SURVIVAL OF ENCAPSULATED PROBIOTIC BACTERIA DURING STORAGE AT LOW WATER ACTIVITY AT AMBIENT TEMPERATURE

A thesis submitted for the degree of Doctor of Philosophy

by

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I dedicate this thesis to my father,

late Mr. Noroyono Artodibyo,

who believed so much in sciences.

Abstract

Probiotics are defined by FAO/WHO (2001) as microorganisms that improve the healthiness of the host when available in sufficient number in the human intestinal tract. Health benefits of probiotic bacteria are correlated with the number of viable bacteria reaching the host and the recommended bacterial population required is $\geq 10^7$ CFU/g. Besides during processing and storage, probiotic bacteria are required to survive during transit in gastrointestinal tract with very low pH (1.8 – 2.0), in bile juice and pancreatin before beneficial effects are realized in the lower small intestine and colon. Microencapsulation is effective in ensuring bacterial viability which is influenced by the type of encapsulating materials, the methods used for encapsulation and storage thereafter for a given bacterium.

The use of microencapsulating materials is to entrap or immobilize probiotic bacteria within microcapsule and to protect the bacteria during the drying process, which is the last stage of microencapsulation. The objectives of this thesis were to study the effect of different types of microencapsulants (alginate or casein-based formulations), and drying methods (freeze and spray drying) on survival of probiotic bacteria for 10 weeks of storage at 25^oC (ambient temperature) at low water activities (a_w). Three probiotic bacteria (*Bifidobacterium animalis* ssp. *lactis* Bb12, *Bifidobacterium longum* and *Lactobacillus acidophilus*) and one strain of most sensitive lactic acid bacteria (*L. lactis* ssp. *cremoris)* to acid and bile environment were encapsulated and selected functional properties, such as survival, acid and bile tolerance, surface hydrophobicity, and retaining of some of the enzyme activities were investigated. The protective mechanism of microencapsulation was studied by determinations of microstructures using Environment Scanning Electron Microscopy (ESEM), interactions with cellular components by Fourier

Transform Infrared Spectroscopy (FTIR), and glass transition temperature (Tg) measurements by Differential Scanning Calorimetry (DSC).

Microencapsulation of *Bifidobacterium animalis* ssp. *lactis* Bb12 using alginate-based formulation (with or without mannitol) was carried out and stored at low a_w (0.07, 0.1 and 0.2) at 25^{o}C for 10 weeks. Storage at a_w of 0.1 improved survival, acid and bile tolerance, surface hydrophobicity and retention of β-glucosidase, β-galactosidase, lactate dehydrogenase, piruvate kinase, hexokinase, ATPase activities irrespective on with or without mannitol. The effectiveness of alginate-mannitol combination decreased upon storage (82.6% survival at 10 weeks at a_w of 0.1). Mannitol interacted with the polar sites of the phospholipid bilayer of *B. animalis* through H-bond interaction; this explained why encapsulation using alginate-mannitol provided higher survival compared to alginate alone after freeze drying. ESEM showed that incorporation of mannitol into alginate provided smooth surface of the microcapsules.

Due to ineffectiveness of alginate, the use of a casein-based formulation combined with glucose-mannitol (GM) or glucose (G) as microencapsulant was investigated to ascertain the effect on the stability of *Bifidobacterium animalis* ssp. *lactis*Bb12 after spray drying and subsequent storage. Survival of microencapsulated *Bifidobacterium* with GM increased after spray drying with viability retained for 10 weeks of storage at a_w of 0.07. FTIR analysis indicated that GM and G interacted with P=O group of phospholipid bilayers of the bacteria. Mannitol in GM formulation preserved the proteins of bacteria as indicated by Amide I and II bands. Large clumps of globular microcapsules upon spray drying were found in mannitol-casein as compared to glucose-casein system. Perhaps, larger clumps provided more protection and less damage to the bacteria leading to increased survival.

Since microencapsulation based on protein (casein) was more effective than alginate, further experimentations focused on using only protein-based formulations supplemented with sugars, dextrins and sugar alcohols for *B. longum* 1941. Stability of *B. longum* 1941 coated with proteins such as skim milk (SM), whey protein concentrate (WPC), casein (CAS), soy protein isolate (SPI) in combination with glycerol (GLY), mannitol (MAN) and maltodextrin (MD) after freeze drying were studied. CAS+MAN formulation was the most effective in increasing survival and tolerance to acid and bile. Retention of high β-glucosidase activity was achieved using SM+MAN as protectant; whereas ATPase and LDH activities were retained by SM+GLY and CAS+MAN formulations. The most effective coating material was found to be casein supplemented with mannitol.

Based on the effectiveness of casein-mannitol formulation in microencapsulating *Bifidobacterium*, the same formulation was investigated further to microencapsulate other probiotic bacteria, *L. acidophilus* and *L. lactis* ssp. *cremoris* and comparing the two drying methods. Storage at low a_w was carried out by placing samples each in a sealed aluminium foil pouch along with desiccants (NaOH, LiCl and silica gel) for 10 weeks at room temperature (25^oC). Freeze dried *L. acidophilus* and *L. cremoris* kept in pouch containing NaOH or LiCl showed higher survival (89–94%), acid and bile tolerance, and surface hydrophobicity than spray dried bacteria (86–90%) kept under the same storage conditions. Silica gel was least effective. The experimental data on viability and storage time were used to predict maximum time of storage to guarantee a minimum required bacterial population of 10^7 CFU/g. The predicted maximum storage times for microencapsulated *L. acidophilus* (freeze dried), *L. acidophilus* (spray dried) and *L. cremoris* (freeze dried) with NaOH as desiccant were 46, 42 and 42 weeks, respectively; while spray dried *L. cremoris* using LiCl as a desiccant was 39 weeks at room

temperature. FTIR showed that the drying process did not affect fatty acids and secondary protein structures of bacteria, however, the type of desiccants affected interactions between phospholipids and microcapsules. Interaction between the exterior of bacterial cell envelopes and sugars occurred after both spray-drying and freeze-drying. T_g of freeze dried encapsulated *L. acidophilus* and *L. cremoris* was higher and the residual moisture was lower than when spray drying was used. The type of desiccant used during storage had significant effect ($P<0.05$) on T_g and moisture content of microencapsulated *L. acidophilus*. Spray and freeze drying significantly influenced T_g of microencapsulated *L. cremoris* after 10 week of storage, but residual moisture contents were unaffected. Since T_gs were above 25^{o}C, it is expected that the changes in fatty acids and secondary structures of proteins of the microencapsulated bacteria may still occur.

In conclusion, this study showed that for assuring a viable count of $>10^7$ CFU/g, the most effective coating formulation of microencapsulation was casein-glucose-mannitol, freeze drying was superior to spray drying, and the best storage water activities were in the range of 0.07 to 0.1 at 25˚C (room temperature).

This thesis contributes a significant insight of the superiority of casein-sugars to alginate-based system as microencapsulating materials, and the importance of mannitol in preserving selected strains of probiotic bacteria. Besides demonstrating high survival of *Bifidobacterium*, *L. acidophilus* and *L. cremoris* ssp. *lactis* using casein-mannitol after spray- or freeze drying, during 10-week of storage at room temperature and during exposure to harsh environment of simulated gastrointestinal tract, this study also confirmed the retention of selected enzyme activities important in bacterial metabolism. Furthermore, the protection mechanism was proposed based on FTIR and DSC measurements confirming the interactions between casein-mannitol and phospholipids of cell membrane. This explained why casein-based formulation

added with mannitol was successful in maintaining high viability of probiotic bacteria after drying process and after the storage at room temperature (25˚C).

Certificate

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This is to certify that the thesis entitled "**SURVIVAL OF ENCAPSULATED PROBIOTIC BACTERIA DURING STORAGE AT LOW WATER ACTIVITY AT AMBIENT TEMPERATURE**" submitted by Dianawati Dianawati in partial fulfillment of the requirement for the award of the Doctor of Philosophy in Food Technology at Victoria University is a record of bonafide research work carried out by her under my personal guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Werribee, Australia

(Dr. Vijay Mishra)

Thesis supervisor

Date:

Declaration

"I, Dianawati Dianawati, declare that the PhD thesis entitled "**SURVIVAL OF ENCAPSULATED PROBIOTIC BACTERIA DURING STORAGE AT LOW WATER ACTIVITY AT AMBIENT TEMPERATURE**" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work."

Dianawati Dianawati Date:

College of Health and Biomedicine

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Acknowledgement

My sincere thanks and gratitude go to my Associate Supervisor Professor Nagendra P. Shah, who was my Principal Supervisor (27th July 2009 – 13th March 2012) till he moved to the University of Hong Kong in 2012. He made my PhD experience exciting and challenging. He improved my confidence and greatly contributed to the progress of this study. He was always available for valuable discussions and suggestions during the study and during preparation of manuscripts and handled all the manuscript very efficiently regardless of whether he was here at Victoria University or overseas. Without his valuable guidance, editorial assistance and swift handling of the manuscripts, these publications would not have been possible.

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My thanks also go to Dr. Frank Sherkat, RMIT University, Melbourne, Australia for letting me using NIRO spray drier and also the technicians who trained me how to operate it, and to Phill Francis, RMIT University, Melbourne, Australia for permitting and assisting me using Environmental Scanning Electron Spectroscopy.

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List of Publications during Candidature

Peer reviewed publications

1. Dianawati, D., and Shah, N. P. (2011). Survival, acid and bile tolerance, and surface hydrophobicity of microencapsulated *B. animalis* ssp. *lactis* Bb12 during storage at room temperature. *Journal of Food Science*, 76(9), M592-M599. doi: 10.1111/j.1750-3841.2011.02422.x
2. Dianawati, D., and Shah, N. P. (2011). Enzyme stability of microencapsulated *Bifidobacterium animalis* ssp. *lactis* Bb12 after freeze drying and during storage in low water activity at room temperature. *Journal of Food Science*, 76(6), M463-M471. doi: 10.1111/j.1750-3841.2011.02246.x
3. Dianawati, D., Mishra, V., and Shah, N. P. (2012) Role of calcium alginate and mannitol in protecting *Bifidobacterium. Applied and Environmental Microbiology,* 78(19), 6914–6921. doi:10.1128/AEM.01724-12
4. Dianawati, D., Mishra, V., and Shah, N. P. (2013) Stability of microencapsulated *Lactobacillus acidophilus* and *Lactococcus lactis* ssp. *cremoris* during storage at room temperature at low $a_{w.}$ *Food Research International*, 50, 259–265. http://dx.doi.org/10.1016/j.foodres.2012.10.023
5. Dianawati, D., Mishra, V., and Shah, N. P. (2013) Effect of drying methods on microencapsulated *L. acidophilus* and *L. cremoris* on secondary protein structure and glass transition temperature as studied by Fourier transform infrared and differential scanning calorimetry. *Journal of Dairy Science*, 96, 1419–1430. http://dx.doi.org/10.3168/jds.2012-6058.
6. Dianawati, D., Mishra, V., and Shah, N. P. (2013) Survival of *Bifidobacterium longum* 1941 microencapsulated with proteins and sugars after freezing and freeze drying. *Food Research International*, 51, 503–509. http://dx.doi.org/10.1016/j.foodres.2013.01.022.
7. Dianawati, D., Mishra, V., and Shah, N. P. (2013) Survival of microencapsulated probiotic bacteria after processing and during storage: a review. Critical Reviews in Food Science and Nutrition. Accepted. Manuscript ID: BFSN-2013-0914.

Papers under review

1. Dianawati, D., Mishra, V., and Shah, N. P. (2013). Stability of *Bifidobacterium animalis* ssp. *lactis* Bb 12 encapsulated with casein-based system after spray drying and during storage at room temperature at low a_w. Submitted to Food Research International.

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PART A: DETAILS OF INCLUDED PAPERS: THESIS BY PUBLICATION

Please list details of each paper included in the thesis submission. Copies of published papers and submitted and/or final draft paper manuscripts should also be included in the thesis submission.

Item/Chapter No.	**Paper Title**	**Publication Status** (e.g. published, accepted for publication, to be revised and resubmitted, currently under review, unsubmitted but proposed to be submitted)	**Publication Title and Details** (e.g. date published, impact factor etc.)
Two	Survival of microencapsulated probiotic bacteria after freeze-drying or spray-drying and during storage: a review	Accepted (Manuscript ID: BFSN-2013-0914)	Critical Reviews in Food Science and Nutrition (IF 4.789, Rank A)
Four	Survival, acid and bile tolerance, and surface hydrophobicity of microencapsulated *B. animalis* ssp. *lactis* Bb12 during storage at room temperature	Published	Journal of Food Science(8/4/2011, IF 1.658, Rank A)
Five	Enzyme stability of microencapsulated *Bifidobacterium animalis* ssp. *lactis* Bb12 after freeze drying and during storage in low water activity at room temperature	Published	Journal of Food Science (4/7/2011, IF 1.658, Rank A)
Six	Role of calcium alginate and mannitol in protecting *Bifidobacterium*	Published	Applied and Environmental Microbiology (27/7/2012, IF 3.829, Rank A*)
Seven	Stability of *Bifidobacterium animalis* ssp. *lactis* Bb 12 encapsulated with casein-based system after spray drying and during storage at room temperature at low a_w	Submitted (currently under review)	Food Research International (IF 3.150, Rank A)
Eight	Survival of *Bifidobacterium longum* 1941 microencapsulated with proteins and sugars after freezing and freeze drying	Published	Food Research International (24/1/2013, IF 3.150, Rank A)
Nine	Stability of microencapsulated *Lactobacillus acidophilus* and *Lactococcus lactis* ssp. *cremoris* during storage at room temperature at low a_w	Published	Food Research International (23/10/2012, IF 3.150, Rank A)
Ten	Effect of drying methods on microencapsulated *Lactobacillus acidophilus* and *Lactococcus lactis* ssp. *cremoris* on secondary protein structure and glass transition temperature as studied by Fourier transform infrared and differential scanning calorimetry	Published	Journal of Dairy Science (28/1/2013, IF 2.564; Rank A)

Declaration by (candidate name): **Signature:** **Date:**

Dianawati Dianawati

Oral Presentations

Dianawati, D., Mishra, V., and Shah, N. P. (2012). Characterization of microencapsulated probiotic bacteria during storage at low water activity at room temperature. FOHES Postgraduate Research Conference, held at Victoria University, St. Albans, Australia, in July 2012.

Dianawati, D., Mishra, V., and Shah, N. P. (2013). ADSA-ASAS Joint Annual Meeting 2013. Indiana Polis, Indiana, in July 8-12, 2013. http://jtmtg.org/2013/abstracts/461.pdf.

Table of Contents

List of Abbreviations and Acronyms

ADP	Adenosine diphosphate
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
a_w	Water activity
CA	Ca-alginate without bacteria
CAB	Ca-alginate microcapsules containing bacteria
CAM	Ca-alginate and mannitol without bacteria
CAMB	Ca-alginate and mannitol microcaspules containing bacteria
CAS	Sodium caseinate
CFU	Colony forming unit
DSC	Differential Scanning Calorimetry
ESEM	Environmental Scanning Electron Microscopy
FTIR	Fourier transform infrared
G	Glucose microcapsule
GIT	Gastrointestinal tract
GLM	General linear model
GLY	Glycerol
GM	Glucose and mannitol microcapsule
HK	Hexokinase
HSD	Honestly significant difference
LAB	Lactic acid bacteria
LDH	Lactate dehydrogenase

M	Molar
MAN	Mannitol
MD	Maltodextrin
Min	Minute
mL	milliliter
MRS	de Man, Rogosa and Sharpe
MSG	Monosodium glutamate
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
NFSM	Non fat skim milk
NMR	Nuclear magnetic resonance
O/W	Oil-in-water
PK	Pyruvate kinase
R^2	Coefficient of determination
r	Correlation coefficient
RH	Relative humidity
RM	Residual moisture content
RMSE	Root-mean-square error
RSM	Reconstituted skim milk
SA	Sodium alginate microcapsule
SAM	Sodium alginate and mannitol microcapsule
SD	Standard deviation
SEM	Standard error of mean
SHb	Surface hydrophobicity
SIJ	Simulated intestinal tract

SIT	Small intestinal tract
SM	Skim milk
SMP	Skim milk powder
SPI	Soy protein isolate
T_g	Glass transition temperature
T_i	Inlet temperature
T_o	Outlet temperature
w/v	Weight/volume
WPC	Whey protein caseinate
β-gal	β-Galactosidase
β-glu	β-Glucosidase
μL	Microliter
oNPG	o-Nitrophenyl-β-D-glucopyranose
ρNPG	ρ-Nitrophenyl-β-D-glucopyranose

Chapter 1 : Introduction

Probiotic bacteria are acknowledged as "good bacteria" due to their capabilities to release beneficial effects to the host such as maintaining the balance of bacteria in the bowel, improving intestinal environment strength, enhancing the host's immune system, reducing intestinal infection, reducing the symptoms of lactose intolerance, the risk of certain cancers and inflammatory bowel disease, counteracting allergies as well as decreasing the cholesterol level (Gilliland, 1990; Shah and Jelen, 1990; Gill and Guarner, 2004; Mottet and Michetti, 2005). In order to exert health benefits, the recommended concentration for probiotic bacteria is $\geq 10^7$ CFU/g of a product (Ouwehand and Salminen, 1998). Probiotic bacteria are required to survive in the stomach and small intestinal tract before exerting their health benefits in large intestinal tract and colon of the hosts.

Probiotic bacteria are commonly found in fermented milk such as yoghurt and Yakult. Currently, the market trend is to offer a probiotic supplement instead of fermented milk products in order to fulfill the need of probiotic demand. Islam et al. (2010) reported that probiotic bacterial viability was poor in the dairy products. It is also impracticable for customers to take along yoghurt or Yakult under some circumstances, for example, during traveling, due to its instability at room temperature. In addition, some probiotic bacteria such as bifidobacteria do not survive below pH 4.0 (Shah, 2000) in gastrointestinal system.

Microencapsulation technology becomes important to attain probiotic bacteria in convenient form with high viability during room temperature storage and during exposure to the digestive system. It helps in solving the problems commonly encountered by probiotics in fermented milk. Some commercial microencapsulated bacteria have been designed to be kept at

room temperature. The products are claimed to have high probiotic population at the time of production, but no qualitative information is provided in terms of their survival throughout the period of storage and after exposure to harsh GIT environments. However, some microencapsulated bacteria are not capable of surviving in the harsh environment of gastrointestinal tract (O'Riordan et al., 2001; Krasaekoopt et al., 2004).

