type*Time	4	3.881	0.9702	1.58	0.183
Error	124	76.011	0.6130		
Lack-of-Fit	4	1.275	0.3187	0.51	0.727
Pure Error	120	74.736	0.6228		
Total	159	414.523			

Notes.
Method: Factor coding: (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Storage Temperature	Fixed	2	1, 2
Wall material type	Fixed	2	1, 2
Packaging-material type	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model Summary:
S = 0.782938, R-sq = 81.66%, R-sq (adj) = 76.49%, R-sq (pred) = 69.47%.
Residual plots:

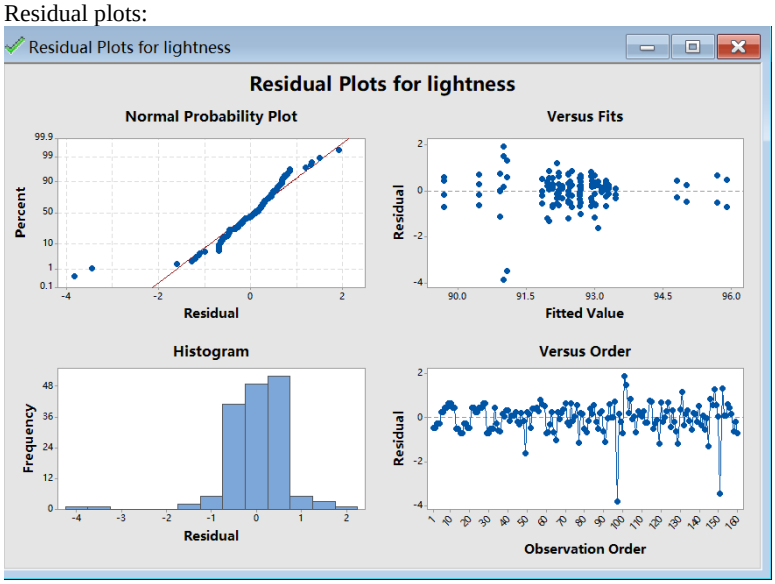


Table 4.15a. Analysis of variance for general linear model: lightness versus full factors (wall-material type, packaging-material type and storage time) and their interactions during storage at 25 ℃

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Wall-material type	1	6.034	6.0335	9.96	0.002
Packaging-material type	1	0.088	0.0878	0.14	0.705
Time	4	106.160	26.5400	43.83	0.000
Wall-material type*Packaging-material type	1	0.686	0.6864	1.13	0.291
Wall-material type*Time	4	5.916	1.4790	2.44	0.056
Packaging-material type*Time	4	2.739	0.6848	1.13	0.351
Wall-material type*Packaging-material type*Time	4	3.085	0.7714	1.27	0.290
Error	60	36.330	0.6055		
Total	79	161.038			

Notes.
Method: Factor coding: (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Wall-material type	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model Summary:
S = 0.778137, R-sq = 77.44%, R-sq (adj) = 70.30%, R-sq (pred) = 59.89%.
Residual plots:

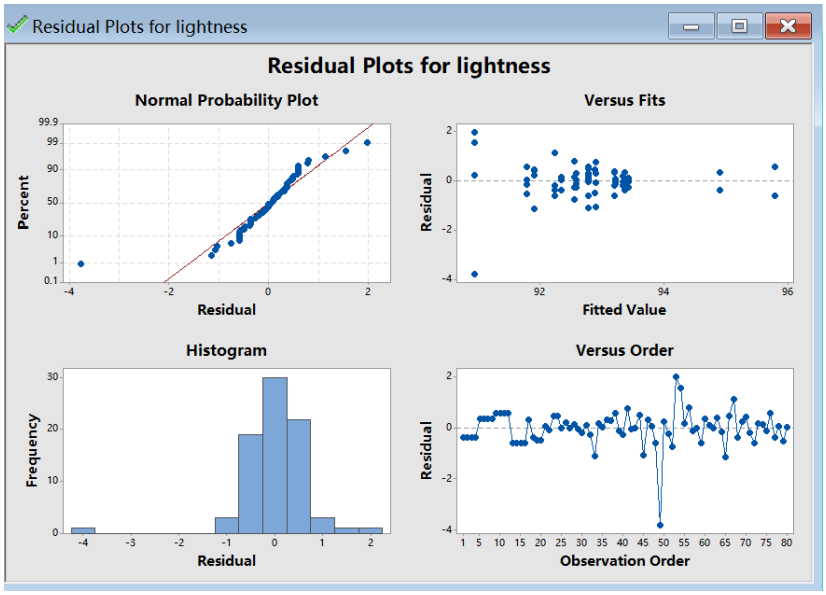


Table 4.15b. Grouping information of the lightness of DPC16 microcapsules made from different types of wall materials during storage at 25 ℃ using the Tukey pairwise comparisons and 95% Confidence

Wall-material type	N	Mean	Grouping
2	40	93.4590	A
1	40	92.9097	B

Notes.
Means that do not share a letter are significantly different.
In the column of Wall-material type, 1 = reconstituted skim milk, 2 = mixed wall material.

Table 4.15c. Grouping information of the lightness of DPC16 microcapsules at different time points during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Time	N	Mean	Grouping
week 0	16	95.3537	A
week 1	16	93.2575	B
week 2	16	92.7656	B C
week 3	16	92.4787	B C
week 4	16	92.0662	C

Notes.
Means that do not share a letter are significantly different.

Table 4.16a. Analysis of variance for general linear model: lightness versus full factors (storage temperature, wall-material type, packaging-material type and storage time) and their interactions during storage at 55 ℃

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Wall-material type	1	3.638	3.6380	5.68	0.020
Packaging-material type	1	11.265	11.2650	17.60	0.000
Time	4	165.027	41.2567	64.45	0.000
Wall-material type*Packaging-material type	1	6.856	6.8562	10.71	0.002
Wall-material type*Time	4	9.138	2.2846	3.57	0.011
Packaging-material type*Time	4	5.864	1.4659	2.29	0.070
Wall-material type*Packaging-material type*Time	4	2.070	0.5175	0.81	0.525
Error	60	38.406	0.6401		
Total	79	242.264			

Notes.
Method: Factor coding: (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Wall-material type	Fixed	2	1, 2
Packaging-material type	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model Summary:
S = 0.800066, R-sq = 84.15%, R-sq (adj) = 79.13%, R-sq(pred) = 71.82%.
Residual plots:

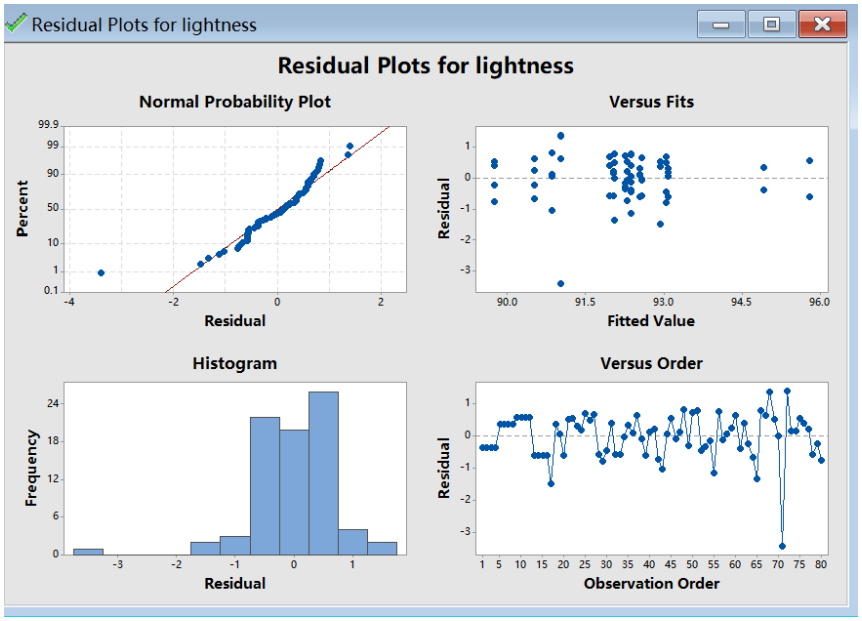


Table 4.16b. Grouping information of the lightness of DPC16 microcapsules made from different types of wall materials during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type	N	Mean	Grouping
1	40	92.8680	A
2	40	92.4415	B

Notes.
Means that do not share a letter are significantly different.
In the column of wall-material type, 1 = reconstituted skim milk, 2 = mixed wall material.

Table 4.16c. Grouping information of the lightness of DPC16 microcapsules packed in different packaging materials during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Packaging-material type	N	Mean	Grouping
1	40	93.0300	A
2	40	92.2795	B

Notes.
Means that do not share a letter are significantly different.
In the column of packaging-material type, 1 = gas-impermeable film, 2 = aluminium foil bag.

Table 4.16d. Grouping information of the lightness of DPC16 microcapsules at different time points during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Time	N	Mean	Grouping
week 0	16	95.3538	A
week 1	16	92.7569	B
week 2	16	92.0688	B C
week 3	16	91.8806	C D
week 4	16	91.2138	D

Notes. Means that do not share a letter are significantly different.

Table 4.16e. Grouping information of the lightness of DPC16 microcapsules in different wall-material type*Packaging material type during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type*Packaging material type	N	Mean	Grouping
2 1	20	93.1095	A
1 1	20	92.9505	A
1 2	20	92.7855	A
2 2	20	91.7735	B

Notes.
Means that do not share a letter are significantly different.
In the column of Wall-material type*packaging-material type, the first number is the type of wall materials and the second number is the type of packaging materials. For example,
1 1 = reconstituted skim milk * gas-impermeable film,
2 2 = mixed wall material * aluminium foil bag.