Type of microencapsulating materials and suitable drying method as a final stage of microencapsulation should be selected in a way to increase bacterial survival during process, storage and exposure to the digestive system. Generally, alginate is used as a coating material for probiotic bacteria (Sultana et al., 2000; Ding and Shah, 2007) combined with freeze drying (Goderska et al., 2003a). However, some studies demonstrated ineffectiveness of alginate (Krasaekoopt et al., 2004; Zohar-Perez et al., 2004); thus, other materials were investigated. Milk proteins (caseins and whey proteins) have been proven effective as coating materials (Crittenden et al., 2006; Reid et al., 2007; Doherty et al., 2011). In addition, some sugar alcohols (glycerol, sorbitol and mannitol) were also found as good protectants (Mugnier and Jung, 1985b; Efiuvwevwere et al., 1999; Santivarangkna et al., 2010); while polysaccharides such as maltodextrin are usually used as inert bulking materials (Oldenhof et al., 2005). Currently, spray drying has also been applied for microencapsulation of probiotics (O'Riordan et al., 2001; Corcoran et al., 2004; Ananta et al., 2005; Kearney et al., 2009) as it offers a cheaper alternative to freeze drying. In addition to the type of microencapsulating materials, water activity (a_w) affected bacterial survival during storage at room temperature (Mugnier and Jung, 1985; Higl et al., 2007; Kurtmann et al., 2009); therefore, storage at low a_w of microencapsulated probiotic bacteria was also studied.

An interaction between the microencapsulating material and phospholipid bilayers of cell envelopes of bacteria (Santivarangkna et al., 2010) and the physical state of microcapsule matrix (Higl et al., 2007) may play an important role in maintaining high survival of the dehydrated bacteria. The amorphous state of the matrix, affected by the glass transition temperature (T_g), with its very high viscosity, is able to retard chemical reactions. Storage at room temperature below T_g could ensure the amorphous state of microcapsule and expected to preserve the viability of probiotic bacteria. The glass transition temperature, T_g is also influenced by the a_w and *vice versa* (Kurtmann et al., 2009) impacting on the viability. Hence, it is crucial to determine whether T_g is below or above observed room temperature (25^{o}C) during storage of the microencapsulated probiotic bacteria at low a_w to predict the physical state of the microcapsule to gain knowledge of the protective mechanism afforded by microencapsulation.

The overall aim of this work was to study the effect of types of microencapsulating materials (alginate-based or milk protein-based formulation) and drying methods (freeze drying and spray drying) on selected functional properties of probiotic bacteria during 10 weeks of storage at 25^{o}C at low water activities (a_ws). The specific objectives were:

1. To establish the survival, acid tolerance, bile tolerance, surface hydrophobicity and retention of enzyme activity of microencapsulated probiotic bacteria after spray- or freeze drying followed by storage for 10 weeks at room temperature (25 ^{o}C) at low a_ws.
2. To investigate the protective mechanism of alginate-based or milk protein-based formulation containing mannitol on bacterial cell envelopes and secondary protein structures of probiotic bacteria; observing their microstructures and their physical state during treatment.

Chapter 2 of this thesis deals with the literature review emphasizing stability of microencapsulated probiotic bacteria (survival, acid and bile tolerance, enzyme retention) after spray- or freeze-drying and during storage, mainly focusing on at room temperature at low a_w. The mechanism of protection by microencapsulating materials is also discussed. Stability of free probiotic bacteria in various environments is also looked over to ascertain the importance of microencapsulation. Chapter 3 describes general materials and methods used in this thesis. Survival, acid tolerance, bile tolerance and surface hydrophobicity of microencapsulated *B. animalis* ssp. *lactis* Bb12 with Ca-alginate crosslinking gelation combined with mannitol after freeze drying and during 10-week storage at low a_ws is described in Chapter 4. Chapter 5 focuses on activity retention of some enzymes of *B. animalis* ssp. *lactis* Bb12 microencapsulated by Ca-alginate-based system. Lactate dehydrogenase, pyruvate kinase and hexokinase were studied due to their important roles in glycolytic pathway; ATPase for its role in maintaining intracellular pH; β-glucosidase for converting the isoflavone glycoside, and β-galactosidase for implication in lactose intolerance. How the mechanism of protection of *B. animalis* by Ca-alginate gel matrix alone or in combination with mannitol after freeze drying and after 10 weeks of storage as investigated by FTIR and ESEM is discussed in Chapter 6. Due to low viability of *Bifidobacterium* coated with alginate-based system after freeze drying, microencapsulation of the bacteria with casein-based system containing glucose (G) or glucose-mannitol (GM) using spray drying method was carried out. The results are reported in Chapter 7. This chapter describes selected functional properties of *B. animalis* after spray drying and after the storage at low a_w. Interactions between microcapsule and bacterial phospholipid bilayers, as well as microstructures of the microcapsules are also described. To confirm the effectiveness of casein and mannitol, encapsulation of *Bifidobacterium longum* 1941 using various protein-based

emulsions combined with sugars followed by freeze drying was studied and results presented as Chapter 8. Chapter 9 discusses application of casein-glucose-mannitol (GM) as microencapsulant for *Lactobacillus acidophilus* and *L. cremoris* ssp. *lactis*. This experiment is based on Chapter 7 and 8 demonstrating the effectiveness of casein-based system combined with mannitol. Stability of both microencapsulated bacteria after spray- or freeze-drying and during 10 weeks of storage kept in aluminium foil pouch containing different desiccants at 25^{o}C was ascertained. Chapter 10 examines protective mechanism of microcapsules on *Lactobacillus acidophilus* and *L. cremoris* ssp. *lactis* after drying and after the storage using FTIR. In addition, the effect of a_w on glass transition of the microcapsules was determined by DSC. The general conclusions of this work, limitations and suggested future directions for the research are presented in Chapter 11.

Overall, this thesis reports the results of the study on the survival of microencapsulated probiotic bacteria during storage at room temperature using two different types of microencapsulation systems. The protection effects of microcapsule materials and drying techniques were compared. In addition, the effect of storage at room temperature at a range of water activity of 0.07 to 0.2 was assessed. The protection mechanism of microencapsulation after the storage at low water activities was reported as interactions resulting from bonding between the microcapsules and bacterial cell envelopes constituents, particularly the phospholipids. The physical state of microcapsules was also reported by measuring the glass transition.

Chapter 2 : Literature review

Introduction

This chapter discusses the literatures related to survival of microencapsulated probiotic bacteria after drying and storage. The coating materials, freeze- or spray-drying are described with respect to their effects on viability, acid and bile tolerances, metabolic enzymes of probiotic bacteria. The effects of microencapsulation on these functionalities are also reviewed in relation to storage stability at cold, frozen and room temperatures.

The paper entitled "Survival of microencapsulated probiotic bacteria after freeze-drying or spray-drying and during storage: a review" by D. Dianawati, V. Mishra and N. P. Shah has been accepted in Critical Reviews in Food Science and Nutrition (Manuscript ID: BFSN-2013-0914).

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PART B: DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by (candidate name): DIANAWATI DIANAWATI **Signature:** [redacted] **Date:** 11/3/2013

Paper Title

Survival of microencapsulated probiotic bacteria after freeze-drying or spray-drying and during storage: a review

In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Dianawati Dianawati	70	Prepared major part of the manuscript
Nagendra P. Shah	20	Contribution to writing of paper and journal submission
Vijay Mishra	10	Contribution to writing of paper including the tables

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
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Survival of microencapsulated probiotic bacteria after freeze-drying or spray-drying and during storage: a review

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Abstract

The use of live probiotic bacteria as food supplement has become popular. Maintaining viability of probiotic bacteria at room temperature is necessary for customer's convenience and cost reduction. Hence, production of dried form of probiotic bacteria is important. Two common drying methods commonly used for microencapsulation are freeze- and spray-drying. However, both methods can adversely influence cell membrane integrity and protein structures resulting in decrease in bacterial viability. Microencapsulation is a promising technology to ensure bacterial stability during microencapsulation process and to preserve their viability during storage without significantly losing their functional properties such acid tolerance, bile tolerance, surface hydrophobicity and enzyme activities. Storage at room temperatures instead of freezing or low temperature is preferable for minimizing costs of handling, transportation and storage. Concepts of water activity and glass transition are important determinants of bacterial survival during the storage. The effectiveness of microencapsulation is also affected by microcapsule materials. Carbohydrate and protein-based microencapsulants and their combination are reviewed in terms of their protecting effect on probiotic bacteria during dehydration, storage and exposure to harsh gastrointestinal environment.

178 words

Keywords: microencapsulation process, gut, room temperature storage

INTRODUCTION

Probiotics are considered as a functional food due to their abilities to provide health benefits (Lin, 2003; Sarkar, 2007) beyond nutrition. The use of probiotic bacteria is not limited to fermented milk, such as yogurt or Yakult, but is extended to other forms of functional foods or beverages (Gibson, 2007; Prado et al., 2008). Consumption of probiotic bacteria in dried form was worth $1.2 billion in 2007 with predictions to achieve $1.7 billion in 2013 (Anonymous, 2008). From the consumer's point of view, it is more convenient to consume dehydrated probiotic bacteria. In fact, most of probiotic bacteria products have to be kept under refrigeration to keep the bacteria alive in high population (Amagase and Ide, 2007), which means high costs of transportation and storage. In addition, there is high expectation that probiotic bacteria should still be alive at a certain population number during passage through gastrointestinal tract before adhering to the host lower intestinal tract and colon. Microencapsulation is designed to cope with these adverse conditions. Studies have been conducted to produce microencapsulated probiotic bacteria which can easily be kept at room temperature and survive during exposure to harsh digestive systems (O'Riordan et al., 2001; Desmond et al., 2002; Sunny-Roberts and Knorr, 2009; Heidebach et al., 2010). The harsh conditions encountered by microencapsulated probiotic bacteria prior to exerting beneficial effects to the hosts are shown in Figure 1.

Microencapsulation methods of probiotic bacteria are based on hydrocolloid system or emulsion system followed by spray drying (O'Riordan et al., 2001; Crittenden et al., 2006), freeze drying (Bruno and Shah, 2003; Capela et al., 2006; Heidebach et al., 2010), vacuum desiccation (Efiuvwevwere et al., 1999; Xiaoyan and Xiguang, 2009), hybridisation system (Ann et al., 2007) and extrusion followed by fluidized bed drying (Kim et al., 1988). Freeze drying method is the most common technique to dehydrate probiotic bacteria within coating materials or

in dairy products (Meng et al., 2008). On the other hand, spray drying is popular in microencapsulation industries due to its economical aspect and flexibility (Kailasapathy, 2002). A combination of encapsulating material(s) and drying method with optimum setting conditions, for instance proportion of formulations, freezing temperature, time, temperature and pressure of freeze drying, or inlet and outlet temperatures of spray drying ensures high viability of probiotic bacteria. Reviews on probiotic microencapsulation technology including application of various substances as protectants have been carried out by Anal and Singh (2007), Mortazavian et al. (2007), Kailasapathy (2002), Poncelet (2006), Rokka et al. (2010) and Poddar et al. (2013). Carvalho (2004) reviewed more specifically the effects of freeze drying on probiotic bacteria, and Peighambardoust et al. (2011) emphasized the spray drying technique and its effect on lactic acid bacterial stability. Storage at ambient temperature has gained more attention due to its low storage cost as compared to that under refrigeration or frozen conditions. In addition, review on effectiveness of spray- and freeze drying as part of microencapsulation in preserving probiotic bacteria protected by hydrocolloids, sugars, emulsion-based system or their combinations during storage is lacking. Therefore, the present article is more focused on the effectiveness of microencapsulation of probiotic bacteria in improving survival including their acid and bile tolerance after freeze- or spray-drying and during subsequent storage particularly that at room temperatures. Studies related to microencapsulation technology of probiotic bacteria are shown in Table 1. Effectiveness of microencapsulation of probiotic bacteria is dependent on the type of microcapsulating materials, method of microencapsulation and bacterial strains.

PROBIOTIC BACTERIA

Probiotics have been defined by FAO/WHO (2001) as microorganisms that when administered in adequate amount provide one or more health benefits to the hosts. The definition

proposed by Tabbers and Benninga (2007) and Boirivant and Strober (2007) is that probiotics are "single or mixed non-pathologic bacteria that have capability to alleviate inflammation when supplied into inflamed intestine". They have abilities to release advantageous effects to the host such as maintaining the balance of bacteria thus improving strength of intestinal environment, enhancing the host's immune system resulting in reduction in intestinal infection, reducing the symptoms of lactose intolerance, reducing the risk of certain cancers, reducing inflammatory bowel disease and counteracting allergies, and providing antioxidants (Gilliland, 1990; Shah and Jelen, 1990; Gill and Guarner, 2004; Mottet and Michetti, 2005). In conjunction with those expectations, probiotic bacteria should be stable in gastric juice and bile salts of intestinal tracts, be able to adhere to human epithelial cells before releasing some benefits such as antimicrobial activity and prohibiting adhesion of pathogen to the epithelial cells (Dicks and Botes, 2010). Most probiotic bacteria belong to the species of *Lactobacillus* and *Bifidobacterium* (Lin, 2003), some *Lactococcus* strains have also been considered as probiotic based on their acid and bile tolerance (Kimoto et al., 1999; Kimoto et al., 2003).

Bifidobacterium

The first invention of *Bifidobacterium* was by Tissier of the Pasteur Institute in France in 1899 with an original name of *Bacillus bifidus communis.* It was classified as genus *Lactobacillus* based on its morphology and its characteristics, but was then declared as a sovereign genus namely *Bifidobacterium* in 1960s (Ishibashi et al., 1997). Currently, more than 30 *Bifidobacterium* species have been isolated from either human or animal intestines. *Bifidobacteria* isolated from human feces include *B. longum, B. breve, B. infantis, B. bifidum, B. adoltescentis* and *B. pseudocatenulatum*, while those isolated from animal feces are *B. pseudolongum, B. thermophilus* and *B. animalis* (Ishibashi et al., 1997). Among *Bifidobacteria,*

B. animalis is more adaptive in acid environment. *B. animalis* that has encountered the genetic changes is known as *B. lactis* strains (Meile et al., 1997).

The bacteria of the genus *Bifidobacterium* are gram positive, immobile and nonsporulated (Ballongue, 1998). They have rod and coccoid forms in the exponential and early stationary growth phases, and the cells are developed into branched and septated filaments, clubbed cell forms in the late stationary and death phases. The glucose fermentation end-products formed during their growth are acetic and lactic acids. They are not acid tolerant, however they are able to develop mechanisms to adjust to the unfavorable environments including pH of 4.0-4.2 (Novik et al., 2001). Some strains of *B. animalis* and *B. thermacidophilum* are still capable of surviving at pH 3.5-4.0 (Dong et al., 2000). Bifidobacteria are also anaerobic, even though some strains such as *Bifidobacterium animalis* subsp. *lactis* and *Bifidobacterium thermophilum* are considered as microaerophilic (Ballongue, 1998; Von-Ah et al., 2007; Li et al., 2010).

Lactobacillus

Lactic acid bacteria are gram-positive, non-sporing, non-respiring cocci or rods, and produce lactic acid as the major end-product during the fermentation of carbohydrates. *L. acidophilus* species is a microaerophilic having ability to ferment sugars (Axelsson, 1998) but some strains are capable of digesting sucrose more efficiently than lactose (Mital and Garg, 1992). Catabolic metabolism through Embden-Meyerhof-Parnas pathway is a key to digest glucose in order to produce lactic acid; thus lactic acid bacteria are categorized as homofermentative bacteria (Axelsson, 1998).

Due to their health-promoting properties, some of these bacteria are classified as probiotic bacteria. *L. acidophilus* has been recognized as probiotic bacteria due to their ability to adhere to animal or human intestines and to release health advantages for the hosts.

Lactobacillus also binds mutagens contributing to a protection mechanism against cancer (Ljungh and Wadström, 2006). *L. acidophilus* and *L. salivarius* have strong probiotic properties as they survive well in harsh environments such as very low pH and high bile concentration. They have ability to control *Salmonella* by preventing the yeast colonization on the epithelium (which is known as co-aggregation mechanism) (Del-Re et al., 2000) as they are able to adhere onto it (Orłowski and Bielecka, 2006).

Lactococcus

Lactococcus strains, besides *Lactobacillus* strains, are also categorized as lactic acid bacteria. *Lactococcus* has an important role as a starter (or mixed cultures) in cheese and other fermented milk products owing to their high proteolytic and acidifying abilities (Monteagudo-Mera et al., 2011). These are gram-positive and non-spore forming; they forms pair or short chain on the media. They are categorized as homo-fermentative bacteria with lactic acid as a main product. They also produce nisin and cytokine which has a role in immune system (Nouaille et al., 2003; Elmarzugi et al., 2010). Two *Lactococcus* strains generally used in milk industry are *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris.* The difference between these strains is *L. lactis* ferments lactose, sucrose, glucose, maltose, galactose and fructose; while *L. cremoris* metabolizes lactose, glucose, galactose and fructose. However, none reacts with mannitol: a characteristic similar to that of *L. acidophilus* (Ahmed and Kanwal, 2004).

L. lactis are potential probiotic bacteria, but their functional properties such as survival in gastrointestinal tract and adherence ability to mucosal surface are highly strain-dependent (Drouault et al., 1999; Kimoto et al., 1999). *Lactococcus lactis* subsp. *lactis* bv. diacetylactis strains N7 and *Lactococcus lactis* subsp. *cremoris* ATCC 19257 can survive in intestinal tract of mice (Kimoto et al., 2003). A study on human feces showed that *L. lactis* was still able to survive

in human gastrointestinal tract up to 3 days (Klijn et al., 1995). *L. lactis* has also the ability to form proteins including antigen in order to improve mucosal vaccines (Nouaille et al., 2003). More detailed studies regarding the functional properties of *L. lactis* as probiotic bacteria have been reported (Drouault et al., 1999; Kimoto et al., 1999; Kimoto et al., 2003; Sabir et al., 2010).

Stability of Probiotic Bacteria in the Gut

Viable probiotic bacteria are expected to improve microflora in the intestinal system and provide health benefits to the hosts. Therefore, probiotic bacteria need to survive during passage through the gastrointestinal tract and to adhere to the mucosal layer of the hosts. Stability of some probiotic bacteria in acid and bile environment is shown in Table 2. In general, they show different responses to those environments depending upon their characteristics. Study on acid stability (pH 1.5-3.0, 3h exposure) and on bile stability (bile salts 0-1.5%, 3 h exposure) of 6 *L. acidophilus* and 9 *Bifidobacterium* showed that the most robust strains surviving in both adverse conditions were *L. acidophilus* strains 2415, *Bifidobacterium pseudolongum* strain 20099 and *B. longum* strain 1941 (Lankaputhra and Shah, 1995). Acid stability of gram-positive bacteria is affected by ATPase activity which has a role in pumping proton out in order to maintain the pH of intracellular cells (Cotter and Hill, 2003; Corcoran et al., 2005). Survival is enhanced by glucose which provides ATP as substrate for F_0F_1-ATPase; the activity of the enzyme results in "proton exclusion" besides the conversion of ATP to ADP + P (Galazzo and Bailey, 1990; Corcoran et al., 2005).