Table 4.16f. Grouping information of the lightness of DPC16 microcapsules in different wall-material type * (storage) time at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type*Time	N	Mean	Grouping
2 0	8	95.7950	A
1 0	8	94.9125	A
1 1	8	93.0100	B
1 2	8	92.5625	B C
2 1	8	92.5038	B C
1 3	8	92.3188	B C
2 2	8	91.5750	C D
1 4	8	91.5363	C D
2 3	8	91.4425	C D
2 4	8	90.8913	D

Notes.
In the column of Wall-material type*Time, the first number is the type of wall materials and the second number is the time points. For example,
1 1 = reconstituted skim milk * gas-impermeable film,
2 2 = mixed wall material * aluminium foil bag.

Table 4.17 Analysis of variance for general linear model: redness versus full factors (storage temperature, wall-material type, packaging-material type and storage time) during storage

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Storage Temperature	1	1.648	1.648	1.49	0.224
Wall-material type	1	138.980	138.980	126.04	0.000
Packaging-material type	1	1.285	1.285	1.17	0.282
Time	4	5.763	1.441	1.31	0.271
Storage Temperature*Wall-material type	1	0.668	0.668	0.61	0.438
Storage Temperature*Packaging-material type	1	2.777	2.777	2.52	0.115
Storage Temperature*Time	4	6.103	1.526	1.38	0.243
Wall-material type*Packaging-material type	1	0.279	0.279	0.25	0.616
Wall-material type*Time	4	1.054	0.264	0.24	0.916
Packaging-material type*Time	4	5.620	1.405	1.27	0.284
Storage Temperature*Wall-material type*Packaging-material type	1	1.073	1.073	0.97	0.326
Storage Temperature*Wall-material type*Time	4	3.462	0.865	0.78	0.537
Storage Temperature*Packaging-material type*Time	4	5.739	1.435	1.30	0.273
Wall-material type*Packaging-material type*Time	4	2.062	0.516	0.47	0.759
Error	124	136.726	1.103		
Lack-of-Fit	4	4.240	1.060	0.96	0.432
Pure Error	120	132.486	1.104		
Total	159	313.239			

Notes.
Method: Factor coding: (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Storage temperature	Fixed	2	1, 2
Wall-material type	Fixed	2	1, 2
Packaging-material type	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model Summary:
S = 1.05006, R-sq = 56.35%, R-sq (adj) = 44.03%, R-sq (pred) = 27.33%.
Residual plots:

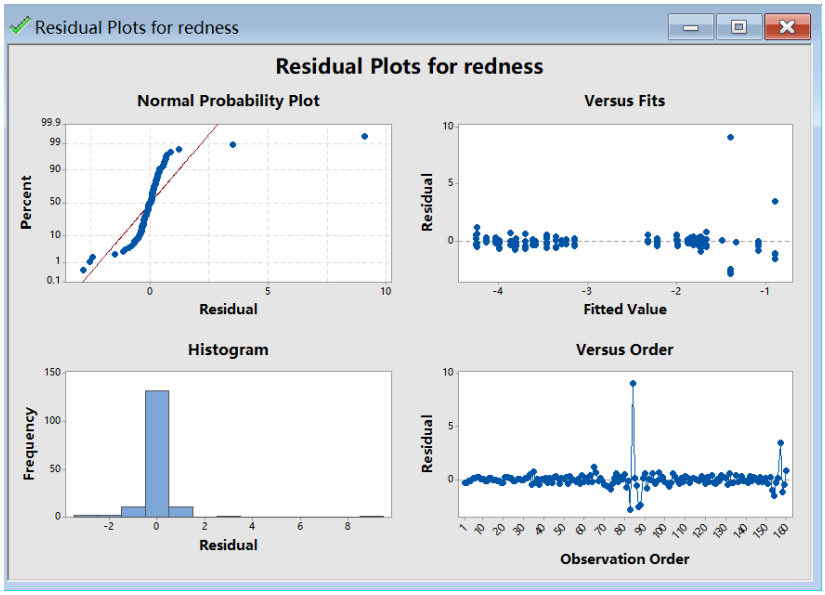


Table 4.18a. Analysis of variance for general linear model: redness versus full factors (wall-material type, packaging-material type and storage time) and their interactions during storage at 25 ℃

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Wall-material type	1	79.4609	79.4609	584.34	0.000
Packaging-material type	1	0.1420	0.1420	1.04	0. 311
Time	4	3.2714	0.8178	6.01	0.000
Wall-material type*Packaging-material type	1	0.1288	0.1288	0.95	0.334
Wall-material type*Time	4	0.8877	0.2219	1.63	0.178
Packaging-material type*Time	4	0.2825	0.0706	0.52	0.722
Wall-material type*Packaging-material type*Time	4	0.7928	0.1982	1.46	0.226
Error	60	8.1591	0.1360		
Total	79	93.1251			

Notes.
Method: Factor coding: (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Wall material type	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model Summary:
S = 0.368761, R-sq = 91.24%, R-sq (adj) = 88.46%, R-sq (pred) = 84.42%.
Residual plots:

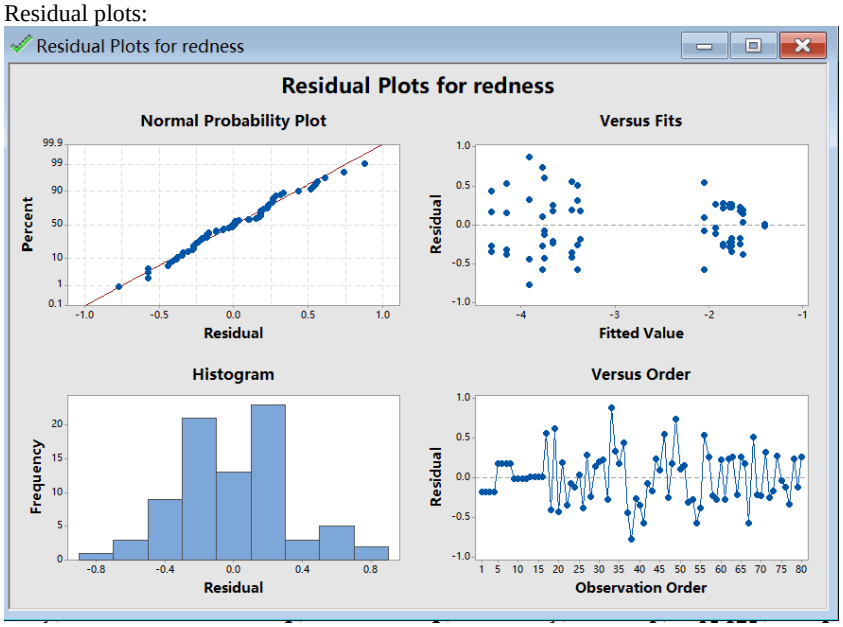


Table 4.18b. Grouping information of the redness of DPC16 microcapsules made from different types of wall materials during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall material	N	Mean	Grouping
2	40	-1.72500	A
1	40	-3.71825	B

Notes.
Means that do not share a letter are significantly different.
In the column of wall-material type, 1 = reconstituted skim milk, 2 = mixed wall material.

Table 4.18c. Grouping information of the redness of DPC16 microcapsules at different time points during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Time	N	Mean	Grouping
week 0	16	-2.39000	A
week 1	16	-2.67625	A B
week 4	16	-2.68812	A B
week 3	16	-2.86750	B
week 2	16	-2.98625	B

Notes. Means that do not share a letter are significantly different.

Table 4.19a. Analysis of variance for general linear model: redness versus full factors (storage temperature, wall-material type, packaging-material type and storage time) and their interactions during storage at 55 ℃

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Wall-material type	1	60.187	60.1872	29.05	0.000
Packaging-material type	1	3.921	3.9206	1.89	0.174
Time	4	8.594	2.1485	1.04	0.396
Wall-material type*Packaging-material type	1	1.223	1.2227	0.59	0.445
Wall-material type*Time	4	3.629	0.9071	0.44	0.781
Packaging-material type*Time	4	11.076	2.7691	1.34	0.267
Wall-material type*Packaging-material type*Time	4	5.510	1.3774	0.66	0.619
Error	60	124.327	2.0721		
Total	79	218.466			

Notes.
Method: Factor coding: (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Wall-material type	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model summary:
S = 1.43949, R-sq = 43.09%, R-sq (adj) = 25.07%, R-sq (pred) = 0.00%.
Residual plots:

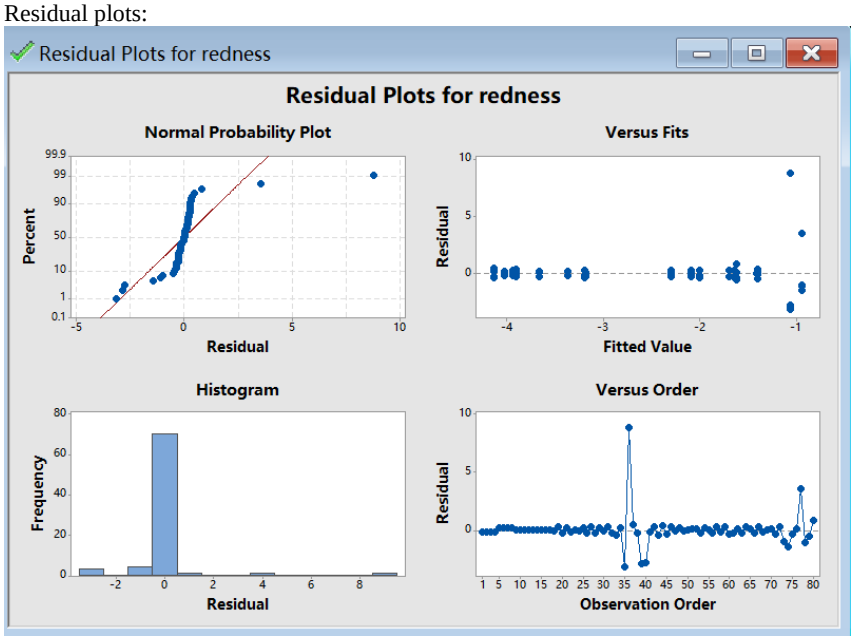


Table 4.19b. Grouping information of the redness of DPC16 microcapsules made from different types of wall materials during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type	N	Mean	Grouping
2	40	-1.65125	A
1	40	-3.69700	B

Notes.
Means that do not share a letter are significantly different.
In the column of wall-material type, 1 = reconstituted skim milk, 2 = mixed wall material.