Survival of bacteria in the bile environment depends on the concentration of bile, exposure time and bacterial species and strains (Vinderola and Reinheimer, 2003). Bile tolerance of some probiotic is also shown in Table 2. In fact, bacteria get partially injured due to very low pH of gastric juice resulting in irregular responses to the new harsh environment of bile salts

(Dicks and Botes, 2010). Bacteria show ability to survive in 0.3% bile but only some are capable of surviving in 1% bile. Most *Lactobacilli* are sensitive to bovine and porcine bile (Ljungh and Wadström, 2006). All bifidobacteria survive well in the medium added with 0.5% conjugated bile salts; however, higher concentrations such as 1% have a deteriorative effect (Vinderola and Reinheimer, 2003; Noriega et al., 2006).

Adherence on epithelial surface is a requirement for probiotic bacteria in conjunction with colonization in the lower intestinal tract or colon (Canzi et al., 2005). Its activity consists of "receptor-specific binding, charge and hydrophobic interaction" (Ljungh and Wadström, 2006). Malagelada (1998) reported that due to the hydrophobic nature of the intestinal mucus layer, the hydrophobic bacterial surface was essential for non-specific interface with mucin. Mucin is the glycoprotein intestinal layer acting as a receptor on the intestinal epithelial cell with fatty acid binding sites (Ballongue, 1998).

Cell surface hydrophobicity (SHb) can be accurately measured by determining the adherence of bacteria to hydrocarbons (hexadecane, octane and xylene) (Rosenberg et al., 1980; Pan et al., 2006; Rahman et al., 2008). Microbial adhesion to hexadecane involves Van der Waals interactions and is affected by pH (Kiely and Olson, 2000). Several *Lactobacillus* species possess a surface layer protein comprising glyco-proteins (Vadillo-Rodrı´guez et al., 2004), the S-proteins as a part of the cell envelopes commonly found in bacteria with relative molecular weight between 40,000 and 200,000 (Sara and Sleytr, 2000), which help *Lactobacillus* to adhere to hexadecane *via* hydrophobic interactions (Greene and Klaenhammer, 1994; van der Mei et al., 2003). In addition, the presence of predominant apolar groups of bacterial membrane such as saturated fatty acids, monoenoic acids (Veerkamp, 1971) and lipoteichoic acids of bifidobacterial membrane (Op-den-Camp et al., 1985) may also support the adherence.

The adhesion ability varies with the type of bacteria and strain. Canzi et al. (2005) revealed that adhesion ability of *B. bifidum* strains with xylene or n-hexadecane was strain-dependent with the highest (76-98%), *B. pseudocatenulatum* the lowest (2-48%), *B. longum* and *B. adolescentis* low to moderate (4-58%). That observation was contradictory to that of Rahman et al. (2008) who found strains of *B. longum* with the highest SHb (surface hydrophobicity) (91.4 – 97.3%) except *B. longum* BB 536 (51.5%). On the other hand, SHb of *B. bifidum* was in the wide range of 51.9 – 92.8% depending on the strains. The highest SHb of *B. asteroides* and *B. pseudocatenulatum* was 37.2% and 32.1%, respectively; it was higher than SHb of *B. longum* (12.5%) and that of *B. animalis* (18.6%) (Pan et al., 2006). Wang et al. (2010) demonstrated that SHb of *B. animalis* Bb12, *L. acidophilus* NCFM and *L. rhamnosus* GG were 50, 8 and 20%, respectively. SHb of *L. acidophilus* was 57-70% (strain-dependent); L. *delbrueckii* subsp. *delbrueckii* and *L. paracasei* were 90 and 90%, respectively; and *L. plantarum* was 65 to 84% (strain-dependent) (Colloca et al., 2000). SHb of *L. acidophilus* M92 was high (71%); pH decrease resulted in decrease in SHb, and SHb of *L. acidophilus* was totally removed due to the activity of pronase and pepsin (Kos et al., 2003). However, any related factors such as different chemical composition of cell membranes, media (compositions, pH) and time of cultivation contributed to a large discrepancy with SHb between strains (Pan et al., 2006). In addition, adhesion might be reduced due to previous exposure to a very low pH of gastric tract and bile salts environment of small intestinal tract (Dicks and Botes, 2010). Zavaglia et al. (2002) showed that surface hydrophobicity of *B. pseudolongum* and *B. bifidum* grown on MRS media (37^{o}C, 15 h) was in the range of 90.3–97%; whereas that of both bifidobacteria grown on MRS media supplied with 0.1% bile (37^{o}C; 24h) decreased to 28 - 49%. Bile interaction with

hydrophobic site of cell membrane of the strains might be the reason for decreased SHb of cells since bile acts as emulsifier (Ding and Shah, 2009b).

MICROENCAPSULATION TECHNOLOGY

A bacterium in spore form is naturally resistant to temperature changes, radiation, toxic chemicals and starvation (Sunde et al., 2009). The outermost part of the spore known as the "coat" consists of several coatings of cross-linked proteins; the inside part is called the "cortex" consisting of cross-linked peptidoglycan matrix. Both layers are responsible for maintaining the dry-state of the "core" and for protection from oxygen, moisture, chemicals and enzyme (Driks, 1999; Henriques and Moran, 2000). Based on this natural encapsulation phenomenon, the concept of microencapsulation of probiotic bacteria has been developed to improve stability during storage and passage through digestive systems (Gibbs et al., 1999).

Microencapsulation is defined as an "entrapment of a compound or a system inside a dispersed material for its immobilization, protection, controlled release, structuration and functionalization" (Poncelet, 2006). According to this definition, microencapsulation can limit contact between a protected substance and other parts in the system or in the environment, can homogenize the small liquid core with a high volume of microencapsulating materials and convert the mixture into powder, can release the active ingredient and display its functionality in the targeted GI tract once the microcapsule is ruptured (Shahidi and Han, 1993). By applying microencapsulation technology, bacterial integrity can be maintained during the drying process and during oral delivery and be released from the microcapsule when they reach their target destination through the gradual breakdown of coating materials (Islam et al., 2010).

A microcapsule comprises a semipermeable, round, thin, and strong membrane bordering a solid/liquid core, with a diameter in a range from a few microns to 1 mm (Anal and Singh,

2007). The substance within the microcapsule is recognized as the core, internal phase, or fill, while the wall is named as the shell, coating, wall material, or membrane. The walls can be single or even multiple, meanwhile the core can be a crystalline material, an emulsion, a suspension of solids, or a suspension of smaller microcapsules (Gharsallaoui et al., 2007). Based on the morphology, microcapsules can be divided into three elementary categories i.e. mono-cored in which single core is coated by protectants, poly-cored in which some cores are within protectants, and matrix types in which protectants form matrices with the core entrapped therein (Yoshizawa, 2004). Sugars, polysaccharides, proteins or their combinations are used as coating agents for probiotic bacteria as shown in Table 1.

Microencapsulating Materials

Loss in cell viability is mainly due to protein denaturation and changes in cell envelopes and removal of water during evaporation. These variables have an important role in stabilizing the structure of cells and in maintaining cell functional integrity (Brennan et al., 1986). Encapsulation of sensitive materials such as proteins, enzymes and probiotic bacteria within a polysaccharide and protein based system using spray drying has been carried out to protect the core from thermal and dehydration inactivity (Broadhead et al., 1994; Adler and Lee, 1999; Desmond et al., 2002; Hsiao et al., 2004; Yadav et al., 2009). The following sections focus on the effectiveness of carbohydrate-based or protein-based systems and their combinations in protecting probiotic bacteria from spray- or freeze-drying and from the harsh gastrointestinal environment.

Carbohydrate-based system

Among polysaccharides, alginate is a common microencapsulation material due to it being nontoxic, relatively cheap and its easiness to create strong beads; thus its use is discussed

more specifically. Calcium alginate in the form of gel beads has been widely used for the immobilization of probiotic bacteria (Sultana et al., 2000) due to its easy handling, nontoxic nature, low cost, gentle process condition, and ease to dissolve in the intestine thus releasing entrapped cells (Reid et al., 2005; Mortazavian et al., 2007). *Lactobacillus bulgaricus* L2 entrapped in 6% of alginate beads showed almost 100% survival, while 1.5% and 3.0% of alginate beads showed 80% survival. However, the high proportion of alginate was too dense to be applied on a commercial scale. Several combinations of alginate with proteins, sugars, or antioxidants have been established to improve bacterial stability (Cui et al., 2006; Gbassi et al., 2009; Kim et al., 2008; Sultana et al., 2000). Alginate 3.6% combined with 6% of glycerol or mannitol as cryoprotectants improved the bacterial survival during freezing at -20^{o}C for 2 weeks (95 and 90%, respectively) (Sheu et al., 1993). Data on acid and bile tolerance of freeze dried *Lactobacillus* strains coated with alginate-base are shown in Table 2. Cui et al. (2006) demonstrated that addition of either yeast extract, cryoprotectants (glycerol or lactose), antioxidants ($NaHSO_3$ or ascorbic acid) or buffering agent ($Mg_3(PO_4)_2$) improved the survival of bifidobacteria-loaded alginate poly-l-lysine microparticles significantly during freeze drying as compared to control (cells entrapped in alginate poly-l-lysine without fortification).

Ineffectiveness of alginate matrix as a cell coating material has also been reported. Zohar-Perez et al. (2004) showed that bacterial distribution in alginate-beads was not homogenous; the cells tend to be on the surface of alginate beads instead of within the beads. Dianawati and Shah (2011b) demonstrated that alginate was not effective in protecting *B. animalis* ssp. *lactis* Bb12 during freeze drying and during exposure to pH 2.0 for 2h; a significant decrease in viability (10^4 log CFU/g) occurred. Alginate beads immersed in peptone solution were not effective in protecting *Streptococcus thermophilus* during freeze drying as a decrease of > 99% of viable

population was observed (Champagne and Gardner, 2001). Similar results were shown for *Lactococcus lactis* in alginate beads coated with poly-L-lysine (Champagne et al., 1992). Andrade et al. (2010) found no difference between alginate alone and alginate combined with non-milk protein isolates in protecting *L. casei* from harsh gastric intestinal environment. Similarly, *B. lactis* encapsulated with alginate was not detected after 14 days of refrigerated storage of yoghurt; however, survival of *L. bulgaricus* encapsulated with alginate was at 85.7% after storage at the same conditions (Grosso and Fávaro-Trindade, 2004). Krasaekoopt et al. (2004) demonstrated that chitosan-coated alginate beads provided good protection only for *L. acidophilus* in acid (pH 1.5) and bile environment (0.6% bile salt) but not for *B. bifidum* (Table 2).

Polysaccharides such as cellulose acetate phthalate (Fávaro-Trindade and C.R.Grosso, 2002), maltodextrin (Johnson and Etzel, 1995; To and Etzel, 1997) and modified waxy maize starch (O'Riordan et al., 2001) have also been applied as microencapsulants (Table 3). The results were highly varied; cellulose acetate phthalate preserved probiotic better than maltodextrin and modified starch. It is because maltodextrin acts as an inactive bulking compound which does not interact with cell envelopes of the bacteria (Oldenhof et al., 2005). However, the results cannot be compared due to difference in probiotic species and strain and microencapsulation techniques applied.

The use of low molecular weight sugars (lactose, trehalose, maltose, sucrose) was effective in protecting *L. rhamnosus* (Miao et al., 2008); but sucrose and trehalose were not effective in protecting *L. salivarius* (Zayed and Roos, 2004) (Table 3). The use of sugar alcohols as protectant has been established by De Valdez et al. (1983) and Carvalho et al. (2003b). The effectiveness of mannitol and sorbitol in protecting bacteria was demonstrated by Mugnier and

Jung (1985); Efiuvwevwere et al. (1999) and Santivarangkna et al. (2010). Berner and Viernstein (2006) (Table 3) reported that higher proportion of mannitol adversely affected bacterial survival of microencapsulated *L. lactis*. This may be due to the formation of crystalline mannitol (Constantino et al, 1998). The mechanism of protection by sorbitol and mannitol through their interactions with the polar site of phospholipid bilayers of bacterial cell envelopes has been explained by Dianawati et al. (2012), Santivarangkna et al. (2010), Oldenhof et al. (2005) and Leslie et al. (1995). However, the use of sugars was not always successful as protectant. For instance, sucrose was not effective in protecting freeze-dried *L. bulgaricus* (Carvalho et al., 2003a). Ineffectiveness of sucrose is in agreement with the result of Miao et al. (2008) and Leslie et al. (1995) but in the contradiction with the result of Oldenhof et al. (2005).

Protein-based system

Skim milk, casein, and whey protein or non-milk proteins have recently been used to protect probiotic bacteria from spray drying or freeze drying (Ananta et al., 2005; Chavez and Ledeboer, 2007; Heidebach et al., 2009; Heidebach et al., 2010). The use protein-based systems combined with relatively short carbon chains of sugars was effective in increasing survival of probiotic bacteria during spray drying, whereas incorporation of polysaccharides was less effective (Gardiner et al., 2000; Corcoran et al., 2004; Ananta et al., 2005; Sunny-Roberts and Knorr, 2009). These are in contradiction with the results of Desmond et al. (2002) and Rodrıguez-Huezo et al. (2007) who found that incorporation of polysaccharides into proteins improved bacterial stability significantly as shown in Table 3. Sodium caseinate provided excellent protection for bifidobacteria during spray drying and storage (Crittenden et al., 2006). Similarly, heat denatured 10% whey protein solution was effective in maintaining viability of bifidobacteria after spray drying (Picot and Lacroix, 2004). Maltodextrin blended with sodium

caseinate, gelatin, or soy protein was also used to protect phospholipids during spray drying (Yu et al., 2007). The authors revealed that combination of maltodextrin and sodium caseinate retained 90% of phospholipids after spray drying (air inlet temperature = 140°C); solid concentration = 20%); feed temperature = 30°C). Successful encapsulation of phospholipids by Yu et al. (2007) suggested the possible application of casein-based system for probiotics, as phospholipid bilayers are part of cell envelopes of bacteria (Crowe et al., 1987).

Cell dehydration can have serious effect on membrane phospholipids such as fusion and transformation from crystal liquid of fatty acids into a gel phase; which increases the membrane permeability (Crowe et al., 1987). Encapsulation using sugars, proteins or their combinations is effective in protecting cells from damaging effects of freezing and freeze drying. This is due to interactions between sugars (sucrose, trehalose) or sugar alcohols (mannitol, sorbitol) and the polar sites of phospholipid bilayer of cell membranes *via* hydrogen bond as demonstrated by Fourier Transform infrared (FTIR) spectroscopy (Leslie et al., 1995; Oldenhof et al., 2005; Santivarangkna et al., 2010; Dianawati et al., 2012).

Some studies related to the use of sugars or their combination with various proteins to protect probiotic bacteria during freeze drying has been compiled in Table 3. Most studies confirmed an increase in bacterial survival due to the use of milk proteins (Zayed and Roos, 2004; Reid et al., 2007; Ming et al., 2009; Golowczyc et al., 2010; Heidebach et al., 2010). An incorporation of polysaccharides did not improve bacterial stability during freeze drying and spray drying (Ananta et al., 2005; Desmond et al., 2002; Heidebach et al., 2010; Oldenhof et al., 2005). Addition of resistant starch might disturb the protein matrix homogeneity thus its effectiveness in cell protection could be reduced (Heidebach et al., 2010).

Stability of Microencapsulated Probiotic Bacteria during Freeze-Drying or Spray-Drying

Drying is used to form a structure of the micro-capsule and reduce the moisture content to ensure desirable shelf life of probiotic bacteria. The effect of drying on the probiotic viability varies with the characteristics of the bacteria, type of drying and the formulation used for microencapsulation. Both freeze- and spray drying are mainly used for this purpose. Freeze drying is preferred due to the use of low temperature avoiding thermal stress regardless of its high production cost. Currently, spray drying is being suggested as an alternative to freeze drying as it offers some advantages such as low cost and high production rate. Spray drying is usually carried out at low a temperature as possible to maintain viability of cells since the reduction in viability is a function of temperature and the resident time used (Oliveira et al., 2007). From industrial point of view, the use of spray drying is more beneficial since its fixed cost and manufacturing cost were 12% and 20%, respectively, of that of freeze drying (Peighambardoust et al., 2011).

Due to sensitivity of probiotic bacteria toward extremely low or high drying temperatures, protecting probiotic bacteria using coating materials has been studied. Comparison between freeze-drying and spray-drying as a final step of microencapsulation technology for probiotic bacteria was carried out by Johnson and Etzel (1995); they found that freeze drying was more effective than spray drying to maintain viability (Table 3). Interestingly, spray drying (T_{outlet} of 82^{o}C) retained high aminopeptidase and β-galactosidase by 85 and 17%, respectively, compared to those of frozen cells which were 15 and 2%, respectively; those enzymes were lost in freeze-dried or spray-dried cells with T_{outlet} of 120^{o}C. Similar results were obtained by Kim and Bhowmik (1990) and Wong et al. (2010). Zamora et al. (2006) found 100% and 66.7% survival of *L. reuteri* after spray drying and freeze drying, respectively (Table 3); whereas Ying

et al. (2010) found no difference between *L. rhamnosus* GG after spray- or freeze-drying. This difference may be due to the effects of various factors such as strains, growth conditions (medium, pH), growth phase, coating materials, and different set-up of freeze- or spray-drying used (Johnson and Etzel, 1995; Ying et al., 2010).

Stability of Microencapsulated Bacteria in Gastrointestinal Tract

Alginate is a common microencapsulant used for protecting probiotic bacteria from harsh acid environment (Table 2). Nevertheless, alginate was not successful in protecting *B. bifidum* from high acidity of gastric juice (pH=1.55) (Krasaekoopt et al., 2004); that study was in agreement with Dianawati and Shah (2011b) who demonstrated that a significant plummeting (>10^4 log CFU/g) of freeze-dried *B. animalis* ssp. *lactis* Bb12 coated with alginate occurred during exposure to pH 2.0 for 2h. It is because alginate is hydrolyzed into D-mannuronic and L-guluronic acid in the acidic environment (Heyraud and Leonard, 1990) resulting in bacteria being released before arrival to the lower intestinal tract or the colon.

Some studies have incorporated proteins to improve the protective effect of alginate on probiotic bacteria. Some strains of *L. plantarum* were successfully protected by Ca-alginate-based microcapsules layered by whey proteins (Gbassi et al., 2009). Similarly, the use of Ca-alginate coated with chitosan also improved survival during exposure to simulated gastrointestinal tract (Chavarri et al., 2010; Li et al., 2011b) (Table 2). Ding and Shah (2009b) found high viability of some species of *Lactobacillus* and *Bifidobacterium* microencapsulated with Ca-alginate coated with poly-L-lysine and palm oil. The application of Ca-alginate combined with other carbohydrates such as starch and glycerol (Sultana et al., 2000) or glycerol and xanthan gum (Kim et al., 2008) was also proven effective in increasing probiotic bacterial bile tolerance. However, some studies demonstrated that the use of polysaccharides such as

alginate and starch as coating materials without any addition of protein was less effective in protecting *Lactobacillus* and *Bifidobacterium* from the acid environment (O'Riordan et al., 2001; Krasaekoopt et al., 2004; Sultana et al., 2000). O'Riordan et al. (2001) stated that starch was not able to protect spray dried *Bifidobacterium* PL1 at very low pH; no survivors was detected after 3 h exposure to pH 2.8 (37^{o}C).

Microencapsulation using "milk protein matrices" induced by rennet was successful in improving stability of *Lactobacillus paracasei* ssp. *paracasei* and *Bifidobacterium lactis* Bb12 during exposure to pH 2.5 for 1.5 h (Heidebach et al., 2009). The use of whey proteins as part of the microcapsule of spray-dried *Bifidobacterium breve* R070 and *Bifidobacterium longum* R023 also improved bacterial stability in simulated GIT; however, the survival level was strain-dependent (Picot and Lacroix, 2004). A similar result was reported by Doherty et al. (2011) using *Lactobacillus rhamnosus* GG as a model. A sudden drop in viability occurred when microencapsulated bifidobacteria strains were exposed to SGJ (Simulated Gastric Juice) containing pepsin (pH 1.9) for 30 min (less than 1.0 log CFU/g), but they were able to grow when exposed to pancreatin pH 7.5 for 6 h (achieving 7.5 and 4.0 log CFU/g for R070 and R023, respectively) (Rodríguez-Huezo et al., 2007).

Combination of proteins and carbohydrates were investigated to increase the effectiveness of microencapsulation. Casein provided the shielding effect on bifidobacteria from the low pH of simulated gastric tract (Charteris et al., 1998; Crittenden et al., 2006). Survival in SGJ (pH 1.6, 60 min, 37^{o}C) of spray dried *L. rhamnosus* GG encapsulated with trehalose-MSG was $1.7x10^{7}$ CFU/mL; but *L. rhamnosus* E800 was not able to survive (Sunny-Roberts and Knorr, 2009). The use of complex formulation comprising cellulose, maltodextrin, prebiotic and reconstituted milk as protectant also increased acid and bile tolerance of spray-dried

Lactobacillus acidophilus and *Bifidobacterium lactis* (Bb-12) (Fávaro-Trindade and C.R.Grosso, 2002); detail is shown in Table 2.

Enzyme activities of microencapsulated bacteria

Determination of activity of enzymes is important since it relates to cell metabolism and fermentation pathways and probiotic functional properties. Enzymes such as β-glucosidase and β-galactosidase are capable of improving health benefits to the hosts by hydrolyzing isoflavones and lactose, respectively (Vasiljevic and Jelen, 2003; Otieno et al., 2007). Lactate dehydrogenase (LDH), piruvate kinase (PK) and hexokinase (HK) are key enzymes of fermentation pathways (Axelsson, 1998; Ballongue, 1998). ATPase stabilizes intracellular pH during exposure to low pH along with production of ATP hydrolysis (Axelsson, 1998; Corcoran et al., 2005).

Sugars combined with proteins have been recognized as effective microcapsule materials to protect enzymes during dehydration. Stability of some enzymes during dehydration due to the presence of protectants has been studied by Burin et al. (2002); Izutsu and Kojima (2002); Vasiljevic and Jelen (2003); Okamoto et al. (2002); Han et al. (2007); Yoshii et al. (2008). Effectiveness of sugars to protect dehydrated enzymes might rely on ability of sugars to interact with proteins during dehydration (Carpenter and Crowe, 1988). Among sugars, mannitol in an amorphous state has been found as an effective protectant for lysozyme, bovine serum albumin, ovalbumin, β-lactoglobulin and LDH (Izutsu and Kojima, 2002; Singh and Singh, 2003). However, the crystalline state of mannitol has a negative influence on enzyme stability (Sharma and Kalonia, 2004). Specific studies on some enzyme activities of microencapsulated probiotic bacteria have been carried out (Dianawati and Shah, 2011a; Li et al., 2011a). Encapsulation of *B. lactis* ssp. *lactis* Bb12 using alginate-based system added with mannitol maintained high activities of β-glu, β-gal, HK and ATPase after freeze drying (98.5, 94.6, 78.9 and 77.7%,

respectively), but not of LDH and PK (68.6 and 69.2%) (Dianawati and Shah, 2011a). Previously, Li et al. (2011a) reported no effect of cryoprotectants on PK and HK activities. However, sucrose 15% (w/v), trehalose 10% (w/v), and RSM 10% (w/v) were able to protect the activities of LDH; and trehalose (10%) protected the activity of ATPase after freeze drying (Li et al. (2011a).

Lactic acid and probiotic bacteria can be grouped into homofermentative or heterofermentative. Even though the end product(s) of both metabolic pathways are different, the main purpose of the cell activities is to produce ATP (Axelsson, 1998). Among glycolytic enzymes taking part into a catabolism pathway, hexokinase, pyruvate kinase and lactate dehydrogenase are connected to the production of ATP. The conversion of D-glucose into glucose-6-P through phosphorylation is due to the activity of HK. This step is in conjunction with dephosphorylation of ATP into ADP (Blei and Odian, 2000). Production of ATP from ADP phosphoryation occurs along with a transformation of phosphoenolpyruvate into pyruvate; this step requires PK (Knowles et al., 2001). At the end of the glycolytic activities, oxidation of NADH takes place along with the formation of lactate from pyruvate; this activity is carried out by LDH (Garvie, 1980). Denaturation and aggregation can occur due to the sensitiveness of these enzymes to high temperature. At 45^{o}C, HK and PK are stable at pH 7.0 (30 min) and at pH 7.6 (10 min), respectively (Anonymous 2013b; Anonymous 2013a). Study on LDH, HK, and PK was important due to their crucial role in EMP pathway related to metabolic activities during freeze drying. Additionally, a decrease in ATPase, a membrane-bound enzyme, activity is related to a decrease in cell membrane integrity (Li et al., 2011). Therefore, the activity of these enzymes is directly or indirectly linked to the viability of probiotic bacteria during microencapsulation process. The activities of β-glucosidase and β-galactosidase are related to

their abilities to exert health benefits (Vasiljevic and Jelen, 2003); these enzyme activities must be retained to assure success of a microencapsulation process.

Studies on enzyme preservation using protectants mostly used LDH as model due to its high sensitivity to the temperature. LDH consisted of two protein sub-units connected by weak force binding. Degree of LDH denaturation in spray-dried product was lower when outlet temperature was below 90^{o}C (Matzinos and Hall, 1993). Remaining activity of freeze dried LDH was 70% when trehalose and hydroxyectoine were used as protectants, while sucrose and maltose contributed about 65 and 60% of remaining LDH activity, respectively (Lippert and Galinski, 1992). In addition, the use of polyethylene glycol above 1% (w/v) as cryoprotectant increased the stability of LDH more than 80% of overall activity (Mi et al., 2004).

β-Galactosidases are categorized as thermo-resistant enzymes; however, they have wide a range of thermal stability from 0 to 80^{o}C depending on bacterial species (Asraf and Gunasekaran, 2010). Stability of β-galactosidase of *B. longum* CCRC 15708 was maximum at 30^{o}C for 40 min exposure (98% enzyme activity remaining); an increase in exposure temperature to 40^{o}C decreased enzyme activity to 80% (Hsu et al., 2006). Stability β-glucosidase can be achieved at 40^{o}C for 150 min when polysaccharide matrices are used as protectants (Rashid, 1997).

Some studies have been carried out in order to preserve β-galactosidase through microencapsulation techniques (Burin et al., 2002; Vasiljevic and Jelen, 2003; Goel et al., 2006). Encapsulation of *S. thermophilus* using calcium alginate, carrageenan and gellan-xanthan enhanced the stability of β-galactosidases of the bacteria at temperature >55^{o}C (Goel et al., 2006). Remaining activity of freeze-dried β-galactosidase protected by skim milk was still 80% after more than 100 h exposure to 85^{o}C, while protection using lactose or whey proteins resulted

in less enzyme activity (62 or 70%, respectively) (Burin et al., 2002). Stability of freeze-dried β-galactosidase was high when trehalose was used as lyoprotectant; the addition of K-citrate or K-acetate to the system increased protective effect by three times (Santagapita and Buera, 2008). Preservation of β-galactosidase using spray drying has been carried out by Vasiljevic and Jelen (2003). High retention of β-galactosidase of *L. delbruekii* subsp. *bulgaricus* ATCC 11842 was obtained when spray drying using sugars or milk proteins as carrier was carried out with an outlet temperature of 40-50^{o}C. In addition, the use of mannitol (5%) was also effective in protecting β-galactosidase during spray drying at T_{outlet} = 50^{o}C (Okamoto et al., 2002). Similarly, high β-gal activity during spray drying was achieved when 10% mannitol, 10% sucrose and 10% trehalose were used (Broadhead et al., 1994). Meanwhile, Woodward et al. (1993) found that microencapsulation of β-glucosidase using propylene glycol alginate/bone gelatin was stable during exposure to 40^{o}C for couple months without losing its effectiveness.

Fermentative microorganisms including probiotic bacteria produce ATP through "substrate level phosphorylation". The main role of ATPase is to hydrolyze ATP into ADP and P. Pumping of protons out of cytoplasm also takes place particularly in the low pH environment (Corcoran et al., 2005). The activity of ATPase (F_1F_0-ATPase has been identified as '*atp*' operon in gram positive bacteria) results in improvement of acid tolerance of *Lactobacillus plantarum* (Duary et al., 2010). Intracellular pH is stable through this exclusion mechanism when *Lactobacillus rhamnosus* GG is exposed to the low pH environment (Corcoran et al., 2005). For *Bifidobacterium*, it has been found that *B. lactis* and *B. animalis* were more acid tolerant reflected in higher ATPase activity than *B. longum*, *B. adolescenti*, and *B. pseudocatenulatum* (Matsumoto et al., 2004). ATPase of *L. reuteri* encapsulated by trehalose or RSM was preserved during freeze drying (Li et al., 2011a).

STORAGE STABILITY

Materials and proportion of microencapsulant, drying temperatures, a_w, and residual moisture after drying are factors that influence stability of microencapsulated bacteria during storage (Zamora et al., 2006; Chavez and Ledeboer, 2007; Higl et al., 2007; Miao et al., 2008; Kurtmann et al., 2009; Coulibaly et al., 2010; Savini et al., 2010; Ying et al., 2010). Spray drying with low outlet temperature (50^{o}C) could be more beneficial than freeze drying in terms of maintaining viability; however, it might result in a high residual moisture content of products due to inadequate drying. This approach could have a harmful effect on bacterial survival during storage, as water could be available as solvent required for physico-chemical reactivity. Removal of remaining free water of dehydrated microcapsules can be carried out through further treatments such as use of desiccants or vacuum drying in a second stage. Freeze- or spray dried microcapsules with low a_w is desired for the purpose of long term storage at room temperatures i.e. between 8-12 weeks (Corcoran et al., 2004; Donthidi et al., 2010) and storage up to 20 months at cold temperature (Bruno and Shah, 2003). Storage at low temperature (4-7^{o}C) certainly ensures high viability of the cells for long periods but results in increased cost of transportation and storage. As a result, transporting microencapsulated bacteria for a long distance becomes impracticable. The following sections explain the survival of dehydrated probiotic bacteria encapsulated with various materials during storage at cold, freezing or room temperatures.

Storage at Cold and Freezing Temperatures

Storage at 4^{o}C (Boza et al., 2004; Lee et al., 2004; Heidebach et al., 2010; Savini et al., 2010) or at -18 ^{o}C (Bruno and Shah, 2003) has always been proven effective in lengthening shelf life of probiotic bacteria. Freeze-dried *Bifidobacterium longum* 1941 protected with

unipectin was only able to survive at freezing or cold temperatures but not at room temperature (Bruno and Shah, 2003). This is in contradiction with the results of Saarela et al. (2005); details of both studies are shown in Table 4. It demonstrated superiority of milk or low MW sugars as protectants as compared to polysaccharides. Similarly, SM + trehalose + ascorbic acid improved survival of freeze-dried *Lactobacillus* sp.; storage at 4^{o}C was preferable (Jalali et al. 2011). Ca-alginate was more effective than whey proteins in preserving viability of *L. acidophilus* and *B. infantis* after 6 weeks of storage at -20^{o}C (Kailasapathy and Sureeta, 2004). However, protein denaturation of whey proteins might occur during storage at freezing temperature (Bedu-Addo, 2004) reducing its effectiveness as microencapsulant. In spite of its benefit in maintaining high survival of microencapsulated bacteria, high transportation and storage costs at low temperature add to the price of products. Hence, storage of microencapsulated probiotic bacteria at room temperature is needed in order to decrease cost of handling and storage.

Storage at Room Temperature

Conventional storage of frozen probiotic bacteria in the freezer increases risk of cell loss associated with thawing. Microencapsulation could elongate storage period at room temperature and reduce the distribution and storage costs. Besides the type of coating materials, water activity (a_w) and glass transition affect microencapsulated probiotic survival during storage at room temperature (Peighambardoust et al., 2011). Water activity determines the accessibility of water for chemical reactions or the growth of microorganisms (Roos, 1995). Glass transition indicates "a physical change in an amorphous material promoted by the addition of heat and/or the uptake of low molecular weight substances" (Bell and Hageman, 1994). Rahman (2010) suggested that both a_w and glass transition concepts are useful in determining food deterioration or food stability, and also in predicting shelf-life of food products during storage.

Low water activity storage at room temperature is crucial for preserving dried probiotic bacteria. Storage at very low a_w such as 0.07 and 0.1 (Mugnier and Jung, 1985; Higl et al., 2007; Kurtmann et al., 2009) improved bacterial survival during storage at room temperature. Kearney et al. (2009) stated that a residual water content of 4% (w/w) corresponding to a_w of 0.2 is required to extend the shelf life of probiotic bacteria in dried yoghurt. Storage at low a_w increases survival of microencapsulated bacteria; survival of freeze-dried *Lactobacillus paracasei* ssp. *paracasei* in lactose matrix was highest during storage at 20^{o}C at a_w of 0.07 for 30 days. The bacterial stability during storage at a_w of 0.23 was lower, even though X-ray diffraction pattern showed that the powder was maintained in glassy state (Higl et al., 2007).

Coating materials such as sugars have a role in maintaining survival of probiotic bacteria by their capability in affecting a_w and T_g during drying and storage (Kurtmann et al., 2009). The authors reported that T_g of freeze-dried *L. acidophilus* during storage at 20^{o}C was influenced by a_w and sugar type. At a_w of 0.11, 0.23 and 0.33, T_g of sucrose matrix containing *L. acidophilus* was 24.6, 16.5 and -29.2 ^{o}C, respectively; while that in a lactose matrix was 36.4; 23.1 and 6.2 ^{o}C, respectively. It showed that storage at 20^{o}C of sucrose matrix at a_w of 0.11 and of lactose matrix at a_w of 0.11 and 0.23 were still below glass transition temperature ($T_{ambient} < T_g$), thus an amorphous state is achieved in these a_w ranges. Survival of freeze dried *L. acidophilus* was affected by a_w, sugar matrices and physical state of the matrices (Kurtmann et al., 2009). However, structural alteration of bacteria during freeze-drying or spray-drying contributed further damage during following storage even with storage temperature below T_g (Chang et al., 1996; Ananta et al., 2005). Some studies on stability of freeze-dried or spray-dried probiotic bacteria during storage at room temperature have been carried out by Miao et al. (2008), Chavez and Ledeboer (2007), Coulibaly et al. (2010), Hsiao et al. (2004) and Ying et al. (2010). The

results showing the survival of probiotic bacteria due to different microencapsulating materials, the drying method and the storage conditions can be seen in Table 4.

A comprehensive study on the effect of various drying methods on survival of bifidobacteria coated with skim milk (SM) or soy protein isolate (SPI) combined with various sugars and on cell survival during storage has been carried out by Chavez and Ledeboer (2007). Detail of drying process and the results were shown in Table 4. High residual moisture of spray-dried bacteria resulted in no bacterial survival (Chavez and Ledeboer, 2007). Storage in aluminium bag under vacuum (a_w powder = 0.2) resulted in the highest bacterial viability. SPI and SPI + sucrose provided the highest T-T_g in which matrix was maintained at glassy state, but bacterial survival during storage was low. A glassy state did not reduce the molecular mobility and ensure the bacterial stability during storage (Ananta et al., 2005; Chavez and Ledeboer, 2007; Higl et al. 2007).

Reconstituted skim milk (RSM) was compared to disaccharides (lactose, trehalose, sucrose, maltose, lactose + maltose and lactose + trehalose) to ascertain their effectiveness in protecting freeze-dried *L. rhamnosus* survival during storage at a_w of 0.0; 0.11; 0.22, 0.33 and 0.76 at room temperature (Miao et al., 2008). Results showed that trehalose and lactose + maltose were the most effective encapsulants protecting viability of bacteria during 38 days storage at 25^{o}C at a_w of 0.00 and 0.11; the protective effect of lactose + maltose was better than that of RSM (Table 4). On the other hand, Zayed and Roos (2004) found that storage at a_w of 0.00 resulted in decrease in survival of freeze-dried *L. salivarius* ssp. *salivarius* protected with skim milk combined with sucrose or trehalose at 44% after 1 week of storage at room temperature. Further, decrease in viability by 72% was observed after 7 weeks of storage at a_w of 0.00; while no significant decline was detected at a_w of 0.11. Higher a_w (0.33) contributed to the

crystallization of disaccharides; thus survival of encapsulated *Lactobacillus* decreased. The authors stated that T_g of disaccharides decreased at higher a_w, and *vice versa*; this result was in agreement with that of Higl et al. (2007) and Kurtmann et al. (2009).

Stability of freeze- or spray-dried bacteria during storage at room temperature depends on many factors. Ying et al. (2010) compared the effectiveness of freeze drying (-18 ^oC of freezing, 48 h of freeze drying) and spray drying (T_i/T_o = 160 and 65 ^oC) on retaining the viability of *L. rhamnosus* GG in whey protein and resistant starch during storage at 25^oC at a_w of 0.32, 0.57, and 0.70. Spray-dried powder containing bacteria was more stable than freeze-dried powder during 37 days of storage. It was because spray-dried capsules had a stronger water-binding energy as measured by NMR spectroscopy. Zamora et al. (2006) encapsulated *L. reuteri* using either 20% non-fat skimmed milk (NFSM), 10% D-glucose or 12% lactose, followed by freeze drying (-80^oC – 24 h for freezing; -15^oC and 15^oC for primary and secondary drying, respectively) or spray drying (T_{inlet} = 170^oC; T_{outlet} = 80–85^oC; product input flow 670 mL/h). The results showed that NFSM was more effective in maintaining bacterial survival compared to glucose or lactose. However, survival of freeze dried *L. reuteri* protected with NFSM was higher than that of spray dried *L. reuteri* within the same material after 60-day storage at 20 ^oC; this is in disagreement with the results of Ying et al. (2010) (Table 4). The difference in the results could be due to probiotic strains and species, microencapsulating materials, set up of freeze drier or spray drier, and a_w.