Table 4.19c. Grouping information of the redness of DPC16 microcapsules at different time points during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Time	N	Mean	Grouping
week 4	16	-2.23625	A
week 0	16	-2.39000	A B
week 3	16	-2.88562	B
week 2	16	-2.92750	B
week 1	16	-2.93125	B

Notes.
Means that do not share a letter are significantly different.

Table 4.20 Analysis of variance for general linear model: yellowness versus full factors (storage temperature, wall-material type, packaging-material type) and their interactions and storage time during storage

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Storage Temperature	1	202.19	202.185	188.51	0.000
Wall-material type	1	368.15	368.146	343.25	0.000
Packaging-material type	1	13.62	13.619	12.70	0.001
Time	4	86.52	21.629	20.17	0.000
Storage Temperature*Wall-material type	1	67.11	67.107	62.57	0.000
Storage Temperature*Packaging-material type	1	12.34	12.343	11.51	0.001
Storage Temperature*Time	4	53.90	13.474	12.56	0.000
Wall-material type*Packaging-material type	1	11.69	11.686	10.90	0.001
Wall-material type*Time	4	65.19	16.296	15.19	0.000
Packaging-material type*Time	4	4.41	1.102	1.03	0.396
Storage Temperature*Wall-material type*Packaging-material type	1	30.84	30.835	28.75	0.000
Storage Temperature*Wall-material type*Time	4	24.30	6.074	5.66	0.000
Storage Temperature*Packaging-material type*Time	4	15.59	3.898	3.63	0.008
Wall-material type*Packaging-material type*Time	4	15.33	3.833	3.57	0.009
Error	124	132.99	1.073		
Lack-of-Fit	4	8.51	2.127	2.05	0.092
Pure Error	120	124.48	1.037		
Total	159	1104.14			

Notes.
Method: Factor coding: (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Storage Temperature	Fixed	2	1, 2
Wall material type	Fixed	2	1, 2
Packaging-material type	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model Summary:
S = 1.03562, R-sq = 87.96%, R-sq (adj) = 84.56%, R-sq (pred) = 79.95%.
Residual plots:

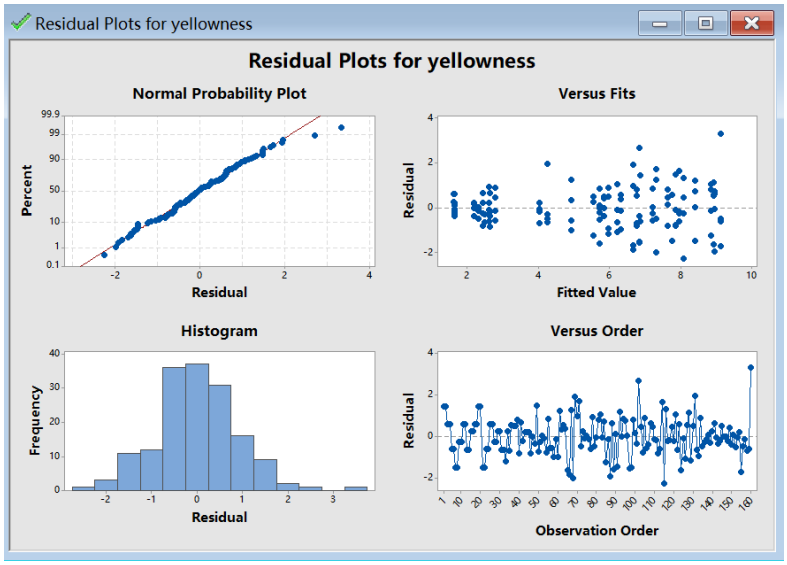


Table 4.21a. Analysis of variance for general linear model: yellowness versus full factors (wall-material type, packaging-material type and storage time) and their interactions during storage at 25 ℃

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Wall-material type	1	374.805	374.805	381.01	0.000
Packaging-material type	1	0.016	0.016	0.02	0.900
Time	4	25.293	6.323	6.43	0.000
Wall-material type*Packaging-material type	1	2.278	2.278	2.32	0.133
Wall-material type*Time	4	7.605	1.901	1.93	0.117
Packaging-material type*Time	4	4.435	1.109	1.13	0.352
Wall-material type*Packaging-material type*Time	4	5.715	1.429	1.45	0.228
Error	60	59.023	0.984		
Total	79	479.170			

Notes.
Method: Factor coding: (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Wall-material type	Fixed	2	1, 2
Packaging-material type	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model summary:
S = 0.991827, R-sq = 87.68%, R-sq (adj) = 84.78%, R-sq (pred) = 78.10%.
Residual plots:

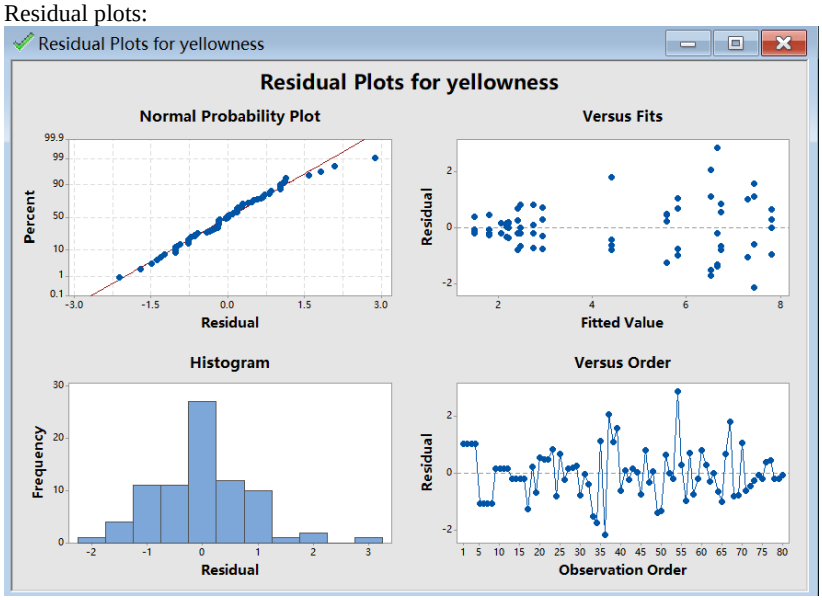


Table 4.21b. Grouping information of the yellowness of DPC16 microcapsules made from different types of wall-materials during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall	N	Mean	Grouping
1	40	6.56275	A
2	40	2.23375	B

Notes.
Means that do not share a letter are significantly different.
In the column of wall-material type, 1 = reconstituted skim milk, 2 = mixed wall material.

Table 4.21c. Grouping information of the yellowness of DPC16 microcapsules at different time points during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Time	N	Mean	Grouping
week 3	16	4.97375	A
week 2	16	4.72187	A
week 0	16	4.68250	A
week 1	16	4.23313	A B
week 4	16	3.38000	B

Notes.
Means that do not share a letter are significantly different.

Table 4.22a. Analysis of variance for general linear model: yellowness versus full factors (wall-material type, packaging-material type and storage time) and their interactions during storage at 55 ℃

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Wall-material type	1	60.45	60.448	55.41	0.000
Packaging-material type	1	25.95	25.946	23.78	0.000
Time	4	115.12	28.780	26.38	0.000
Wall-material type*Packaging-material type	1	40.24	40.243	36.89	0.000
Wall-material type*Time	4	81.88	20.469	18.76	0.000
Packaging-material type*Time	4	15.56	3.891	3.57	0.011
Wall-material type*Packaging-material type*Time	4	18.13	4.532	4.15	0.005
Error	60	65.46	1.091		
Total	79	422.78			

Notes.
Method: Factor coding: (-1, 0, +1).
Factor Information:

Factor	Type	Levels	Values
Wall-material type	Fixed	2	1, 2
Packaging-material type	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model Summary:
S = 1.04450, R-sq = 84.52%, R-sq (adj) = 79.61%, R-sq (pred) = 72.47%.
Residual plots:

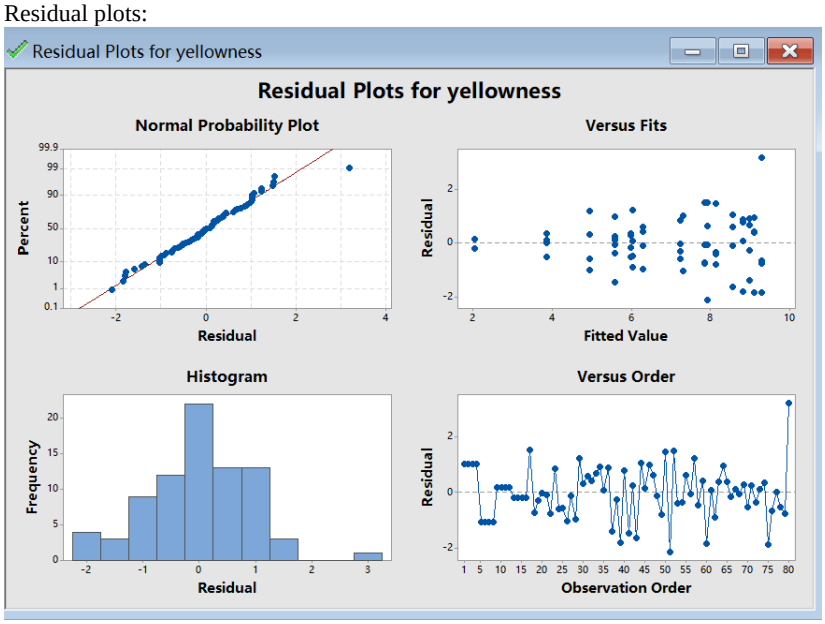


Table 4.22b. Grouping information of the yellowness of DPC16 microcapsules made from different types of wall materials

during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type	N	Mean	Grouping
1	40	7.51575	A
2	40	5.77725	B

Notes.
In the column of wall-material type, 1 = reconstituted skim milk, 2 = mixed wall material.
Means that do not share a letter are significantly different.