Besides skim milk, the use of sodium caseinate as coating material has also been proven effective in improving freeze-dried *Lactobacillus* F19 and *Bifidobacterium* Bb12 during storage at 25^oC (Heidebach et al., 2010). The authors examined stability of freeze-dried *Bifidobacterium* Bb12 encapsulated with enzymatically cross-linked casein during storage at different

temperature and a_w. Incorporation of resistant starch into microcapsule formulation made from enzymatic cross-linked casein resulted in viability decrease of freeze-dried *Bifidobacterium* Bb12 and did not improve stability of *Lactobacillus*. Storage at 4^{o}C of encapsulated freeze-dried *Bifidobacterium* Bb12 resulted in higher survival than that at 25^{o}C and was not affected by a_w (Heidebach et al., 2010). Spray-dried probiotic bacteria coated with casein-based system, however, showed less survival as compared to freeze-dried bacteria after the drying process and after storage at 25^{o}C at low a_w (Dianawati et al., 2013). This finding is in agreement with that of Wong et al. (2010), Zamora et al. (2006) and Johnson and Etzel (1995).

Reconstituted skim milk (RSM) combined with prebiotic (raftilose or polydextrose) and RSM alone as control (20% total solids) has been compared to establish their effectiveness in improving survival of *Lactobacillus rhamnosus* GG (ATCC 53103) during spray drying and during storage at room temperature (Ananta et al., 2005). Incorporation of prebiotics did not influence bacterial survival; partial replacement of prebiotics by RSM had an adverse impact on bacterial survival during 6 weeks of storage at 25 and 37^{o}C compared to RSM only. Similarly, polysaccharides did not improve bacterial survival during storage at room temperature (Corcoran et al.,2004; Desmond et al., 2002; O'Riordan et al., 2001).

The role of polysaccharides with high molecular weight raises the question whether they can function as encapsulating materials, just as an “inactive bulking compound” or space filler without any interaction with the core (Oldenhof et al., 2005). Polysaccharides may have an adverse effect on bacterial survival as proposed by Hincha et al. (2002) and Ananta et al. (2005). Large sized polymers (such as prebiotic) might cause the “steric hindrance” preventing them interacting with dehydrated proteins and membrane lipids (Hincha et al., 2002); this is in agreement with Heidebach et al. (2010). It was hypothesized that skim milk was capable of

interacting with polar headgroups of membrane phospholipids, thus protecting cell membranes during spray drying and storage (Ananta et al. 2005). However, this mechanism could be difficult due to high number of proline residues and lack of tertiary structures of caseins, as the main proteins of skim milk, resulting in their tendency to be hydrophobic (Anonymous, 2012). On the other hand, sorbitol and mannitol as sugar alcohol are able to interact with P=O of cell envelopes of probiotic bacteria *via* hydrogen-bond (Santivarangkna et al. 2010; Dianawati et al. 2012). The effectiveness of sugar alcohols such as glycerin, mannitol and sorbitol in protecting freeze dried probiotic bacteria at room temperature for five months has been proven by Savini et al. (2010). Mugnier and Jung (1985) found mannitol more effective in protecting dehydrated gram positive bacteria than glycerol, reducing sugars or higher MW sugars during storage at room temperature at a_w of 0.07. That result was in agreement with that of Carvalho et al. (2003a) who reported the inability of sucrose to increase the survival of freeze-dried *L. bulgaricus* during storage at 20^{o}C.

Other studies also demonstrated that mostly combinations of proteins, low MW sugars and high MW sugars were effective in protecting bifidobacteria, but the results were strain-dependent. Chavez et al. (2007) found that soy proteins combined with maltodextrin contributed to produce the highest viability of *B. lactis* BB12 during 3 months of storage at 30^{o}C, while skim milk + trehalose and skim milk + arabic gum provided lower stability. Crittenden et al. (2006) found that high survival of spray-dried *Bifidobacterium infantis* Bb-02 was achieved when they were encapsulated using formulation containing sodium caseinate, fructooligosaccharides and resistant starch. However, survival of bifidobacteria after spray drying and after storage at 25^{o}C is strain-dependent (Table 4) (Simpson et al., 2005). Trehalose + MSG was also effective in protecting *Lactobacillus rhamnosus* E80 during 4-week storage at 25^{o}C but not GG (Sunny-

Roberts and Knorr, 2009; Table 4). The protection of MSG is likely due to its anti-oxidation potential.

In addition to proteins combined with sugars, alginate is a common material used for microencapsulating probiotic bacteria (Mortazavian et al., 2007, Sultana et al., 2000). However, the effectiveness of alginate in protecting probiotic bacteria during storage at room temperature was only studied by Donthidi et al. (2010). The authors demonstrated that after 24 weeks of storage, some probiotic bacteria encapsulated with alginate+starch+lecithin kept at 23^{o}C did not survive (Table 4). Incorporation of chitosan into alginate improved survival of *L. bulgaricus* during 4 weeks storage (4^{o}C) (Lee et al., 2004) (Table 4). Mannitol incorporation into alginate improved bifidobacterial survival and acid and bile tolerance after storage (25^{o}C, 10 weeks) at a_w of 0.1. In fact, the viability was only 5.46 log CFU/g at the end of the storage period (Dianawati and Shah, 2011b). This suggests that even though alginate has been widely applied as microencapsulant for probiotic bacteria, its use as single material may not be effective in improving survival during storage at room temperature.

The diffusion of oxygen through microcapsule might still occur resulting in adverse effects such as changes in cell membrane structure (Hsiao et al., 2004). Among probiotic organisms, bifidobacteria were the most susceptible to oxygen due to their anaerobic characteristic (Talwalkar and Kailasapathy, 2003). The addition of resistant starch to casein decreased the stability of *Bifidobacterium* during storage due to its adverse effects on protein-matrix stability (Heidebach et al. 2010). Survival of spray-dried bifidobacteria ($T_{inlet/outlet}$ = 100/50^{o}C) in skim milk, gum arabic, gelatin or soluble starch with or without oxygen absorber and desiccant was observed (Hsiao et al., 2004); the results were presented in Table 4.

CONCLUSION

Microencapsulation of probiotic bacteria is effective in improving the bacterial stability during transportation and storage and during passage through harsh environment of gastrointestinal tract before adhering onto colon of the host in order to provide several health benefits. Various encapsulating materials prepared from carbohydrates (sugar alcohols, reducing sugars, polysaccharides, hydrocolloids) or proteins (milk- or non-milk based) or their combination have been studied to ascertain their effectiveness in protecting probiotic bacteria during the process of microencapsulation, storage and passage through simulated gastric or bile juice. Skim milk and casein and/or sugars such as mannitol, sorbitol, trehalose and sucrose were proven effective as encapsulating materials for *Lactobacillus* and *Bifidobacterium*. The use of polysaccharides showed varied results depending on bacterial strains, microencapsulating materials and their concentrations, and drying methods. Each of these must be optimized to increase the shelf life of probiotic bacteria including their functional characteristics.

Storage temperature is the key to maintain stability of microencapsulated bacterial properties; low temperature storage ensures higher stability. However, maintaining low temperature requires a high cost of transportation and storage. Storage at room temperature (usually between 20 and 30^{o}C) can be a cheap alternative required to be developed. Storage water activity of and glass transition are critical factors that influence physiology of microencapsulated probiotic bacteria. Viability is favored in an amorphous state as influenced by glass transition temperature and can only be achieved at low a_w storage. Storage at ambient temperature at low a_w of 0.07, 0.1 and under vacuum is effective in improving the survival of probiotic strains used for long storage periods assuring health benefits.

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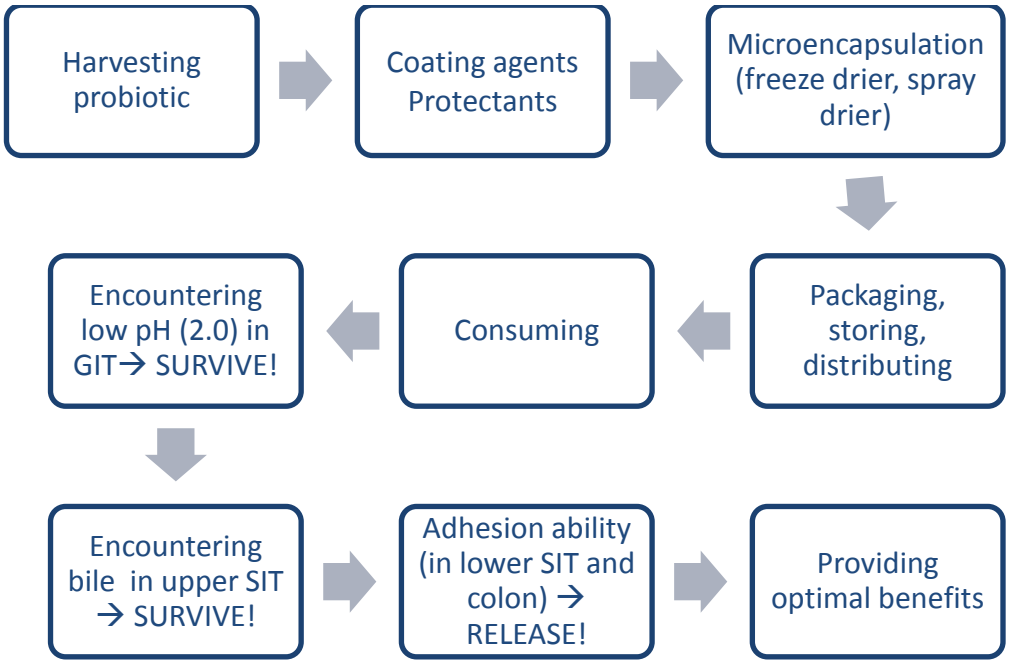

Figure 1. The "long journey" of microencapsulated probiotic bacteria prior to exerting beneficial effects to the hosts

Table 1. Microencapsulation technology of some probiotic bacteria using some combination of encapsulants

Probiotic / LAB bacteria	Microencapsulation Materials	Microencapsulation techniques	Functionality	References
L. acidophilus ATCC 43121	fructooligosaccharide, lactulose or raffinose and A poly (vinylacetate) phthalate-based aqueous enteric coating system	hybridisation system	Acid, salt and heat tolerance	Ann et al. (2007)
Lactobacillus casei 81	sodium alginate blended with low-methoxyl pectin or modified starch	Gel beads	Viability in yoghurt	Calleros et al. (2007)
Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium bifidum, and *Bifidobacterium longum*	fructooligosaccharides or isomaltooligosaccharides, sodium alginate, peptides	Gel beads	Storage, acid and bile tolerance	Chen et al. (2005)
L. rhamnosus type Lr-32, *B. longum* type Bl-05, *L. salivarius* type Ls-33, *L. plantarum* Lpc-37, *L. acidophilus* NCFM, *L. paracasei* Lp-115, *B. lactis type* Bl-04, *and B. lactis* type Bi-07	Alginate emulsion	Gel beads	Acid, bile and heat tolerance	Ding and Shah (2007)
Lactobacillus rhamnosus, Bifidobacterium longum, L. salivarius, L. plantarum, L. acidophilus, L. paracasei, B. lactis type Bl-04, *B. lactis* type Bi-07, *HOWARU L. rhamnosus,* and *HOWARU B.*	alginate, guar gum, xanthan gum, locust bean gum, and carrageenan gum	Gel beads	acid and bile tolerance	Ding and Shah (2009a)

bifidum				
Lactobacillus rhamnosus	Alginate and hydroxypropyloammonium starch	Freeze dried beads	Survival in acid and pH 7.0, 8.0	Goderska et al. (2003)
Lactobacillus acidophilus and *Bifidobacterium lactis*	alginate and Hi- Maize starch	Gel beads	Application to yoghurt, storage at 4^{o}C	Kailasapathy (2006)
Lactobacillus rhamnosus	alginate, pectin, carrageenan, whey protein isolate	Freeze dried gel beads	Survival in gastrointestinal tract model	Reid et al. (2005)
LAB cells	alginate, gelatin and trehalose	gel beads	Survival in acid-bile tract, storage at 4^{o}C	Xiaoyan and Xiguang (2009)
Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus rhamnosus and *Bifidobacterium spp.*	Unipectine^{TM} RS 150, Hi-maize, FOS, or inulin, alginate	gel beads	application to yoghurt (fresh and freeze dried), survival during storage	Capela et al. (2006)
Lactobacillus F19 and *Bifidobacterium* Bb12	Casein, high- amylose maize starch , sun flower oil	W/O emulsion technique - freeze dried microcapsules	Survival after drying and storage	Heidebach et al. (2010)
Lactobacillus paracasei ssp. *paracasei*	Lactose	Freeze dried pellet	Storage stability at low a_w	Higl et al. (2007)
Lactobacillus rhamnosus	reconstituted skim milk, polydextrose, prebiotic	Spray dried powder	Storage stability	Corcoran et al. (2004)
Bifidobacterium infantis	Glucose, sodium caseinate, canola oil, fructooligosaccharides	Spray dried powder	Acid-bile tolerance, storage stability	Crittenden et al. (2006)
Lact. paracasei NFBC 338	reconstituted skim milk, gum acasia	Spray dried powder	survival in gastric juice, storage stability	Desmond et al. (2002)
Bifidobacterium PL1	modified waxy maize starch	Spray dried powder	Acid tolerance, storage, stability in food	O'Riordan et al. (2001)
Bifidobacterium bifidum	whey protein concentrate, gum arabic, mesquite gum, maltodextrin, local prebiotic	Spray dried powder	storage stability	Rodríguez-Huezo et al. (2007)

Table 2. Stability of free and microencapsulated probiotic bacteria in acid and bile environment

Probiotic	Products	Microencapsulant	acid tolerance	acid condition	bile tolerance	bile condition	References
L. acidophilus 2409	Freeze dried beads	2% alginate + Hi-maize resistant starch	56.2%	pH 2.0, 3 h	80.2%	1%, 6 h	Sultana et al. (2000)
L. acidophilus	Freeze dried beads	sodium alginate 2% + xanthan gum 5% + glycerol 0.15% (w/v)	56.0%	pH 1.5, 3 h, 37°C	86.4%	0.3% bile, after 24 h, 37°C	Kim et al. (2008)
B. bifidum		Free	0%	3.0 g/L pepsin, pH 2.0, 120 min, 37°C	0%	Bile salt 3.0 g/L, pH 7.5, 120 min, 37°C	Chavarri et al. (2010)
		20 g/L of sodium alginate coated with chitosan 0.4% (w/v) solution	>90%		95.6%		
L. gasseri		free	0%		0%		
		20 g/L of sodium alginate coated with chitosan 0.4% (w/v) solution	>85%		98.9%		
L. acidophilus		Free	36.1%	pH 1.55, 2 h	21.9%	0.6% bile salt , pH 8.25, 37°C, 2 h	Krasaekoopt et al. (2004)
		Chitosan 1%	75.1%		55.5%		
		alginate	60.4%		57.6%		
		poly-L-lysine 0.05% + alginate solution 0.17%	56.6%		52.2%		
B. bifidum		Free	All not detected		27.8%		
		Chitosan 1%			84.9%		
		alginate			49.5%		
		poly-L-lysine 0.05% + alginate solution 0.17%			39.1%		
L. acidophilus sp.	Free	-	3.4 - 5.6	pH 2.0, 3 h, 37°C	45.7-79.9%	1%, 72 h	Vinderola and Reinheimer (2003)
L. delbrueckii			6.0		0.0-5.1%		

subsp. *bulgaricus*							
L. lactis strains			6.0		0.9-61.3%		
B. bifidum			3.3 – 4.9		18.9-41.0%		
B. longum			>6.0		7.1-43.1%		
			(all in log CFU/mL decrease)				
L. acidophilus Z1L			79%	pH 2.0, 1 h	81%	0.3%, 2 h, 37°C	Sabir et al. (2010)
L. cremoris			71%		74%		
L. acidophilus	free		35.3%	pH 2.0, 2 h	40.2%	3%, 8 h	Ding and Shah (2009)
	wet beads	3% alginate	67.3%		63.0%		
L. bulgaricus KFRI 673	free		0%	pH 2, 1h	83.3%	phosphate-buffered saline solution (pH 7.4) without pancreatin, 37°C, 120 min	Lee et al. (2004)
	Freeze dried beads	2% (w/v) sodium alginate, 5.5% (w/v) MRS broth, 5% (v/v) glycerol, 0.26% xanthan gum, 0.1% Tween 20, coated with chitosan	46.4%	pH 2.0, 3 h, 37°C	77.9%		
L. plantarum 299	free		0%	pH 1.8, 90 min	-	0.9% sodium chloride + pancreatin + 1% trypsin + 0.3% of bile salts, pH 6.5, 180 min, 37°C	Gbassi et al. (2009)
	Freeze dried beads	alginate 0.2% coated with whey proteins 0.2% (all w/v)	67%	pH 1.8, 2 h	34%		
L. acidophilus CSCC 2400	free		42.6%	pH 2.0; 3 h	87.1%	1%, 6h, 37°C	Chandramouli et al. (2004)
	wet beads	alginate 1.5%	71.1%	pH 2.0, 3 h, 37°C	89.0%		

L. casei NCDC-298	free		44.7%	pH 1.5, 3 h	77.1%	1% bile salt, 12 h	Mandal et al. (2006)
	wet beads	2% alginate	63.8%		84.7%		
		4% alginate	80.1%		86.7%		
L. rhamnosus	free		84.70%	pH 2.0, 3 h	84.7%	MRS broth pH 7.0	Goderska et al. (2003)
	freeze dried	Alginate and Hydroxypropyloammonium starch (No concentration given)	95.30%		100%		
L. casei	Dried under control air-flow, 4°C	Alginate bead (1.5%)	7.1	pH 2.0, 120 min	4.1	Bile 0.5% 6 h	Li et al. (2011b)
		Alginate bead–chitosan solution (1%)	7.4		7.3		
		Alginate bead–chitosan solution–carboxymethyl chitosan solution (0.5%) (all in w/v)	7.9 (log CFU/g; no initial CFU given)		7.9 (log CFU/g; no initial CFU given)		
L. acidophilus (La-05)	Spray dried powder (T_i/T_o=130/75)	free	95.6%	pH 2.0, 120 min	103.1%	Bile 2%, 12 h	Fávaro-Trindade and Grosso (2002)
		RSM 10% + cellulose acetate phthalate 10% + Glycerol 3.5% + Maltodextrin 2.0% + Raftilose 1.0% + Tween 80 0.1%	95.5%		103.1%		
B. lactis (Bb-12)	Spray dried powder (T_i/T_o =130/75)	free	87.5%		94.4%		
		RSM 10% + cellulose acetate phthalate 10% + Glycerol 3.5% + Maltodextrin 2.0% + Raftilose 1.0% + Tween 80 0.1% (all in w/v)	100.0%		95.6%		
L. paracasei NFBC 338	Spray dried powder	RSM: Gum Acasia (10:10)%	49.1%	pH 3.0, 120 min, 37°C			Desmond et al. (2002)