Table 4.22c. Grouping information of the yellowness of DPC16 microcapsules packed in different packaging materials during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Packaging-material type	N	Mean	Grouping
2	40	7.216	A
1	40	6.077	B

Notes.
Means that do not share a letter are significantly different.
In the column of packaging-material type, 1 = gas-impermeable film, 2 = aluminium foil bag.

Table 4.22d. Grouping information of the yellowness of DPC16 microcapsules at different time points during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Time	N	Mean	Grouping
week 2	16	7.98937	A
week 3	16	7.79312	A
week 1	16	6.58563	B
week 4	16	6.18187	B
week 0	16	4.68250	C

Notes.
Means that do not share a letter are significantly different.

Table 4.22e. Grouping information of the yellowness of DPC16 microcapsules at different types of wall material*packaging material during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type*Packaging-material type	N	Mean	Grouping
1 1	20	7.6555	A
1 2	20	7.3760	A
2 2	20	7.0560	A
2 1	20	4.4985	B

Notes.
Means that do not share a letter are significantly different.
In the column of Wall-material type*Packaging-material type, the first number is the type of wall materials and the second number is the type of packaging materials. For example,
1 1 = reconstituted skim milk * gas-impermeable film,
2 2 = mixed wall material * aluminium foil bag.

Table 4.22f. Grouping information of the yellowness of DPC16 microcapsules at different types of wall-material type*time points during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type*Time	N	Mean	Grouping
1 2	8	8.91125	A
1 3	8	8.02250	A B
2 3	8	7.56375	A B
1 1	8	7.54500	A B
1 0	8	7.31250	A B C
2 2	8	7.06750	B C
2 4	8	6.57625	B C
1 4	8	5.78750	C
2 1	8	5.62625	C
2 0	8	2.05250	D

Notes.
Means that do not share a letter are significantly different.
In the column of Wall-material type*Time, the first number is the type of wall materials and the second number is the time points. For example,
1 1 = reconstituted skim milk * week 1,
2 2 = mixed wall material * week 2.

Table 4.22g. Grouping information of the yellowness of DPC16 microcapsules at different types of packaging-material

type*time points during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Packaging-material type*Time	N	Mean	Grouping
2 2	8	8.68875	A
2 3	8	8.50500	A
2 4	8	7.43250	A B
1 2	8	7.29000	A B
1 3	8	7.08125	A B
2 1	8	6.77125	B
1 1	8	6.40000	B C
1 4	8	4.93125	C D
1 0	8	4.68250	D
2 0	8	4.68250	D

Notes.
Means that do not share a letter are significantly different.
In the column of Packaging-material type*Time, the first number is the type of packaging materials and the second number is the time points. For example,
1 1 = gas-impermeable film * week 1,
2 2 = aluminium foil bag * week 2.

Table 4.22h. Grouping information of the yellowness of DPC16 microcapsules at different types of wall-material type*packaging-material type*time points during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type*Packaging-material type*Time	N	Mean	Grouping
2 2 4	4	9.2850	A
2 2 3	4	9.0950	A
1 1 2	4	8.9975	A B
1 2 2	4	8.8250	A B
2 2 2	4	8.5525	A B C
1 1 3	4	8.1300	A B C D
1 2 3	4	7.9150	A B C D
1 1 1	4	7.8425	A B C D
1 1 0	4	7.3125	A B C D E
1 2 0	4	7.3125	A B C D E
1 2 1	4	7.2475	A B C D E
2 2 1	4	6.2950	B C D E F
2 1 3	4	6.0325	C D E F
1 1 4	4	5.9950	C D E F
2 1 2	4	5.5825	D E F
1 2 4	4	5.5800	D E F
2 1 1	4	4.9575	E F
2 1 4	4	3.8675	F G
2 2 0	4	2.0525	G
2 1 0	4	2.0525	G

Notes.
Means that do not share a letter are significantly different.
In the column of Wall-material type*Packaging-material type*Time, the first number is the type of wall materials, the second number is the type of packaging materials and the third number is time points. For example,
1 1 1 = reconstituted skim milk * gas-impermeable film * week 1,
2 2 2 = mixed wall material * aluminium foil bag * week 2.

d. Moisture content of microcapsules

Table 4.23 Analysis of variance for general linear model: The moisture content of DPC16 microcapsules versus full factors (storage temperature, wall-material type, packaging-material type and storage time) and their interactions during storage

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Storage temperature	1	0.007107	0.007107	1633.48	0.000
Wall-material type	1	0.001912	0.001912	439.39	0.000
Packaging-material type	1	0.000006	0.000006	1.45	0.231
Time	4	0.000670	0.000168	38.50	0.000
Storage temperature*Wall-material type	1	0.000492	0.000492	113.03	0.000
Storage temperature*Packaging-material type	1	0.003078	0.003078	707.46	0.000
Storage temperature*Time	4	0.001981	0.000495	113.84	0.000
Wall-material type*Packaging-material type	1	0.000275	0.000275	63.10	0.000
Wall-material type*Time	4	0.000118	0.000030	6.79	0.000
Packaging-material type*Time	4	0.000033	0.000008	1.89	0.117
Storage temperature*Wall-material type*Packaging-material type	1	0.000000	0.000000	0.04	0.849
Storage temperature*Wall-material type*Time	4	0.000127	0.000032	7.31	0.000
Storage temperature*Packaging-material type*Time	4	0.000818	0.000205	47.00	0.000

Wall-material type*Packaging-material type*Time	4	0.000095	0.000024	5.44	0.000
Error	124	0.000540	0.000004		
Lack-of-Fit	4	0.000025	0.000006	1.46	0.220
Pure Error	120	0.000515	0.000004		
Total	159	0.017252			

Notes.
Factor coding: (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Storage temperature	Fixed	2	1, 2
Wall-material type	Fixed	2	1, 2
Packaging-material type	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model Summary:
S = 0.0020859 , R-sq = 96.87%, R-sq (adj) = 95.99%, R-sq (pred) = 94.79%
Residual plots:

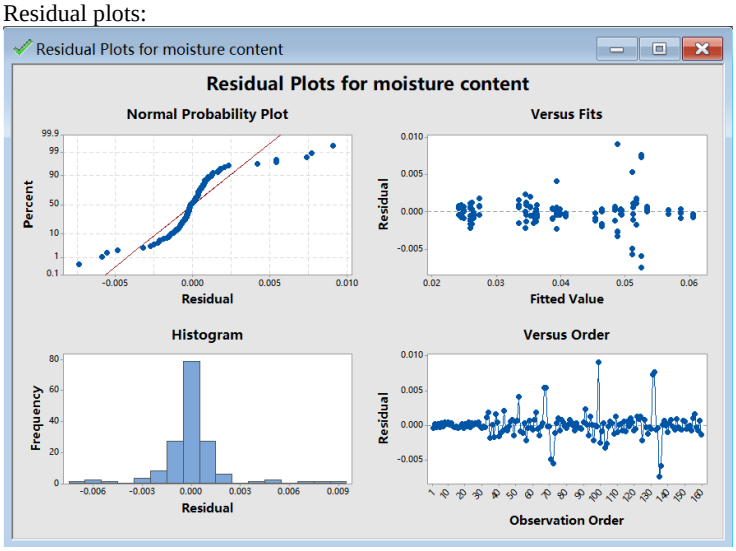


Table 4.24a. Analysis of variance for general linear model: moisture versus full factors (wall-material type, packaging-material type and storage time) and their interactions during storage at 25 ℃

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Wall-material type	1	0.002171	0.002171	289.85	0.000
Packaging-material type	1	0.001403	0.001403	187.27	0.000
Time	4	0.000462	0.000116	15.42	0.000
Wall-material type*Packaging-material type	1	0.000131	0.000131	17.45	0.000
Wall-material type*Time	4	0.000025	0.000006	0.84	0.507
Packaging-material type*Time	4	0.000429	0.000107	14.30	0.000
Wall-material type*Packaging-material type*Time	4	0.000078	0.000019	2.60	0.045
Error	60	0.000450	0.000007		
Total	79	0.005148			

Notes.
Factor coding: (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Wall-material type	Fixed	2	1, 2
Packaging-material type	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model Summary:
S = 0.0027371, R-sq = 91.27%, R-sq (adj) = 88.50%, R-sq (pred) = 84.48%
Residual plots:.

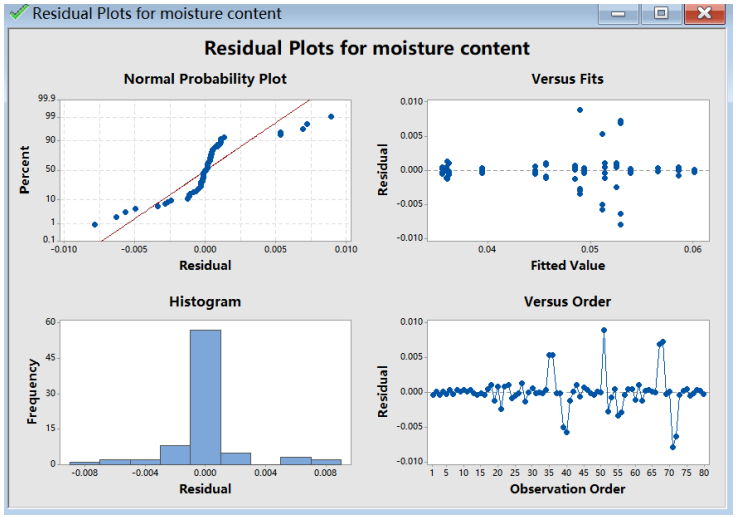


Table 4.24b. Grouping information of the moisture content of DPC16 microcapsules made from different types of wall materials during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type	N	Mean	Grouping
1	40	0.0525438	A
2	40	0.0421240	B

Notes.
Means that do not share a letter are significantly different.
In the column of wall-material type, 1 = reconstituted skim milk, 2 = mixed wall material.

Table 4.24c. Grouping information of the moisture content of DPC16 microcapsules packed in different types of packaging materials during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Packaging-material type	N	Mean	Grouping
1	40	0.0515216	A
2	40	0.0431462	B

Notes.
Means that do not share a letter are significantly different.
In the column of packaging-material type, 1 = gas-impermeable film, 2 = aluminium foil bag.

Table 4.24d. Grouping information of the moisture content of DPC16 microcapsules at different time points during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Time	N	Mean	Grouping
week 4	16	0.0506437	A
week 3	16	0.0487437	A
week 2	16	0.0481375	A
week 1	16	0.0447070	B
week 0	16	0.0444375	B

Notes.
Means that do not share a letter are significantly different.

Table 4.24e Grouping information of the moisture content of DPC16 microcapsules packed at different Wall-material type and Packaging-material type during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type*Packaging-material type	N	Mean	Grouping
1 1	20	0.0554530	A
1 2	20	0.0496346	B
2 1	20	0.0475902	B
2 2	20	0.0366578	C

Notes.
Means that do not share a letter are significantly different.
In the column of Wall-material type*Packaging-material type, 1 1 = RSM * gas-impermeable film; 2 2 = MWM * aluminium foil bags.

Table 4.24f Grouping information of the moisture content of DPC16 microcapsules packed at different packaging-material

type*storage time during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Packaging-material type*Time	N	Mean	Grouping
1 4	8	0.0570125	A
1 3	8	0.0550125	A
1 2	8	0.0525750	A B
1 1	8	0.0485705	B C
2 0	8	0.0444375	C D
1 0	8	0.0444375	C D
2 4	8	0.0442750	C D
2 2	8	0.0437000	D
2 3	8	0.0424750	D
2 1	8	0.0408435	D

Notes.
Means that do not share a letter are significantly different.
In the column of Packaging-material type*Time, 1 1 = gas-impermeable film*week 1; 2 2 = aluminium foil bags*week 2.

Table 4.24g Grouping information of the moisture content of DPC16 microcapsules packed at different wall-material type* packaging-material type*storage time during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type*Packaging-material type*Time	N	Mean	Grouping
1 1 4	4	0.0601250	A
1 1 3	4	0.0585750	A B
1 1 2	4	0.0566250	A B C
2 1 4	4	0.0539000	A B C D
1 2 4	4	0.0529608	A B C D
1 1 1	4	0.0525151	B C D E
2 1 3	4	0.0514500	B C D E F
1 2 2	4	0.0511750	C D E F
1 2 0	4	0.0494250	D E F
1 1 0	4	0.0494250	D E F
1 2 3	4	0.0489750	D E F
2 1 2	4	0.0485250	D E F
1 2 1	4	0.0456370	E F G
2 1 1	4	0.0446259	F G
2 2 0	4	0.0394500	G H
2 1 0	4	0.0394500	G H
2 2 2	4	0.0362250	H
2 2 1	4	0.0360500	H
2 2 3	4	0.0359750	H
2 2 4	4	0.0355891	H

Notes.
Means that do not share a letter are significantly different.
In the column of Wall-material type*Packaging-material type*Time, 1 1 1 = RSM*gas-impermeable film*week 1; 2 2 2 = MWM*aluminium foil bags*week 2.

Table 4.25a. Analysis of variance for general linear model: moisture content versus wall-material type, packaging-material type and storage time at 55 ℃

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Wall-material type	1	0.000232	0.000232	214.09	0.000
Packaging-material type	1	0.001682	0.001682	1550.68	0.000
Time	4	0.002189	0.000547	504.73	0.000
Wall-material type*Packaging-material type	1	0.000144	0.000144	132.73	0.000
Wall-material type*Time	4	0.000220	0.000055	50.80	0.000
Packaging-material type*Time	4	0.000422	0.000106	97.34	0.000
Wall-material type*Packaging-material type*Time	4	0.000042	0.000010	9.64	0.000
Error	60	0.000065	0.000001		
Total	79	0.004996			

Notes.
Factor coding: (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Wall-material-type	Fixed	2	1, 2
Packaging-material-type	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model summary:

S = 0.0010413, R-sq = 98.70%, R-sq (adj) = 98.29%, R-sq (pred) = 97.68%.
Residual plots:

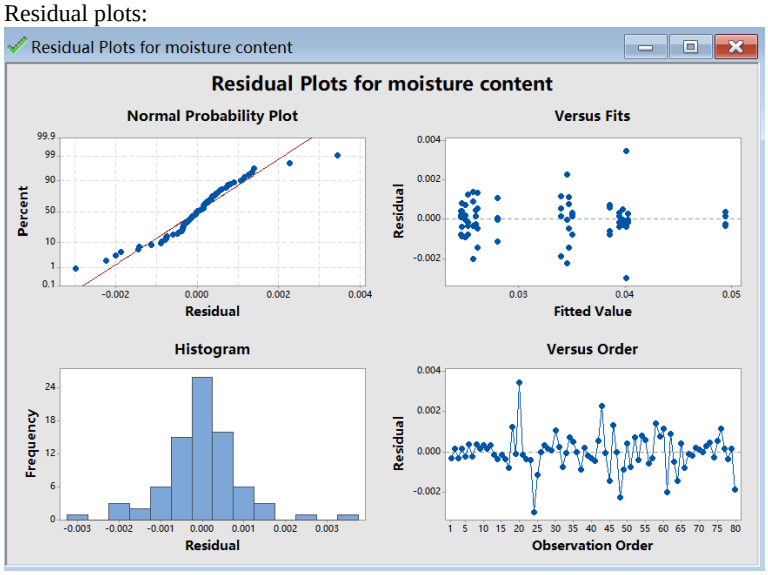


Table 4.25b Grouping information of the moisture content of DPC16 microcapsules made from different types of wall materials during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type	N	Mean	Grouping
1	40	0.0357076	A
2	40	0.0323006	B

Notes.
Means that do not share a letter are significantly different.
In the column of wall-material type, 1 = reconstituted skim milk, 2 = mixed wall material.

Table 4.25c Grouping information of the moisture content of DPC16 microcapsules packed in different types of packaging materials during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Packaging-material type	N	Mean	Grouping
2	40	0.0385888	A
1	40	0.0294194	B

Notes.
Means that do not share a letter are significantly different.
In the column of packaging-material type, 1 = gas-impermeable film, 2 = aluminium foil bag.

Table 4.25d Grouping information of the moisture content of DPC16 microcapsules at different time points during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Time	N	Mean	Grouping
week 0	16	0.0444375	A
week 1	16	0.0320997	B
week 2	16	0.0313669	B C
week 4	16	0.0311964	B C
week 3	16	0.0309200	C

Notes.
Means that do not share a letter are significantly different.

Table 4.25e Grouping information of the moisture content of DPC16 microcapsules at different wall-material type*packaging-material type during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type*Packaging-material type	N	Mean	Grouping
1 2	20	0.0416336	A
2 2	20	0.0355439	B
1 1	20	0.0297816	C
2 1	20	0.0290572	C

Notes.
Means that do not share a letter are significantly different.
In the column of Wall-material type*Packaging-material type, 1 1 = RSM*gas-impermeable film; 2 2 = MWM*aluminium foil bags.

Table 4.25f Grouping information of the moisture content of DPC16 microcapsules at different wall-material type*storage

time during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type*Time	N	Mean	Grouping
1 0	8	0.0494250	A
2 0	8	0.0394500	B
1 1	8	0.0326620	C
1 4	8	0.0324436	C
1 2	8	0.0323900	C
1 3	8	0.0316174	C D
2 1	8	0.0315375	C D
2 2	8	0.0303437	D
2 3	8	0.0302227	D
2 4	8	0.0299491	D

Notes.
Means that do not share a letter are significantly different.
In the column of Wall-material type*Time, 1 1 = RSM*week 1; 2 2 = MWM*week2.

Table 4.25g Grouping information of the moisture content of DPC16 microcapsules at different packaging-material type and time during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Packaging-material type*Time	N	Mean	Grouping
1 0	8	0.0444375	A
2 0	8	0.0444375	A
2 1	8	0.0375658	B
2 2	8	0.0371620	B
2 4	8	0.0371271	B
2 3	8	0.0366514	B
1 1	8	0.0266336	C
1 2	8	0.0255717	C
1 4	8	0.0252656	C
1 3	8	0.0251887	C

Notes.
Means that do not share a letter are significantly different.
In the column of Packaging-material type*Time, the first number is the type of packaging materials and the second number is the time points. For example,
1 1 = gas-impermeable film * week 1,
2 2 = aluminium foil bag * week 2.