	(T_i/T_o =170/95-100)	RSM 20%	18.2%				
B. PL1	Spray dried powder (T_i/T_o =100/45)	modified waxy maize starch 10% (w/v)	29.6% 0%	pH 2.8; 0h; 37°C pH 2.8; 3h; 37°C			O'Riordan et al. (2001)
L. rhamnosus GG	Spray dried powder (T_o =65-70)	Trehalose 20% Tre20%+12.5 MSG (g/L)	n.d. 0.6%	pH 1.6, 37°C, 90 min	97.5% 68.5%	5 mg/mL lysozyme + 1% bile	Sunny-Roberts and Knorr (2009)
L. rhamnosus E-97800	Spray dried powder (T_o =65-70)	Tre 20% Tre20%+12.5 MSG (g/L)	n.d. 0.01%	pH 1.6, 37°C, 90 min	80.6% 69.2%	5 mg/mL lysozyme + 1% bile	
L. casei	free		45.8%	pH 2.0, without pepsin, 37°C, 100 rpm	75.7%	porcine bile 1%, 37°C	Nag (2011)
	free		55.1%	pH 2.0, pepsin 0.32%, 37°C, 100 rpm			
	wet microcapsule	0.25% (w/w) gellan gum+10%(w/w) sodium caseinate acidified by glucono-δ-lactone	72.0%	pH 2.0, without pepsin, 37°C, 100 rpm	100%	porcine bile 1%, 37°C	
			66.4%	pH 2.0, pepsin 0.32%, 37°C, 100 rpm			
B. lactis Bb12	free		64.1%	pH 2.0, without pepsin, 37oC			Heidebach et al. (2009)
	wet microcapsule	SM 35% (w/w) incubated with rennet	91.3%				
L.paracasei ssp. paracasei F19	free		51.5%				
	wet microcapsule	SM 35% (w/w) incubated with rennet	59.2%				
B. breve R070	SD (T_i/T_o=185/85)	free harvested cells+denatured WPI 10% (w/v)	25.3% 12.7%	pH 1.9; pepsin, 30'	63.3% 91.1%	bile 15% + pancreatin, pH 7.5, 360'	Picot and Lacroix (2004)
B. longum R023		free	0.0%		15.0%		

		harvested cells+denatured WPI 10% (w/v)	0.0%		55.9%		
B. animalis ssp. *lactis* E-012010	Freeze dried powder	free	65.2%	pH 2.5; no pepsin; 2h	70.4%	bile extract 1%; 3h	(Saarela et al. (2005)
		sucrose (5% w/w)	69.6%		73.9%		
		RSM (5% w/w)	69.6%		-		
L. acidophilus (LAC 4)	free		70.5%	pH 1.0; 3 h			Oliveira et al. (2007)
	Freeze dried powder	pectin and casein (1 : 1, total solids content of 8%, w/v)	97.7%				
B. lactis (BI 01)	free		58.8%				
	Freeze dried powder	pectin and casein (1 : 1, total solids content of 8%, w/v)	89.3%				

Table 3. Survival of free and microencapsulated probiotic bacteria after spray or freeze drying (SD = spray drying; FD = Freeze drying)

Probiotic	Protectant	Drying method	Survival after drying	a_w/MC	References
B. bifidum	20 g/L of sodium alginate coated with chitosan 0.4% (w/v) solution	FD	94.8%		Chavarri et al. (2010)
L. gasseri	As above	FD	96.1%		
L. lactis	mannitol 10% - sucrose 10% mannitol 5% - sucrose 10% mannitol 10% - skim milk 10% mannitol 5% - MRS broth	FD	16% 26% 10% 62%		Berner and Viernstein (2006)
L. acidophilus (La-05)	RSM 10% + cellulose acetate phthalate 10% + Glycerol 3.5% + Maltodextrin 2.0% + Raftilose 1.0% + Tween 80 0.1%	SD (Ti/To=130/75)	98.3%	0.23 / 5.3%	Fávaro-Trindade and Grosso (2002)
B. lactis (Bb-12)	As above		78.7%		
Lactobacillus helveticus	maltodextrin (19% w/v)	SD To = 82°C To = 120°C FD Freezing	 15% 0.08% 48% 54%		Johnson and Etzel (1995)
L. reuteri-PS77	non fat skim milk (20%w/v)	FD SD; T_i/T_o = 170/85	66.7% 100%	6.5-7.0% 5.8-6.7%	Zamora et al. (2006)
L. lactis ssp. *cremoris*	230g maltodextrin, 76g lactose, 3.5g NaH2PO4zH2O, 7.1g Na2HPO4 and 685 mL deionized water	FD SD (T_o = 65°C) SD (T_o = 90°C)	63% 2.95% 0.35%		To and Etzel (1997)
L. pseudoplantarum	As above	FD SD (T_o = 65°C) SD (T_o = 70°C)	71% 14.7% 13%		
B. lactis (Bb-12)	SPI-MD (1:1) of total 20% (w/v)	FD SD; T_i/T_o =80/48 SD; T_i/T_o =80/48 + vacuum (45°C)	79.0% 44% 23%	0.13 / 3.58 0.35 / 7.51 0.18 / 4.97	Chavez and Ledeboer (2007)

B. pseudocatenulatum G4	SM (10% w/v)	SD; T_i/T_o =160/75	0.26%	9.2%	Wong et al. (2010)
		SD; T_i/T_o =160/85	0.05%	6.2%	
	free	FD	87%	12.7%	
	SM (10%)	FD	82.1%	4.4%	
	SM (10%) + Lactose (5%)	FD	81.1%	5.1%	
B. lactis Bb12	Free	FD	43%	0.092	Heidebach et al. (2010)
	Casein 15% (w/w) + transglutaminase (10 U/g casein)	FD	42%	0.109	
	As above + Resistant Starch (1% w/w)	FD	45%	0.118	
				(MC 3-4%)	
L. paracasei ssp. *paracasei* F19	Free	FD	71%		
	Casein 15% (w/w) + transglutaminase (10 U/g casein)	FD	34%		
	As above + Resistant Starch (1% w/w)	FD	16%		
B. breve A71	Lactose 5%, gelatine 1.5% and glycerol 1% (all w/v)		83.3%		Trsic-Milanovic et al. (2001)
	Saccharose 8%, gelatine 1.5% and skim milk 10% (all w/v)		66.6%		
B. bifidum BbTD	Lactose 5%, gelatine 1.5% and glycerol 1% (all w/v)		73.9%		
	Saccharose 8%, gelatine 1.5% and skim milk 10% (all w/v)		68.2%		
L. salivarius I 24	Distilled water (control)		0.08%	not given	Ming et al. (2009)
	Skim milk (20% w/v)		13.03%		
	Sucrose (20% w/v)		9.00%		
	Glycerol (5% w/v)		0.005%		
	Calcium carbonate (Ca^{2+}) (0.5% w/v)		0.00%		
L. lactis	egg yolk	FD	44.6%	not given	Nanasombat and Sriwong (2007)
	glucose		46.3%		

	lactose		64.2%		
	skim milk		61.6%		
	sorbitol		59.7%		
	soy milk		60.4%		
	sucrose		61.0%		
	trehalose		55.7%		
	(each 9% w/v distilled water)				
L. paracasei subsp. *tolerance*	SM/Tre/asc(6/8/4)w/v	FD	82%	not given	Jalali et al. (2011)
L. delbrueckii subsp. *bulgaricus*	SM/Tre/asc(6/8/4)w/v	FD	74%		
L. plantarum ATCC 8014	Control	FD	8%		De-Valdez et al. (1983)
	Adonitol (0.8 M)		72%	All <1%	
	Dulcitol (0.8 M)		8%		
	Glycerol (1 M)		33%		
	m-Inositol (0.8 M)		10%		
	Mannitol (0.8 M)		9.50%		
	Sorbitol (1 M)		11%		
	(all dissolved in 10% non fat skim milk; final concentration of each polyol was 0.32 M)				
L. rhamnosus GG	Free	FD	87.90%		Miao et al. (2008)
	Lactose		93.7	not given	
	Trehalose		97.1		
	Maltose		96.4		
	Sucrose		88.4		
	Lactose-Trehalose (1:1)		97.5		
	Lactose-maltose (1:1)		98.7		
	(all 15% w/v)				
L. salivarius subsp. *salivarius*	free	FD	4%	not given	Zayed and Roos (2004)

	Sucrose 4% (w/v)		13%		
	SM 18% (w/v)		22.4%		
	Trehalose 4% (w/v)		34%		
L. rhamnosus GG	RSM 20% (w/v)	SD;T_o=80	65%	MC = 3.7%	Ananta et al. (2005)
	RSM/polydextrose (1:1; total 20% w/v)		56%	MC=3.4%	
	RSM/raftilose P95 (1:1; total 20% w/v)		65%	MC=3.7%	
L. rhamnosus GG	RSM (20%)	SD; T_o =85–90	50%		Corcoran et al. (2004)
	RSM:raftilose (10:10)%		43%		
	inulin (20%)		0.25%		
L. paracasei NFBC 338	20% (wt/vol) RSM supplemented with 0.5% (wt/vol) yeast extract	SD; T_o =70-75	97%	7.30%	Gardiner et al. (2000)
		SD; T_o = 80-85	65%	5%	
L. salivarius UCC 118	20% (wt/vol) RSM supplemented with 0.5% (wt/vol) yeast extract	SD; T_o =70-75	11.30%	8.80%	
		SD; T_o = 80-85	1%	2.10%	
L. plantarum CIDCA 83114	SM 11%(wt/vol)	SD; Ti/T_o =160/70	98.9%		Golowczyc et al. (2010)
L. paracasei NFBC 338	RSM + 1% glucose	SD; Ti/T_o =170/80-85	11.80%		Kearney et al. (2009)
L. paracasei NFBC 338	RSM: Gum Acasia (10:10)%	SD; Ti/T_o =170/95-100	1.40%	MC=2.8%	Desmond et al. (2002)
		SD; Ti/T_o =170/100-105	0.90%	MC=2.5%	
	RSM 20%	SD; Ti/T_o =170/95-100	1.70%	MC=3.2%	
		SD; Ti/T_o =170/100-105	0.01%	MC=2.8%	
Bifidobacterium PL1	free	SD; Ti/T_o =100/45	29.60%		O'Riordan et al. (2001)
	Modified starch 10% (w/v) : cells (10:1)		30.20%		
	Modified starch 10% (w/v) : cells (5:1)		26.84%		

B. bifidum	WPC, whey protein concentrate; GA, gum arabic; MG, mesquite gum; MD, maltodextrin DE 10.				Rodrıguez-Huezo et al. (2007)
	WPC 17% + MG 17% + MD 66% + aguamiel 1.4%	SD; Ti/T_o =155/70	25.10%		
	GA 17% +MG 66% +MD 17% + aguamiel 1.4%		19.90%		
	GA 50% + MG 50% + aguamiel 1.4%		10%		
	WPC 17% +MG 17% + MD 66%		19.90%		
	GA 17% + MG 66% + MD 17%		1.60%		
	GA 50% + MG 50% (all in w/w; aguamiel in w/v as cell re-suspension)		1.30%		
L. rhamnosus GG	Tre 20%	SD; T_o =65-70	68.8%	3.8-4.1%	Sunny-Roberts and Knorr (2009)
	Tre20%+12.5 MSG (g/L)		80.8%		
	SM 20%		75%		
L. rhamnosus E-97800	Tre 20%	SD; T_o =65-70	23.4%		
	Tre20%+12.5 MSG (g/L)		89.3%		
	SM 20%		55%		
L. acidophilus DSM 20079	mod. Starch Hylon VII 30% (w/v)	SD; Ti/T_o =185/85	123.3%		Goderska and Czarnecki (2008)
B. bifidum DSM 20239	mod. Starch N-Tack 30% (w/v)	SD; Ti/T_o =185/85	121.6%		
L. rhamnosus R011	WPI gelled by $CaCl_2$, 20% (w/w) SM, 5%(w/w) sucrose, 1%(w/w) bacto casitone 0.35% (w/v) ascorbic acid	FD	9.5×10^{8}		Reid et al. (2007)
	SM (control)		1.5×10^{10}		
	un-gelled WPI + 25.6% (w/w) lactose + 13.9% (w/w) sucrose		2.3×10^{10}		
			(no initial population given)		
B. breve R070	harvested cells+denatured WPI 10%	SD; Ti/T_o =185/85	25.67%	0.16/1.95	Picot and Lacroix

					(2004)
	harvested cells+milk fat+denatured WPI 10%		10.58%	0.16/1.40	
	freeze dried cells+milk fat+denatured WPI 10%		0.71%	0.14/1.28	
B. longum R023	harvested cells+denatured WPI 10%		1.44%	0.18/2.05	
	harvested cells+milk fat+denatured WPI 10%		0.03%	0.16/2.07	
	freeze dried cells+milk fat+denatured WPI 10%		0.03%	0.14/1.42	
B. animalis ssp. *lactis* E-012010	sucrose (5% w/w)	FD	122.3%	2.70%	Saarela et al. (2005)
	RSM (5% w/w)		122.3%	2.80%	
L. casei	2% alginate + 2% rice starch + 1% lecithin	FD	95.3%		Donthidi et al. (2010)
	2% alginate + 2% hylon + 1% lecithin		92.5%		
	2% alginate + 2% maize + 1% lecithin		91.1%		
	2% alginate + 2% potato + 1% lecithin		83.3%		
	2% alginate + 2% wheat + 1% lecithin		96.1%		
B. lactis (BI 01)	pectin and casein (1 : 1, total solids content of 8%, w/v)	SD; Ti/T_o =70/46	93.0%	11%	Oliveira et al. (2007)
L. acidophilus (LAC 4)	pectin and casein (1 : 1, total solids content of 8%, w/v)		95.3%	9.60%	
B. animalis ssp. *lactis* DSMZ 20105	RSM (20%, w/v)	SD; T_i/T_o = 170/90	87%	2.47%	Simpson et al. (2005)
B. animalis ssp. *lactis* BB12			79%	3.16%	
B. breve			23-38%	3.05-3.23%	
B. longum			20 %	4.18%	
B. thermophilum			22-26%	3.38-3.78%	

Table 4. Stability of microencapsulated probiotic bacteria during storage

Probiotic	protectant	drying method	survival after storage	storage conditions	a_w/MC (%)	References
B. lactis (Bb-12)	SPI-MD (1:1) of total 20% (w/v)	FD	4.9%	2 mo, 30°C	0.13	Chavez and Ledeboer (2007)
		SD; Ti/T_o =80/48	n.d.		0.35	
		SD; Ti/T_o =80/48 + vacuum (45°C)	5.1%		0.18	
L. reuteri-PS77	NFSM (20%)	FD	85.9%	5°C, 60 days		Zamora et al. (2006)
	NFSM (20%) + 0.5% yeast extract	SD; Ti/T_o =170/85	33.1%			
L. reuteri-PS77	NFSM (20%)	FD	66.6%	20°C, 60 days		
	NFSM (20%) + 0.5% yeast extract	SD; Ti/T_o =170/85	10.7%			
L. rahmnosus GG	WPI (20%) + High amylose maize starch (20%) + sunflower oil (0%) + commercial freeze dried *L. rhamnosus* GG (20%)	FD	~53%	25°C, 5 weeks	0.32	Ying et al. (2010)
		SD; Ti/T_o = 160/65	~80%			
L. paracasei subsp. *tolerance*	SM/Tre/asc(6%/8%/4%)w/v	FD	76.0%	3 mo, 4°C		Jalali et al. (2011)
			37.0%	3 mo, 23°C		
L. delbrueckii subsp. *bulgaricus*	SM/Tre/asc(6%/8%/4%)w/v	FD	72.0%	3 mo, 4°C		
			35.0%	3 mo, 23°C		
B. longum 1941	Unipectin 2% (w/v)	FD	85.3%	-18 °C, 20 mo		Bruno and Shah (2003)
			46%	4 °C, 20 mo		
			n.d.	20°C, 5 mo		
L. bulgaricus KFRI 673	free cells in 10% skim milk solution	FD	94.7%	4 weeks, 4°C		Lee et al. (2004)
	alginate		89.0%			

	alginate 2% + chitosan (MW 3.852 x 10^3)		91.3%		
	alginate 2% + chitosan (MW 1.824 x 10^4)		94.0%		
	alginate 2% + chitosan (MW 1.709 x $10^{5)}$		94.0%		
	free cells in 10% skim milk solution		69.9%	4 weeks, 22^{o}C	
	alginate		72.6%		
	alginate 2% + chitosan (MW 3.852 x 10^3)		79.4%		
	alginate 2% + chitosan (MW 1.824 x 10^4)		83.6%		
	alginate 2% + chitosan (MW 1.709 x $10^{5)}$		90.4%		
B. bifidum	free	FD	0%	28 days, 4^{o}C	Chavarri et al. (2010)
	20 g/L of sodium alginate coated with chitosan 0.4% (w/v) solution		87.2%		
L. gasseri	free		0%	28 days, 4^{o}C	
	20 g/L of sodium alginate coated with chitosan 0.4% (w/v) solution		88.5%		
Lactobacillus ssp. *paracasei* LMG9192T	free	FD	20.0%	Vacuum-sealed aluminium foil, 150 days, 25^{o}C	Coulibaly et al. (2010)
	Glycerol 3% (w/v) + Sorbitol 1.2% (w/v)		40.0%		
	Glycerol 3% (w/v) + MSG 1.2% (w/v)		25.0%		
L. plantarum	free		30.0%		
CWBI-B1419	Glycerol 3% (w/v) + sorbitol 1.2% (w/v)		60.0%		
	Glycerol 3% (w/v) + MSG 1.2% (w/v)		43.0%		