Table 4.25h Grouping information of the moisture content of DPC16 microcapsules at different wall-material type* packaging-material type*storage time during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type*Packaging-material type*Time	N	Mean	Grouping
1 1 0	4	0.0494250	A
1 2 0	4	0.0494250	A
1 2 4	4	0.0403101	B
1 2 1	4	0.0400817	B
1 2 2	4	0.0397914	B
2 1 0	4	0.0394500	B
2 2 0	4	0.0394500	B
1 2 3	4	0.0385598	B
2 2 1	4	0.0350500	C
2 2 3	4	0.0347430	C
2 2 2	4	0.0345326	C
2 2 4	4	0.0339441	C
2 1 1	4	0.0280250	D
2 1 2	4	0.0261548	D E
2 1 4	4	0.0259541	D E
2 1 3	4	0.0257023	D E
1 1 1	4	0.0252422	E
1 1 2	4	0.0249886	E
1 1 3	4	0.0246750	E
1 1 4	4	0.0245772	E

Notes.
Means that do not share a letter are significantly different.
In the column of Wall-material type*Packaging-material type*Time, 1 1 1 = RSM*gas-impermeable film*week 1; 2 2 2 = MWM*aluminium foil bags*week 2.

e. Bulk density of microcapsules

Table 4.26 Analysis of variance for general linear model: The bulk density of DPC16 microcapsules versus full factors (storage temperature, wall-material type, packaging-material type and storage time) and their interactions during storage

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Storage temperature	1	0.112944	0.112944	566.76	0.000
Wall-material type	1	0.122711	0.122711	615.77	0.000
Packaging-material type	1	0.000050	0.000050	0.25	0.619
Time	4	0.003501	0.000875	4.39	0.002
Storage temperature*Wall-material type	1	0.005869	0.005869	29.45	0.000
Storage temperature*Packaging-material type	1	0.033264	0.033264	166.92	0.000
Storage temperature*Time	4	0.033416	0.008354	41.92	0.000
Wall-material type*Packaging-material type	1	0.000879	0.000879	4.41	0.038
Wall-material type*Time	4	0.001149	0.000287	1.44	0.224
Packaging-material type*Time	4	0.001937	0.000484	2.43	0.051
Storage temperature*Wall-material type*Packaging-material type	1	0.003177	0.003177	15.94	0.000
Storage temperature*Wall-material type*Time	4	0.001740	0.000435	2.18	0.075
Storage temperature*Packaging-material type*Time	4	0.011503	0.002876	14.43	0.000
Wall-material type*Packaging-material type*Time	4	0.000852	0.000213	1.07	0.375
Error	124	0.024711	0.000199		
Lack-of-Fit	4	0.001496	0.000374	1.93	0.109
Pure Error	120	0.023214	0.000193		
Total	159	0.357702			

Notes.
Factor coding: (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Storage temperature	Fixed	2	1, 2
Wall-material type	Fixed	2	1, 2
Packaging-material type	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model Summary:
S = 0.0141166, R-sq = 93.09%, R-sq (adj) = 91.14%, R-sq (pred) = 88.50%
Residual plots:

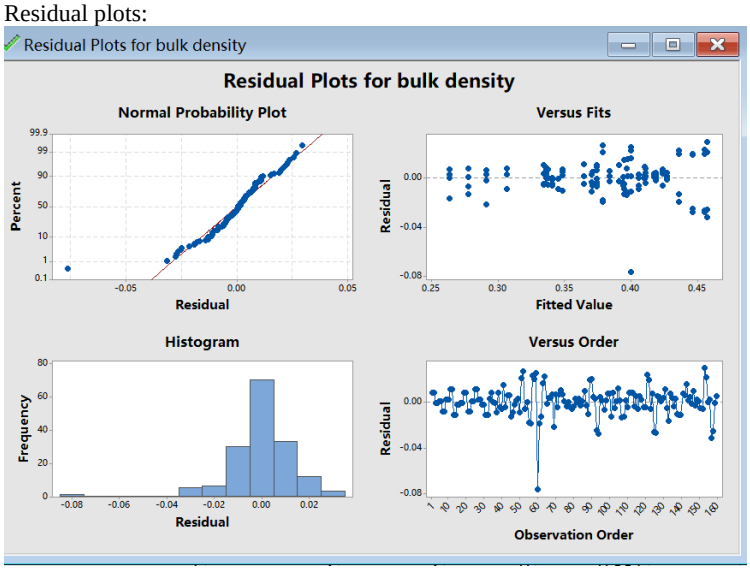


Table 4.27a. Analysis of variance for general linear model: The bulk density of DPC16 microcapsules versus full factors (wall-material type, packaging-material type and storage time) and their interactions during storage at 25 ℃

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Wall-material type	1	0.091125	0.091125	1340.57	0.000
Packaging-material type	1	0.017940	0.017940	263.92	0.000
Time	4	0.009744	0.002436	35.84	0.000
Wall-material type*Packaging-material type	1	0.003699	0.003699	54.42	0.000
Wall-material type*Time	4	0.001009	0.000252	3.71	0.009
Packaging-material type*Time	4	0.009517	0.002379	35.00	0.000
Wall-material type*Packaging-material type*Time	4	0.001002	0.000251	3.69	0.009
Error	60	0.004078	0.000068		
Total	79	0.138114			

Notes.
Factor coding: (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Wall-material type	Fixed	2	1, 2
Packaging-material type	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model Summary:
S = 0.0082447, R-sq = 97.05%, R-sq(adj) = 96.11%, R-sq (pred) = 94.75%.
Residual plots:

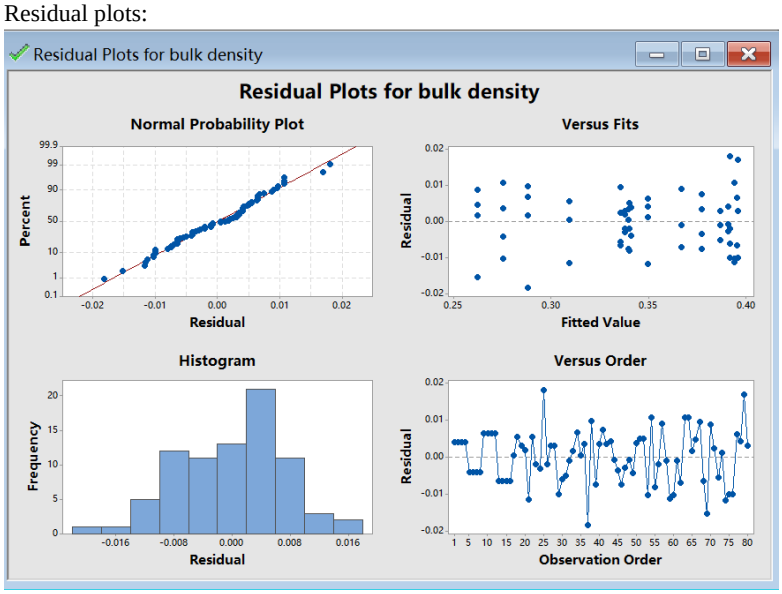


Table 4.27b. Grouping information of the lightness of DPC16 microcapsules made from different types of wall materials during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type	N	Mean	Grouping
2	40	0.384525	A
1	40	0.317025	B

Notes.
Means that do not share a letter are significantly different.
In the column of wall-material type, 1 = reconstituted skim milk, 2 = mixed wall material.

Table 4.27c. Grouping information of the bulk density of DPC16 microcapsules made from different types of wall materials during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Packaging-material type	N	Mean	Grouping
2	40	0.36575	A
1	40	0.33580	B

Notes.
Means that do not share a letter are significantly different.
In the column of packaging-material type, 1 = gas-impermeable film, 2 = aluminium foil bag.

Table 4.27d. Grouping information of the bulk density of DPC16 microcapsules at different time points during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Time	N	Mean	Grouping
week 0	16	0.368250	A
week 1	16	0.356625	B
week 2	16	0.349000	B C
week 3	16	0.344125	C
week 4	16	0.335875	D

Notes.
Means that do not share a letter are significantly different.

Table 4.27e Grouping information of the bulk density of DPC16 microcapsules at different wall materials * packaging material during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type*Packaging-material type	N	Mean	Grouping
2 2	20	0.39270	A
2 1	20	0.37635	B
1 2	20	0.33880	C
1 1	20	0.29525	D

Notes.
Means that do not share a letter are significantly different.
In the column of Wall-material type*packaging-material type, the first number is the type of wall materials and the second number is the type of packaging materials. For example,
1 1 = reconstituted skim milk*gas-impermeable film,
2 2 = mixed wall material*aluminium foil bag.

Table 4.27f Grouping information of the bulk density of DPC16 microcapsules in different wall-material type*(storage) time during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type*Time	N	Mean	Grouping
2 0	8	0.395500	A
2 1	8	0.389500	A B
2 2	8	0.384125	A B C
2 3	8	0.380625	B C
2 4	8	0.372875	C
1 0	8	0.341000	D
1 1	8	0.323750	E
1 2	8	0.313875	E F
1 3	8	0.307625	F G
1 4	8	0.298875	G

Notes.
In the column of Wall-material type*Time, the first number is the type of wall materials and the second number is the time points. For example,
1 1 = reconstituted skim milk * week 1,
2 2 = mixed wall material * week 2.

Table 4.27g Grouping information of the bulk density of DPC16 microcapsules at different types of packaging-material type*time points during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Packaging-material type*Time	N	Mean	Grouping
1 0	8	0.368250	A
2 0	8	0.368250	A
2 3	8	0.367125	A
2 4	8	0.365750	A
2 2	8	0.365125	A
2 1	8	0.362500	A B
1 1	8	0.350750	B
1 2	8	0.332875	C
1 3	8	0.321125	C
1 4	8	0.306000	D

Notes.
Means that do not share a letter are significantly different.
In the column of Packaging-material type*Time, the first number is the type of packaging materials and the second number is the time points. For example,
1 1 = gas-impermeable film * week 1,
2 2 = aluminium foil bag * week 2.

Table 4.27h Grouping information of the bulk density of DPC16 microcapsules at different types of wall-material type*packaging-material type*time points during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type*Packaging-material type*Time	N	Mean	Grouping
2 2 4	4	0.39600	A
2 2 0	4	0.39550	A
2 1 0	4	0.39550	A
2 2 3	4	0.39425	A
2 1 1	4	0.39200	A
2 2 2	4	0.39075	A
2 2 1	4	0.38700	A B
2 1 2	4	0.37750	A B
2 1 3	4	0.36700	B C
2 1 4	4	0.34975	C D
1 1 0	4	0.34100	D

1 2 0	4	0.34100	D
1 2 3	4	0.34000	D
1 2 2	4	0.33950	D
1 2 1	4	0.33800	D
1 2 4	4	0.33550	D
1 1 1	4	0.30950	E
1 1 2	4	0.28825	E F
1 1 3	4	0.27525	F G
1 1 4	4	0.26225	G

Notes.
Means that do not share a letter are significantly different.
In the column of Wall-material type*Packaging-material type*Time, the first number is the type of wall materials, the second number is the type of packaging materials and the third number is time points. For example,
1 1 1 = reconstituted skim milk * gas-impermeable film * week 1,
2 2 2 = mixed wall material * aluminium foil bag * week 2.

Table 4.28a. Analysis of variance for general linear model: The bulk density of DPC16 microcapsules versus full factors (wall-material type, packaging-material type and storage time) and their interactions during storage at 55 ℃

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Wall-material type	1	0.037455	0.037455	117.44	0.000
Packaging-material type	1	0.015374	0.015374	48.20	0.000
Time	4	0.027173	0.006793	21.30	0.000
Wall-material type*Packaging-material type	1	0.000357	0.000357	1.12	0.294
Wall-material type*Time	4	0.001881	0.000470	1.47	0.221
Packaging-material type*Time	4	0.003923	0.000981	3.08	0.023
Wall-material type*Packaging-material type*Time	4	0.001346	0.000337	1.06	0.387
Error	60	0.019136	0.000319		
Total	79	0.106644			

Notes.
Method: Factor coding: (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Wall-material type	Fixed	2	1, 2
Packaging-material type	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model Summary
S = 0.0178586, R-sq = 82.06%, R-sq (adj) = 76.37%, R-sq (pred) = 68.10%.
Residual plots:

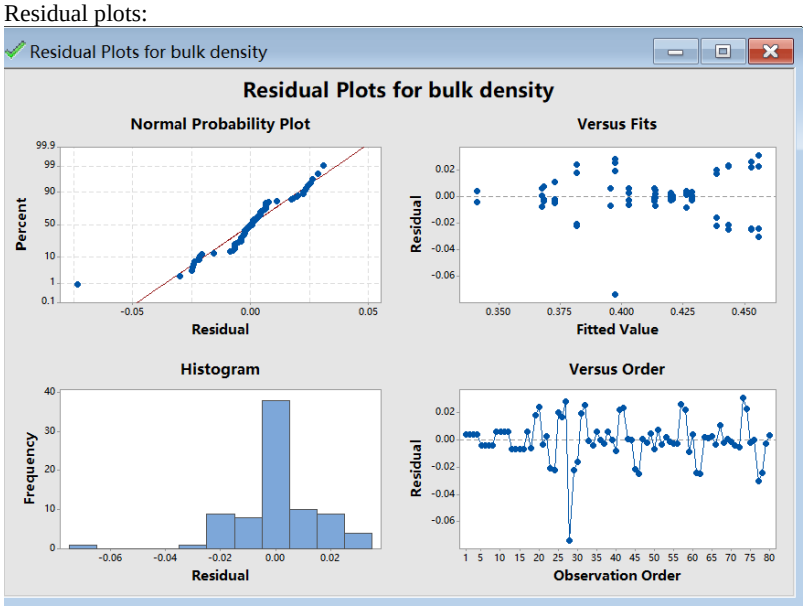


Table 4.28b. Grouping information of the bulk density of DPC16 microcapsules made from different types of wall materials during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type	N	Mean	Grouping
2	40	0.425550	A
1	40	0.382275	B

Notes.
Means that do not share a letter are significantly different.
In the column of Wall-material type, 1 = reconstituted skim milk, 2 = mixed wall material.

Table 4.28c. Grouping information of the bulk density of DPC16 microcapsules packed in different types of packaging materials during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Packaging-material type	N	Mean	Grouping
1	40	0.417775	A
2	40	0.390050	B

Notes.
Means that do not share a letter are significantly different.
In the column of packaging-material type, 1 = gas-impermeable film, 2 = aluminium foil bag.

Table 4.28d Grouping information of the bulk density of DPC16 microcapsules at different time points during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Time	N	Mean	Grouping
week 4	16	0.419375	A
week 3	16	0.415312	A
week 2	16	0.411375	A
week 1	16	0.405250	A
week 0	16	0.368250	B

Notes.
Means that do not share a letter are significantly different.

Table 4.28e Grouping information of the bulk density of DPC16 microcapsules in different wall-material type*Packaging material type during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type*Packaging-material type	N	Mean	Grouping
2 1	20	0.43730	A
2 2	20	0.41380	B
1 1	20	0.39825	C
1 2	20	0.36630	D

Notes.
Means that do not share a letter are significantly different.
In the column of Wall-material type*packaging-material type, the first number is the type of wall materials and the second number is the type of packaging materials. For example,
1 1 = reconstituted skim milk * gas-impermeable film,
2 2 = mixed wall material * aluminium foil bag.

Table 4.28f Grouping information of the bulk density of DPC16 microcapsules in different wall-material type * (storage) time at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type*Time	N	Mean	Grouping
2 4	8	0.442250	A
2 3	8	0.439625	A
2 2	8	0.432250	A
2 1	8	0.418125	A B
1 4	8	0.396500	B
2 0	8	0.395500	B
1 1	8	0.392375	B
1 3	8	0.391000	B
1 2	8	0.390500	B
1 0	8	0.341000	C

Notes.
In the column of Wall-material type*Time, the first number is the type of wall materials and the second number is the time points. For example,
1 1 = reconstituted skim milk * week 1,
2 2 = mixed wall material * week 2.

Table 4.28g Grouping information of the bulk density of DPC16 microcapsules at different types of packaging-material type*time points during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Packaging-material type*Time	N	Mean	Grouping
1 4	8	0.438000	A

1 3	8	0.433250	A
1 2	8	0.428500	A B
1 1	8	0.420875	A B C
2 4	8	0.400750	B C D
2 3	8	0.397375	C D E
2 2	8	0.394250	C D E
2 1	8	0.389625	D E
1 0	8	0.368250	E
2 0	8	0.368250	E

Notes.
Means that do not share a letter are significantly different.
In the column of Packaging-material type*Time, the first number is the type of packaging materials and the second number is the time points. For example,
1 1 = gas-impermeable film * week 1,
2 2 = aluminium foil bag * week 2.

Table 4.28h Grouping information of the bulk density of DPC16 microcapsules at different types of wall-material type*packaging-material type*time points during storage at 55 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall-material type*Packaging-material type*Time	N	Mean	Grouping
2 1 4	4	0.45600	A
2 1 3	4	0.45275	A
2 1 2	4	0.44350	A B
2 1 1	4	0.43875	A B C
2 2 4	4	0.42850	A B C D
2 2 3	4	0.42650	A B C D
2 2 2	4	0.42100	A B C D
1 1 4	4	0.42000	A B C D
1 1 3	4	0.41375	A B C D E
1 1 2	4	0.41350	A B C D E
1 1 1	4	0.40300	B C D E
2 2 1	4	0.39750	B C D E
2 1 0	4	0.39550	C D E
2 2 0	4	0.39550	C D E
1 2 1	4	0.38175	D E F
1 2 4	4	0.37300	E F
1 2 3	4	0.36825	E F
1 2 2	4	0.36750	E F
1 1 0	4	0.34100	F
1 2 0	4	0.34100	F

Notes.
Means that do not share a letter are significantly different.
In the column of Wall-material type*Packaging-material type*Time, the first number is the type of wall materials, the second number is the type of packaging materials and the third number is time points. For example,
1 1 1 = reconstituted skim milk * gas-impermeable film * week 1,
2 2 2 = mixed wall material * aluminium foil bag * week 2.

f. Particle size of microcapsules at the storage stage

Table 4.29a Analysis of variance for general linear model: The particle size of DPC16 microcapsules versus full factors (storage temperature, wall-material type, packaging-material type and storage time) and their interactions during storage

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Storage temperature	1	10.18	10.1807	4.10	0.043
Wall-material type	1	3.27	3.2714	1.32	0.251
Packaging-material type	1	16.77	16.7747	6.75	0.009
Time	4	9.93	2.4832	1.00	0.407
Storage temperature*Wall-material type	1	3.19	3.1912	1.28	0.257
Storage temperature*Packaging-material type	1	0.48	0.4802	0.19	0.660
Storage temperature*Time	4	17.46	4.3647	1.76	0.135
Wall-material type*Packaging-material type	1	2.80	2.8004	1.13	0.289
Wall-material type*Time	4	18.68	4.6691	1.88	0.112
Packaging-material type*Time	4	12.09	3.0228	1.22	0.302
Storage temperature*Wall-material type*Packaging-material type	1	4.40	4.3984	1.77	0.184
Storage temperature*Wall-material type*Time	4	9.04	2.2599	0.91	0.458
Storage temperature*Packaging-material type*Time	4	15.53	3.8819	1.56	0.182
Wall-material type*Packaging-material type*Time	4	34.88	8.7189	3.51	0.007

Error	1164	2892.81	2.4852		
Lack-of-Fit	4	11.04	2.7597	1.11	0.350
Pure Error	1160	2881.77	2.4843		
Total	1199	3051.51			

Notes.
Method: Factor coding (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Temperature	Fixed	2	1, 2
Packaging material	Fixed	2	1, 2

Model Summary:
S = 1.57646, R-sq = 5.20%, R-sq(adj) = 2.35%, R-sq(pred) = 0.00%.
Residual plots:

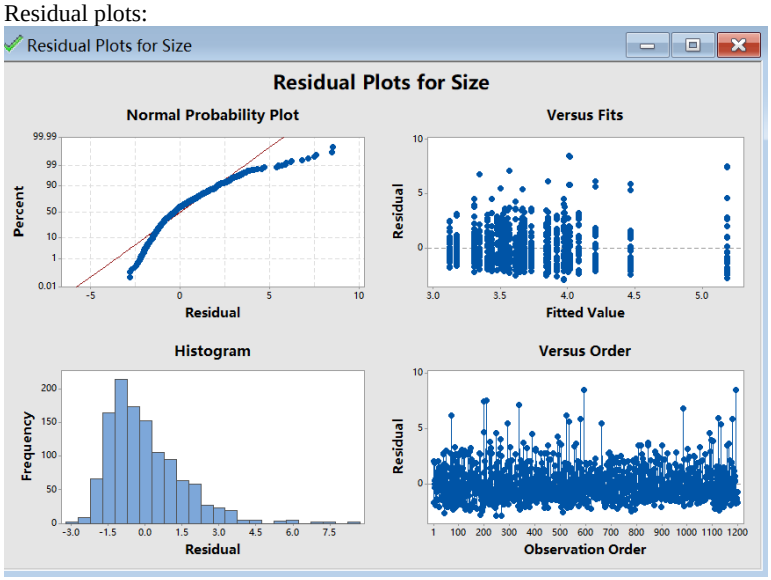


Table 4.29b. Grouping information of the particle size of DPC16 microcapsules stored under different temperatures using the Tukey pairwise comparisons and 95% confidence

Temperature	N	Mean	Grouping
1	600	3.80209	A
2	600	3.61787	B

Notes. Means that do not share a letter are significantly different.
In the column of temperature, 1 = 25 ℃, 2 = 55 ℃.