Lactobacillus rhamnosus GG	lactose + trehalose (1:1; total 15% w/v)	FD	42.7%	38 days, 25°C	a_w = 0.0	Miao et al. (2008)
	lactose + maltose (1:1; total 15% w/v)		86.4%	38 days, 25°C	a_w = 0.0	
	lactose + maltose (1:1; total 15% w/v)		100.0%	38 days25°C	, a_w = 0.11	
	lactose+trehalose (1:1; total 15% w/v)		18.0%	38 days, 25°C	a_w = 0.11	
	RSM 15% (w/v)		65.4%	38 days, 25°C	a_w = 0.11	
L. acidophilus CSCC 2409	free	FD	44.0%	6 weeks, -20°C		Kailasapathy and Sureeta (2004)
	Ca-alginate 2% (w/v)		71.3%			
	WP 10% (w/v)		66.6%			
B. infantis CSCC 1912	free	FD	31.8%	6 weeks, -20°C		
	Ca-alginate 2% (w/v)		70.8%			
	WP 10% (w/v)		52.6%			
L. paracasei NFBC 338	20% (wt/vol) RSM supplemented with 0.5% (wt/vol) yeast extract	SD; T_o =80-85	92.0%	4°C, 2 months		Gardiner et al. (2000)
			11.0%	15°C, 2 months		
L. salivarius UCC 118	20% (wt/vol) RSM supplemented with 0.5% (wt/vol) yeast extract	SD; T_o =80-85	13.0%	4°C, 2 months		Kearney et al. (2009)
			2.0%	15°C, 2 months		
			86.0%	15°C, 42 days		
			n.d.	37°C, 42 days		
L. paracasei NFBC 338	RSM: Gum Acasia (10:10)%	SD; Ti/T_o =170/95-100	68.0%	4°C, 8 weeks	0.37	Desmond et al. (2002)
		SD; Ti/T_o =170/100-105	19.0%		0.35	
	RSM 20%	SD; Ti/T_o =170/95-100	3.5%		0.36	

		SD; Ti/T_o =170/100-105	11.0%		0.36	
	RSM: Gum Acasia (10:10)%	SD; Ti/T_o =170/95-100	0.0%	30°C, 8 weeks	0.44	
		SD; Ti/T_o =170/100-105	0.0%		0.34	
	RSM 20%	SD; Ti/T_o =170/95-100	0.0%		0.41	
		SD; Ti/T_o =170/100-105	0.0%		0.34	
Bifidobacterium infantis CCRC 14633	SM 15%	SD; Ti/T_o =100/50	54.0%	Glass, 25°C, deoxidant+desiccant		Hsiao et al. (2004)
			44.6%	Polyester, 25°C, deoxidant+desiccant		
			96.1%	Glass, 4°C, deoxidant+desiccant		
			95.6%	Polyester, 4°C, deoxidant+desiccant		
	gum arabic 15%		0.0%	Glass, 25°C, deoxidant+desiccant		
			0.0%	Polyester, 25°C, deoxidant+desiccant		
			81.2%	Glass, 4°C, deoxidant+desiccant		
			73.4%	Polyester, 4°C, deoxidant+desiccant		
	Gelatin 15%		41.2%	Glass, 25°C, deoxidant+desiccant		
			34.8%	Polyester, 25°C, deoxidant+desiccant		
			72.4%	Glass, 4°C, deoxidant+desiccant		
			69.9%	Polyester, 4°C, deoxidant+desiccant		
	Soluble starch (proportion was not given)		30.9%	Glass, 25°C, deoxidant+desiccant		
			19.8%	Polyester, 25°C, deoxidant+desiccant		

			62.3%	Glass, 4°C, deoxidant+desiccant	
			59.8%	Polyester, 4°C, deoxidant+desiccant (all kept for 42 days)	
Bifidobacterium PL1	Free	SD; Ti/T_o =100/45	56.4%	20 days, (19-24°C)	O'Riordan et al. (2001)
	modified waxy maize starch 10% (w/v); coating polymer : core = 10:1		44.9%		
B. animalis ssp. *lactis* DSMZ 20105	RSM (20%, w/v)	SD; T_i/T_o=170/90	59%	90 days, 25°C	Simpson et al. (2005)
B. animalis ssp. *lactis* BB12			49%		
B. breve			47%		
B. longum			<25%		
B. thermophilum			32%		
B. bifidum	WPC, whey protein concentrate; GA, gum arabic; MG, mesquite gum; MD, maltodextrin DE 10.				Rodrıguez-Huezo et al. (2007)
	WPC 17% + MG 17% + MD 66% + aguamiel 1.4%	SD; Ti/T_o =155/70	2.0%	5 weeks, 4°C, a_w 0.32	
	GA 17% +MG 66% +MD 17% + aguamiel 1.4%		1.0%		
	GA 50% + MG 50% + aguamiel 1.4%		0.6%		
	WPC 17% +MG 17% + MD 66%		<0.1%		
	GA 17% + MG 66% + MD 17%		0.0%		
	GA 50% + MG 50% (all in w/w; aguamiel in w/v as cell re-suspension)		<0.1%		
L. acidophilus DSM 20079	mod. Starch Hylon VII	SD; Ti/T_o =185/85	60.2%	4°C, 4 months	Goderska and Czarnecki (2008)
	mod. Starch N-Tack		34.1%		

	mod. Starch N-Lock		33.3%		
B. bifidum DSM 20239	mod. Starch N-Tack	SD; Ti/To =185/85	72.3%		
	mod. Starch N-Lock		36.1%		
	(all 30% w/v)				
L. acidophilus DSM 20079	SM	FD	100.0%		
	SM+5% sacch+0.35% ascorbic acid		92.5%		
	saccharides (20%)		90.4%		
B. bifidum DSM 20239	SM		100.0%		
	SM+5% sacch+0.35% ascorbic acid		92.5%		
	saccharides (20%)		93.3%		
Bifidobacterium animalis ssp. *lactis* E-012010	sucrose (5% w/w)	FD	91.3%	37^{o}C, 2 mo	Saarela et al. (2005)
	RSM (5% w/w)		80.0%		
	sucrose (5% w/w)		99.1%		
	RSM (5% w/w)		98.3%		
	sucrose (5% w/w)		99.1%		
	RSM (5% w/w)		96.5%		
L. casei NCFB 161	alginate (2% w/v) + gelatinized starches (2% w/v) + lecithin (1% w/v)	FD	73.5% / 24.9%	12 weeks / 24 weeks (23^{o}C)	Donthidi et al. (2010)
L. plantarum DSM 12028			69.3% / 46.7%		
L. acidophilus NCFB 1748			88.4% / n.d.		
L. gasseri NCFB 2233			65.4% / n.d.		
L. bulgaricus NCFB 1489			72.2% / n.d.		
B. adolescentis NCIMB 702204			62.2% / n.d.		

Lactococcus lactis NCIMB 6681			93.8% / 68.8%			
L. rhamnosus GG	RSM 20%	SD; T_o =85–90	88.9%	4°C, 8 weeks		Corcoran et al. (2004)
			74.4%	37°C, 8 weeks		
	RSM 10%+inulin 10%		43.4%	37°C, 8 weeks		
	Inulin 20%		42.7%	37°C, 1 week		
L. rhamnosus GG	Tre20%+12.5 MSG (g/L)	SD; T_o =65-70	59%	25°C, 4 weeks	a_w 0.11	(Sunny-Roberts and Knorr, 2009)
L. rhamnosus E80			74%			

Chapter 3 : General materials and methods

This section describes general materials and methods used in the thesis. More detailed description of the procedures can be found in Chapter 4 – Chapter 10.

Materials

Microencapsulating materials were sodium alginate, mannitol, sodium caseinate, fructooligosaccharides (FOS), D-glucose (all from Sigma Aldrich Corp.), glycerol (Merck), whey protein concentrate (UMT, Spreyton, Tasmania), skim milk (Murray Goulburn, Victoria), maltodextrin (GPC, Muscatine, Iowa) and soy protein isolate (SPI) (Pure Supplement Powders Inc., Bentleigh, Victoria). Canola oil (Crisco; Goodman Fielder, Australia) was obtained from the local market.

Pure culture of *B. animalis* ssp. *lactis* Bb12 in dried form was received from Chr. Hansen (Hørsholm, Denmark). Pure culture of *B. longum* 1941, *L. acidophilus* 2401 and *L. lactis* subsp. *cremoris* R-704 were received from Victoria University stock culture. Media of de Man, Rogosa, Sharpe (MRS) and M17 were obtained from Oxoid Ltd., Hampshire, U.K; L-cysteine hydrochloride was purchased from Sigma Chemical Co., Castle Hill, Australia.

Bacterial preparation

B. animalis ssp. *lactis* Bb12 was grown in MRS broth (Oxoid Ltd., Hampshire, U.K.) supplemented with filter sterilized 0.05% w/v L-cysteine-hydrochloride at 37^{o}C for 18 h using 1.0% (v/v) inoculum (Ding and Shah, 2009b). Bacterial activation, confirmation and preparation of concentrated cells were explained in Chapter 4. Similar preparation was carried out for *B. longum* 1941; detailed description was in Chapter 8. *L. acidophilus* was grown in de Man

Rogosa Sharpe (MRS) broth at 37^{o}C for 18 h using 1% (v/v) inoculum (Riveros et al., 2009); while *L. lactis* ssp. *cremoris* was grown in M17 supplemented with 0.5% glucose (w/v) at 30^{o}C for 18 h using 1% (v/v) inoculum (Kimoto et al., 2003). Details of preparation of concentrated *Lactobacillus acidophilus* 2401 and *L. lactis* ssp. *cremoris* could be found in Chapter 9.

Microencapsulation of bacteria

Microcapsules using an alginate-based system were prepared (Ding and Shah, 2009b) with or without incorporation of mannitol as follows. The first system consisted of sodium alginate (2.5% w/v) and the second system of sodium alginate (2.5% w/v) and mannitol (2.5% w/v). Ten percent of canola oil containing 0.5% (w/v) Tween 80 was used to develop an emulsion. Pasteurization of each formulation was then carried out (70^{o}C, 30 min) and subsequently cooled to 10^{o}C. One fourth of concentrated *B. animalis* ssp. *lactis* Bb12 cells were added directly into the formulation. Each formulation containing the bacteria was then dropped thoroughly into 0.1 M $CaCl_2$ solution using a sterile burette to produce gel beads with uniform size; two types of beads were then freeze dried (freeze drier model FD-300, Airvac Engineering Pty. Ltd., Dandenong, Australia) with setting of −100 Torr for internal pressure before freeze drying at a temperature of −88 ◦C, 44 h for primary freeze drying, and 4 h for secondary freeze drying. Once freeze drying was completed, moisture content and a_w of beads, survival, acid tolerance, bile tolerance as well as SHb of freeze dried bacteria were determined (Chapter 4). Details of determination of β-Gal, β-glu, LDH, PK, HK and ATPase activities of *B. animalis* ssp. *lactis* Bb12 after freeze drying were shown in Chapter 5, whereas that of FTIR and ESEM observations of the bacteria were described in Chapter 6.

Microencapsulation of *B. animalis* ssp. *lactis* Bb12 using casein-based formulation (Crittenden et al., 2006) was prepared with or without mannitol (Chapter 7). Sodium caseinate

(6% w/v) was combined with either glucose (6% w/v) (G formulation) or glucose : mannitol (3% : 3%, w/v) (GM formulation) and dissolved in sterilized mili-Q water. Fructooligosaccharides (FOS) (2% w/v) were added to each formulation, and then canola oil (10% w/v) was added to develop the emulsion system. Each formulation was heated to 95^{o}C (30 min) to promote Maillard substances and then cooled to 10^{o}C before incorporation of the *Bifidobacterium.* Microencapsulation was carried out by spray drying the mixture using NIRO atomizer serial no. 1902 (Copenhagen, Denmark) with T_i / T_o = 120^{o}C / 50$\pm$5^{o}C; flow rate of sample input = 300 mL/h (peristaltic pump Masterflex Model 7518-00).

Effectiveness of proteins (sodium caseinate, SC; whey protein concentrate, WPC; skim milk, SM; or soy protein isolate, SPI) combined with sugars (glycerol, GLY; mannitol, MAN; maltodextrin, MD) as cryoprotectant to protect *Bifidobacterium longum* 1941 after freeze drying was tested. SC (12%), WPC (12% w/v), WPC:CAS (6%:6% w/v), SM (12% w/v), SPI (12% w/v) were diluted into sterilized milliQ water. GLY (3% w/v), MAN (3% w/v) or MD (3% w/v) were added into each of protein solution. Inulin (2% w/v) was incorporated, followed by canola oil 10% (w/v). Each formulation was pasteurized (70^{o}C, 30 minutes) then cooled to 10^{o}C. One fifth (100 mL) of concentrated cells (8×10^{10} CFU/mL) were added into 400 mL of the formulation. Each mixture was then freeze dried using the above procedure. Dependent variables measured after freeze drying comprised a_w of freeze dried products, survival, acid tolerance, bile tolerance, SHb, retention of β-glu, LDH and ATPase of the bacteria (Chapter 8).

Chapter 9 reports GM formulation to protect *Lactobacillus acidophilus* 2401 or *Lactococcus lactis* subsp. *cremoris* R-704; spray- or freeze-drying was applied as the last stage of microencapsulation. Spray drying was carried out using Buchi Mini spray drier B290 Switzerland with Dehumidifier B296 (humidity 86%; temperature −3 °C). To achieve the outlet

temperature of 50 °C, inlet temperature was set up to 99 °C with peristaltic pump set at 27% (feeding rate=7.1 mL/min) for emulsion system containing *L. acidophilus* and was set up to 80 °C with peristaltic pump set at 20% (feeding rate=3.0 mL/min) for emulsion system containing *L. cremoris*. Residual moisture content and a_w of spray or freeze dried products, survival, acid tolerance, bile tolerance, SHb and retention of β-gal of the bacteria after drying were determined (Chapter 9). Changes in cell envelopes and protein structures of *L. acidophilus* and *L. cremoris* after drying and glass transition temperature of microcapsules were observed using FTIR and DSC, respectively (Chapter 10).

Storage at low a_w at room temperature (25 ^oC)

All microencapsulated bacteria were kept at 25 ^oC for 10 weeks at low water activities. SA and SAM beads (Chapter 4, 5 and 6), or G and GM (Chapter 7) powders containing *B. animalis* ssp. *lactis* Bb12 were put on plates and kept in desiccators with a_w maintained at 0.07, 0.1 and 0.2 using saturated solution of NaOH, LiCl and CH_3COOK, respectively. Equilibrium of a_{ws} was achieved within two weeks as the sample weights were constant. Storage of 0 week of storage was corresponding to 2 weeks of initial equilibration of a_w. Control was the samples stored in an aluminium foil pouch without a_w adjustment. The a_w of freeze dried beads (SA and SAM), that of powder (G and GM) as well as that of saturated salt solutions was measured at ambient temperature using a water activity meter (Decagon, CX-2 Serial I/O, Pullman, Wash., U.S.A.) following method previously described by Kurtmann and others (2009). The details are provided in Chapter 4 (page 88).

In the second part of the storage experiment, instead of carrying out the experiment inside the desiccators (Chapter 4, 5, 6, 7), the equilibrated microcapsules with different a_ws were transferred into aluminium foil pouches containing packs of NaOH, LiCl and silica gel in a semi-

permeable membrane to maintain the a_ws constant during storage (Chapter 9 and 10). All the packed products were put in the incubators (25^oC) and were considered as 0 week. Spray- or freeze-dried samples kept in aluminium foil pouches without any incorporation of desiccant in foil packages were used as control.

Survival

One gram of spray- or freeze-dried bacteria within the protein-based system was suspended in peptone water 0.15% (w/v) and serial dilutions were carried out. In case of alginate-based microencapsulation, the bacteria were released using 0.4 M sodium phosphate buffer before being suspended in the peptonized water. Dilutions were vortexed for 30s individually before plating using MRS agar in the presence of 0.05% (w/v) L-cysteine-hydrochloride for *B. animalis* and *B. longum* (Ding and Shah, 2009). Plates were incubated at 37°C for 72 h in an anaerobic jar containing an anaerobic gas generating kit. In case of *L. lactis* ssp. *cremoris* or *L. acidophilus*, plating was carried out using M17 agar supplemented with 0.5% (w/v) glucose (Kimoto et al., 2003) or using MRS agar (Riveros et al., 2009), respectively. Plates were incubated at 30 ^oC and 37 ^oC for *L. lactis* ssp. *cremoris* or *L. acidophilus*, respectively, for 48 h. Details of calculation of survival percentage of microencapsulated bacteria during 10 week storage was described in Chapter 4, 7 and 9.

Acid and Bile Tolerance

Analysis of acid and bile tolerance was carried out according to the method of Ding and Shah (2009). Acid environment was prepared by adjusting the pH of MRS broth or M17 broth to pH 2.0 using 5 M HCl. One gram of encapsulated bacteria was inoculated into 9 mL of acidified MRS broth or M17 broth depending upon the bacteria; incubated at 37^oC for 2 h.

Bile environment was prepared by incorporating 0.3% w/v of taurocholic acid to MRS broth or M17 broth with pH adjusted to 5.8 using 5 M HCl. Preparation of encapsulated bacteria was as mentioned above, incubation was at 37^{o}C for 8 h (Chapter 4, 7 and 9). Details of percent calculation of survival in acid and bile of microencapsulated bacteria during 10 week storage were described in Chapter 4, 7 and 9.

Surface Hydrophobicity

The surface hydrophobicity (SHb) measurement was carried out according to the modified method of Vinderola and Reinheimer (2003) and Riveros et al. (2009). Microorganisms were released from microcapsules and centrifuged, detailed procedures were shown in Chapter 4. Concentrated bacteria were washed twice and were resuspended in 50 mM K_2HPO_4 to achieve an absorbance value of about 0.5 at 560 nm of bacterial suspension. Five milliliter of the suspension was mixed with 1 mL of n-hexadecane by vortexing for 120 s and incubated for 1 h at room temperature (25^{o}C). Changes in the absorbance of probiotic bacterial suspension were recorded at 560 nm using a UV-Vis spectrophotometer. Details of surface hydrophobicity (SHb%) determination and its retention are described in Chapter 4, 7 and 9.

Enzyme Retention

Retention of some enzymes such as β-glu, β-gal, LDH, PK, HK and ATPase is described in Chapter 5, 7, 8, 9. The steps included cell extraction; analysis of enzyme activities and calculation of enzyme retention after spray- or freeze-drying and after 10 weeks of storage. Specific study in conjunction with analysis of the six enzymes in detail is shown in Chapter 5.

Fourier Transform Infrared (FTIR)

Measurement of spectra of functional groups was carried out at room temperature (25^{o}C) using an FTIR combined with IRsolution software (type 8400S, Shimadzu, Kyoto, Japan). All FTIR spectra were recorded using a resolution of 4 cm^{-1} and 20 scans. A reference spectrum was recorded prior to each experiment to correct for background effects. The instrument was purged with dry nitrogen gas to reduce interference of water vapour and CO_2 in all the FT-IR measurement.