Table 4.29c. Grouping information of the particle size of DPC16 microcapsules packed in different packaging materials using the Tukey pairwise comparisons and 95% confidence

Packaging-material type	N	Mean	Grouping
2	600	3.82821	A
1	600	3.59175	B

Notes. Means that do not share a letter are significantly different.
In the column of packaging-material type, 1 = gas-impermeable film, 2 = aluminium foil bag.

Table 4.29d. Grouping information of the particle size of DPC16 microcapsules packed at different wall-material type*packaging-material type*storage time using the Tukey pairwise comparisons and 95% confidence

Wall-material type*Packaging-material type*Time	N	Mean	Grouping
1 2 1	60	4.51455	A
2 2 2	60	4.33663	A B
2 2 4	60	4.01342	A B
1 1 4	60	3.87955	A B
1 2 2	60	3.81852	A B
1 1 2	60	3.75682	A B
1 2 3	60	3.75427	A B
2 2 3	60	3.69881	A B
1 1 3	60	3.62872	A B
2 2 0	60	3.61619	A B
2 1 0	60	3.61619	A B

1 1 1	60	3.61092	A	B
1 2 0	60	3.58533	A	B
1 1 0	60	3.58533	A	B
2 1 4	60	3.56505	A	B
2 1 1	60	3.53817	A	B
1 2 4	60	3.48791		B
2 2 1	60	3.45649		B
2 1 2	60	3.38933		B
2 1 3	60	3.34738		B

Notes.
Means that do not share a letter are significantly different.
In the column of Wall-material type*Packaging-material type*Time, the first number is the type of wall materials, the second number is the type of packaging materials and the third number is time points. For example,
1 1 1 = reconstituted skim milk * gas-impermeable film * week 1,
2 2 2 = mixed wall material * aluminium foil bag * week 2.

Table 4.30a Analysis of variance for general linear model: The particle size of DPC16 microcapsules versus full factors (wall-material type, packaging-material type and storage time) and their interactions during storage at 25 ℃

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Wall	1	6.46	6.4624	2.29	0.131
Packaging	1	11.47	11.4655	4.06	0.044
Time	4	21.71	5.4283	1.92	0.106
Wall*Packaging	1	0.09	0.0898	0.03	0.859
Wall*Time	4	24.72	6.1805	2.19	0.069
Packaging*Time	4	19.40	4.8497	1.72	0.145
Wall*Packaging*Time	4	38.36	9.5908	3.39	0.009
Error	580	1639.77	2.8272		
Total	599	1761.99			

Notes.
Wall = wall-material type; Packaging = packaging-material type; Time = storage time.
Method: Factor coding (-1, 0, +1).
Factor Information:

Factor	Type	Levels	Values
Wall	Fixed	2	1, 2
Packaging	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model Summary:
S = 1.68143, R-sq = 6.94%, R-sq(adj) = 3.89%, R-sq(pred) = 0.41%.
Residual plots:

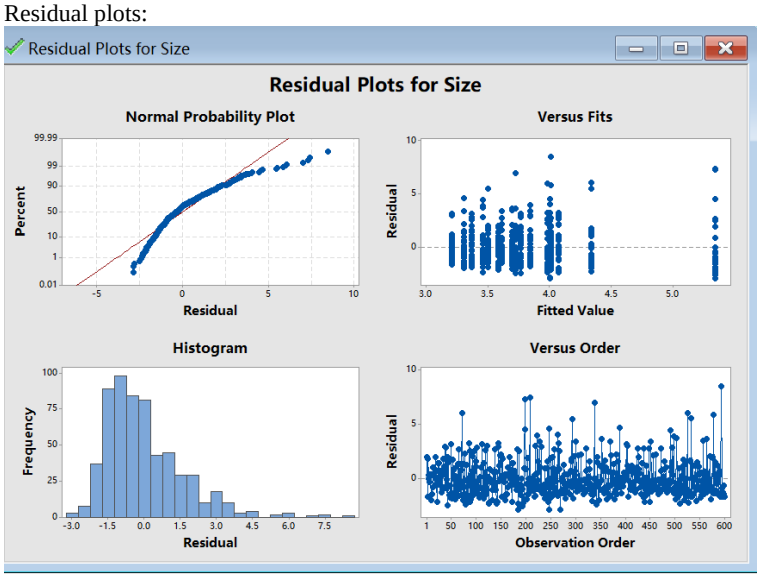


Table 4.30b. Grouping information of the particle size of DPC16 microcapsules vacuum-packed in different packaging materials during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Packaging-material type	N	Mean	Grouping
2	300	3.94032	A
1	300	3.66385	B

Notes. Means that do not share a letter are significantly different.
In the column of packaging-material type, 1 = gas-impermeable film, 2 = aluminium foil bag.

Table 4.30c. Grouping information of the particle size of DPC16 microcapsules under different wall material*packaging material*storage time during storage at 25 ℃ using the Tukey pairwise comparisons and 95% confidence

Wall*Packaging*Time	N	Mean	Grouping
1 2 1	30	5.34132	A
2 2 2	30	4.33663	A B
1 1 4	30	4.07641	A B
2 2 4	30	4.01342	A B
2 1 4	30	4.01103	A B
1 2 3	30	4.00522	A B
1 1 2	30	3.98287	A B
1 2 2	30	3.84487	A B
1 1 1	30	3.76342	B
2 1 1	30	3.72389	B
2 2 3	30	3.69881	B
2 1 0	30	3.61619	B
2 2 0	30	3.61619	B
1 2 0	30	3.58533	B
1 1 0	30	3.58533	B
1 2 4	30	3.50494	B
2 2 1	30	3.45649	B
1 1 3	30	3.36896	B
2 1 2	30	3.30339	B
2 1 3	30	3.20701	B

Notes. Means that do not share a letter are significantly different.
In the column of Wall*Packaging*Time, the first number is the type of wall materials, the second number is the type of packaging materials and the third number is storage time. For example,
1 1 1 = reconstituted skim milk * gas-impermeable film * week 1,
2 2 2 = mixed wall material * aluminium foil bag * week 2.

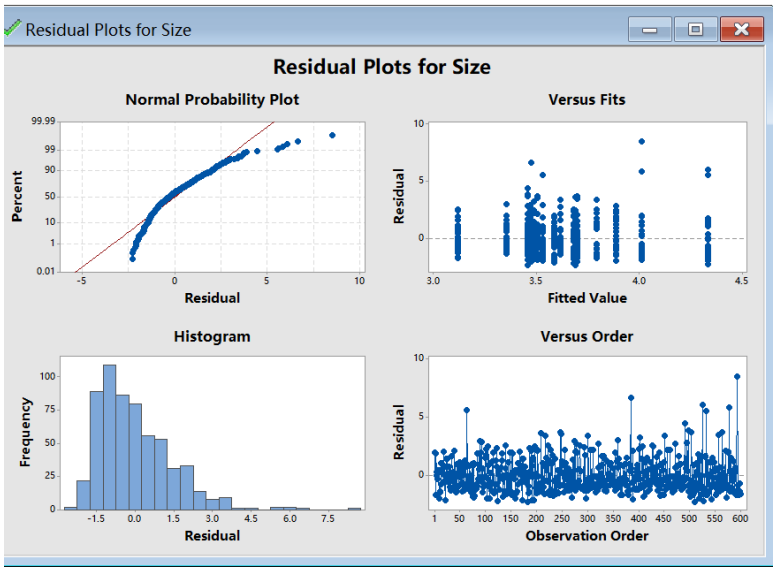
Table 4.31. Analysis of variance for general linear model: particle size of DPC16 microcapsules versus wall material, packaging materials and storage time during storage at 55 ℃

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Wall-material type	1	0.00	0.00025	0.00	0.991
Packaging-material type	1	5.79	5.78938	2.70	0.101
Time	4	5.68	1.41962	0.66	0.618
Wall-material type*Packaging-material type	1	7.11	7.10907	3.32	0.069
Wall-material type*Time	4	2.99	0.74844	0.35	0.844
Packaging-material type*Time	4	8.22	2.05497	0.96	0.429
Wall-material type*Packaging-material type*Time	4	7.55	1.88776	0.88	0.475
Error	580	1242.00	2.14138		
Total	599	1279.34			

Notes.
Wall = wall-material type; Packaging = packaging-material type; Time = storage time.
Method: Factor coding (-1, 0, +1)
Factor Information:

Factor	Type	Levels	Values
Wall	Fixed	2	1, 2
Packaging	Fixed	2	1, 2
Time	Fixed	5	0, 1, 2, 3, 4

Model Summary
S = 1.46334, R-sq = 2.92%, R-sq (adj) = 0.00%, R-sq (pred) = 0.00%
Residual plots:


Residual Plots for Size
Residual Plots for Size
Normal Probability Plot
Percent
Residual
Versus Fits
Residual
Fitted Value
Histogram
Frequency
Residual
Versus Order
Residual
Observation Order