Solid sample preparation was carried out according to Izutsu and Kojima (2002) and Oldenhof et al. (2005). Detailed FTIR analysis procedures of alginate-based microcapsules are described in Chapter 6, while those of casein-based microcapsules are explained in Chapter 7 and Chapter 10. Spectra of fresh *B. animalis* ssp. *lactis* Bb12 (Chapter 6, 7), *Lactobacillus acidophilus* 2401 and *Lactococcus lactis* subsp. *cremoris* R-704 (Chapter 10) harvested after 18 h were used as a control to recognize band alterations due to the treatments. Interaction between microencapsulating materials and phospholipid bilayers of cell envelopes, changes in cell envelopes and secondary protein structures were observed in the range of frequencies in the FTIR spectra. Assignment of some functional groups observed in the frequencies of FTIR spectra is described in Chapter 6, 7, and 10.

Environmental Scanning Electron Microscopy (ESEM)

Morphology of microcapsules after drying and after 10 weeks of storage at low a_w was observed using FEI Quanta 200 environmental scanning electron microscope (ESEM; FEI Burlington Mass, USA) (Chapter 6 and 7). The working distance was 10.2 mm and beam energy was 20.0 kV, spot 5.0, 500 × magnification and pressure 0.98 torr. All bars presented 200.0 µm of size. The microcapsule was loaded on a double sided carbon tape put on multiple studs before

ESEM examination. The number of field viewed was three for each sample to observe the whole matrix as such.

Differential Scanning Calorimetry (DSC) analysis

Differential scanning calorimetry (DSC) was performed using a PerkinElmer DSC 7 (PerkinElmer, San Jose, CA) to determine T_g of the samples. Microcapsules (8-12 mg) were pressed in a standard sealed aluminum DSC pans. Samples were scanned from 5 to 170°C at a heating rate of 5°C/min. Glass transition temperature was determined from the temperature of the midpoint of the change in heat capacity scanned at 10°C/min (Chapter 10). Measurements were carried out in duplicates.

Experimental Design

A randomized full factorial design was applied to the experiments (Chapter 4, 5, 7, 8, 9, 10). In Chapter 4 and 5, the experiments consisted of two independent variables namely type of alginate-based microcapsule (alginate (A) and alginate-mannitol (AM)) and a_w of storage in desiccators (0.07, 0.1 and 0.2). Sampling was carried out fortnightly during 10 weeks of storage. The same experimental design was carried out (Chapter 7) for casein-based formulation (added with glucose (G) or glucose-mannitol (GM)). In Chapter 8, the experiments were carried out using combinations of the type of proteins at 12% w/v as protectant (sodium caseinate (CAS), whey protein concentrate (WPC), skim milk (SM), soy protein isolate (SPI) and WPC-CAS) with different types of sugars at 3% w/v, (mannitol (MAN), maltodextrin (MD) and, glycerol (GLY)). A three-factor experiment was carried out (Chapter 9 and 10) using casein-based formulation supplied with glucose and mannitol. The factors were drying method (spray- or freeze-drying), type of desiccant (NaOH, LiCl or silica gel), and 10-week storage period (with 2-week sampling interval). All treatments were assigned a random order as picked up by complete

randomisation suggested by Mintiab (version 16). This is to assure individual to have the same chance to be chosen. Each sample was thoroughly homogenization before sampling. The powder kept in aluminium foil pouch with adjusted to assigned a_{ws} was shaken thoroughly to avoid variations. Each experiment was repeated three times independently. Details of experimental design of each experiment are presented in Table 1 as follows.

Tabel 1. Summary of experimental design of each chapter

Chapter	Factors	Treatments	Treatment Combinations	Total Experimental Units (3 repetition each)
4 and 5	type of alginate-based microcapsule	- alginate (A) - alginate-mannitol (AM)	48 (2 types of alginate-based microcapsules, 4 levels of a_w including the control and 6 numbers of fortnightly sampling). The controls were A and AM without a_w adjustment.	48 x 3 = 144
	a_w of storage	- 0.07 - 0.1 - 0.2		
	storage period (0 – 10 weeks with 2-week interval)	- Week 0 - Week 2 - Week 4 - Week 6 - Week 8 - Week 10		
7	type of casein-based microcapsule	- Added with glucose (G) - glucose-mannitol (GM)	48 (2 types of casein-based microcapsules, 4 levels of a_w including the control and 6 numbers of fortnightly sampling). The controls were G and GM without a_w adjustment.	48 x 3 = 144
	a_w of storage	- 0.07 - 0.1 - 0.2		
	storage period (0 – 10 weeks with 2-week interval)	- Week 0 - Week 2 - Week 4 - Week 6 - Week 8 - Week 10		
8	type of proteins at 12% w/v	- sodium caseinate (CAS) - whey protein concentrate (WPC) - skim milk (SM) - soy protein isolate (SPI) - WPC-CAS	15 (5 types of proteins and 3 types of sugars). The control is freeze-dried bacteria without any protectant.	15 x 3 = 48
	types of sugars at 3% w/v	- mannitol (MAN) - maltodextrin (MD) - glycerol (GLY)		
9 and 10	Drying method	- spray drying	48 (2 types of drying	48 x 3 = 144

		(SD) - freeze drying (FD)	method, 4 types of desiccant including the control, and 6 weeks of fortnight sampling). The control was the spray- or freeze dried bacteria kept in foil pouch without desiccant.	
	Type of desiccant	NaOH LiCl silica gel		
	storage period (0 – 10 weeks with 2-week interval)	- Week 0 - Week 2 - Week 4 - Week 6 - Week 8 - Week 10		

Statistical Analysis

Results from at least three repetitions were used for ANOVA analysis (α=0.05) to ascertain whether independent variables (such as type of formulation, drying method, a_w of storage) had a significant effect on dependent variables such as survival, acid tolerance, bile tolerance, retention of SHb, retention of some enzymes, glass transition and residual moisture. For survival, acid and tolerance assays, each replication was analysed in duplicate (n=6) (Otieno and Shah, 2007). ANOVA was also used to examine whether there was an interaction between factors. All the experiments used one-, two- or three ways ANOVA depending upon the number of independent variables, and were performed using GLM procedure. The difference between means was determined by Tukey HSD test. Pearson correlation was also applied if necessary. All statistical analysis was carried out using Minitab 16 released in 2010 (Minitab Pty Ltd., Sydney NSW, Australia).

Hierarchical cluster analysis was carried out in Chapter 10 based on collected spectra from FTIR analysis. The Ward's algorithm method was used to analyze the similarities between bacterial spectra determining phospholipid bilayers of cell envelopes and secondary protein structures of *Lactobacillus acidophilus* or *Lactococcus lactis* ssp. *cremoris* after drying and after 10 week of storage at room temperature using various desiccants.

Chapter 4 : Survival, acid and bile tolerance, and surface hydrophobicity of microencapsulated *B. animalis* ssp. *lactis* Bb12 during storage at room temperature

Introduction

Chapter 4 examines the effectiveness of Ca-alginate matrix with or without mannitol incorporation as microencapsulant. Survival, acid and bile tolerance, and surface hydrophobicity of microencapsulated *Bifidobacterium animalis* ssp. *lactis* Bb12 after freeze drying and during storage at room temperature (25 ^{o}C) at low water activity (0.07, 0.1, and 0.2) are discussed.

The paper titled "Survival, acid and bile tolerance, and surface hydrophobicity of microencapsulated *B. animalis* ssp. *lactis* Bb12 during storage at room temperature" by D. Dianawati, and N. P. Shah was published in the peer review journal, *Journal of Food Science*, 76(9), M592-M599, 2011.

Published as: Dianawati, Dianawati and Shah, Nagendra P (2011) Survival, Acid and Bile Tolerance, and Surface Hydrophobicity of Microencapsulated B. animalis ssp. lactis Bb12 during Storage at Room Temperature. Journal of Food Science, 76 (9).
Available from: https://doi.org/10.1111/j.1750-3841.2011.02422.x

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PART B: DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by (candidate name): DIANAWATI DIANAWATI

Signature: [redacted]

Date: 11/3/2013

Paper Title

Survival, acid and bile tolerance, and surface hydrophobicity of microencapsulated *B. animalis* ssp. *lactis* Bb12 during storage at room temperature

In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Dianawati Dianawati	70	Designed the experiment Perform the sample analyses Performed statistical analysis using MINITAB 16 Prepared major part of the manuscript
Nagendra P. Shah	30	Contribution to writing of paper and journal submission

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to **a)** granting bodies, **b)** the editor or publisher of journals or other publications, and **c)** the head of the responsible academic unit; and
5. The original data is stored at the following location(s):

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and will be held for at least five years from the date indicated below:

		Date
Signature 1	[redacted]	18/2/2013
Signature 2	[redacted]	18/2/2013
Signature 3		

Chapter 5 : Enzyme stability of microencapsulated *Bifidobacterium animalis* ssp. *lactis* Bb12 after freeze drying and during storage in low water activity at room temperature

Introduction

This experiment examines the effect of coating materials (alginate and alginate-mannitol) on stabilizing β-galactosidase (β-gal), β-glucosidase (β-glu), lactate dehydrogenase (LDH), pyruvate kinase (PK), hexokinase (HK), and ATPase of *Bifidobacterium animalis* ssp. *lactis* Bb12 after freeze drying and after 10 weeks of storage at low a_w (25^oC).

The paper titled "Enzyme stability of microencapsulated *Bifidobacterium animalis* ssp. *lactis* Bb12 after freeze drying and during storage in low water activity at room temperature" by D. Dianawati, and N. P. Shah was published in the peer review journal, *Journal of Food Science*, 76(6), M463-M471.

Published as: Dianawati, Dianawati and Shah, Nagendra P (2011) Enzyme Stability of Microencapsulated Bifidobacterium animalis ssp. lactis Bb12 after Freeze Drying and during Storage in Low Water Activity at Room Temperature. Journal of Food Science, 76 (6).

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Declaration by (candidate name): DIANAWATI DIANAWATI **Signature:** [redacted] **Date:** 11/3/2013

Paper Title

Enzyme stability of microencapsulated *Bifidobacterium animalis* ssp. *lactis* Bb12 after freeze drying and during storage in low water activity at room temperature

In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Dianawati Dianawati	70	Designed the experiment Perform the sample analyses Performed statistical analysis using MINITAB 16 Prepared major part of the manuscript
Nagendra P. Shah	30	Contribution to writing of paper and journal submission

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4. Potential conflicts of interest have been disclosed to a) granting bodies, b) the editor or publisher of journals or other publications, and c) the head of the responsible academic unit; and
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Chapter 6 : Role of calcium alginate and mannitol in protecting *Bifidobacterium*

Introduction

The aims of this study were to ascertain the protective mechanism of Ca-alginate combined with mannitol on phospholipid bilayers of cell envelopes and secondary protein structures of *Bifidobacterium animalis* subsp. *lactis* Bb12 as well as to observe microstructures of microcapsules after freeze-drying and after 10 weeks of storage at room temperature (25°C) at low a_w.

The paper titled "Role of calcium alginate and mannitol in protecting *Bifidobacterium*" by D. Dianawati, V. Mishra and N. P. Shah was published in the peer review journal, *Applied and Environmental Microbiology,* 78(19), 6914–6921.

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Declaration by (candidate name): DIANAWATI DIANAWATI

Signature: [redacted]

Date: 11/3/2013

Paper Title

Role of calcium alginate and mannitol in protecting *Bifidobacterium*

In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Dianawati Dianawati	70	Designed an experimental Performed the sample analyses Performed all data presentation Prepared major part of the manuscript
Nagendra P. Shah	20	Contribution to writing of paper and journal submission
Vijay Mishra	10	Contribution to writing of paper

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1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
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Signature 2	[redacted]	18/2/2013
Signature 3	[redacted]	18/1/2013

Applied and Environmental Microbiology

Role of Calcium Alginate and Mannitol in Protecting Bifidobacterium

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Role of Calcium Alginate and Mannitol in Protecting *Bifidobacterium*

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Fourier transform infrared (FTIR) spectroscopy was carried out to ascertain the mechanism of Ca-alginate and mannitol protection of cell envelope components and secondary proteins of *Bifidobacterium animalis* subsp. *lactis* Bb12 after freeze-drying and after 10 weeks of storage at room temperature (25°C) at low water activities (a_w) of 0.07, 0.1, and 0.2. Preparation of Ca-alginate and Ca-alginate-mannitol as microencapsulants was carried out by dropping an alginate or alginate-mannitol emulsion containing bacteria using a burette into $CaCl_2$ solution to obtain Ca-alginate beads and Ca-alginate-mannitol beads, respectively. The wet beads were then freeze-dried. The a_w of freeze-dried beads was then adjusted to 0.07, 0.1, and 0.2 using saturated salt solutions; controls were prepared by keeping Ca-alginate and Ca-alginate-mannitol in aluminum foil without a_w adjustment. Mannitol in the Ca-alginate system interacted with cell envelopes during freeze-drying and during storage at low a_ws. In contrast, Ca-alginate protected cell envelopes after freeze-drying but not during 10-week storage. Unlike Ca-alginate, Ca-alginate-mannitol was effective in retarding the changes in secondary proteins during freeze-drying and during 10 weeks of storage at low a_ws. It appears that Ca-alginate-mannitol is more effective than Ca-alginate in preserving cell envelopes and proteins after freeze-drying and after 10 weeks of storage at room temperature (25°C).

Microencapsulation of probiotic bacteria using alginate or alginate fortified with mono-, di-, or polysaccharides or proteins has been widely studied (1, 9, 28, 30, 37, 44, 52). The use of sugars or proteins to fortify alginate was effective in improving bacterial survival; however, Krasaekoopt and others (30) reported that alginate combined with chitosan was not successful in protecting *Bifidobacterium* in very-low-pH environment. Similarly, Zohar-Perez et al. (60) found that alginate was not effective as a bacterial encapsulant. Stabilization of alginate-based microencapsulated bacteria was influenced by many factors, such as type and concentration of microencapsulants and storage conditions, i.e., temperature, water activity, and the presence of oxygen. The use of mannitol was found to be effective in maintaining bacterial viability during drying and during storage at room temperature (15, 27, 38) and also in stabilizing protein during freeze-drying (26) and during storage (8). Mannitol protected probiotic bacteria during storage, likely due to its role as a hydroxyl radical scavenger (15, 49).

Water activity (a_w) is a critical factor for bacterial stabilization during storage at room temperature. Storage at low a_ws at room temperature maintained the glassy state and minimized chemical reactions, and hence, the survival of bacteria improved (24). Storage at low a_ws (0.07 and 0.1) maintained high viability of bacteria during long-term storage at room temperature; however, this phenomenon depended on the type of sugars used as protectants: incorporation of mannitol in Ca-alginate improved the survival of *Bifidobacterium animalis* subsp. *lactis* Bb12 and preserved some glycolytic enzymes during long-term storage at room temperature and low a_w (11, 12).

Even though Ca-alginate is known as an encapsulant for probiotic bacteria (25), the mechanism of its protection of probiotic bacteria has not been established. Changes in functional groups of polypeptides, lipids, and secondary proteins can be observed by Fourier transform infrared (FTIR) spectroscopy (51). FTIR spectroscopy has been successfully applied in identifying microorganisms such as probiotic bacteria and fungi (14, 16, 57, 58). Characterization of lactic acid bacteria (LAB) encapsulated with alginate using FTIR spectroscopy has been carried out by Le-Tien and others (34). The effect of drying and as well as of sugars on bacterial membrane structure and on secondary proteins has been studied by several other groups (26, 40, 45, 46, 47). Trehalose and sorbitol were effective in protecting cell envelopes and secondary proteins of probiotic bacteria during drying (33, 40, 45). However, polysaccharides such as maltodextrin act as inert bulking substances instead of bacterial protectants (33). Lyophilization causes reversible changes to secondary proteins (21) as well as affecting the stability of phosphatidylcholine and liposomes (54). However, freeze-drying is a common method of dehydration of probiotic bacteria. In this study, FTIR spectroscopy was used to establish the mechanism of the protection provided by encapsulants such as Ca-alginate with or without mannitol to cell envelope components and secondary proteins of probiotic bacteria during freeze-drying and after 10-week storage at low a_ws. Interaction between Ca-alginate and mannitol was also observed in order to understand the effectiveness of mannitol as a protectant in a Ca-alginate matrix.

MATERIALS AND METHODS

***B. animalis* subsp. *lactis* Bb12 cultivation.** *B. animalis* subsp. *lactis* Bb12 was cultivated out as described by Ding and Shah (13). The freeze-dried pure culture of *B. animalis* subsp. *lactis* Bb12 was obtained from Chr. Hansen (Bayswater, Victoria, Australia). The organism was grown in MRS broth (Oxoid Ltd., Hampshire, United Kingdom) supplemented with filter-sterilized 0.05% (wt/vol) L-cysteine-hydrochloride (Sigma Chemical Co., Castle Hill, Australia) at 37°C using a 1% inoculum. The organism was propagated three times successively, and its presence was confirmed by Gram staining.

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The cells of *B. animalis* subsp. *lactis* Bb12 were concentrated by centrifugation of the broth at 14,000 × *g* for 15 min at 4°C using a Sorvall centrifuge (56), and the cell pellet was washed twice with 0.85% sterilized saline solution. The cell pellet was then resuspended in one-fourth the original volume (5-ml cell pellet added to 15 ml of saline solution). The initial population of bacteria in the suspension was ~2.5×10^{10} CFU/ml.

Microencapsulation of probiotic bacteria. Microencapsulation was carried out using two types of alginate emulsions as per Dianawati and Shah (12). The first formulation contained sodium alginate (at 2.5% [wt/vol] of the emulsion), and the second formulation contained sodium alginate and mannitol (each at 2.5% [wt/vol] of the emulsion). Canola oil containing 0.5% Tween 80 at 10% was used to develop an emulsion system for each formulations. Each emulsion was pasteurized by heating at 70°C for 30 min and cooled to 10°C before *B. animalis* subsp. *lactis* Bb12 was incorporated into the emulsion system (250 ml cells in a total of 1,000 ml of emulsion). Each mixture containing the bacteria was then dropped into 0.1 M $CaCl_2$ solution using a burette to create either Ca-alginate beads or Ca-alginate-mannitol beads of a uniform size. Wet beads of both types were then freeze-dried (freeze-drier model FD-300; Airvac Engineering Pty. Ltd., Dandenong, Australia), with the instrument set to achieve −100 torr of internal pressure before freeze-drying at −88°C, including 44 h of primary freeze-drying and 4 h of secondary freeze-drying. Once the freeze-drying process was completed, two types of dehydrated beads, namely Ca-alginate containing *B. animalis* subsp. *lactis* Bb12 (CAB) and Ca-alginate-mannitol containing *B. animalis* subsp. *lactis* Bb12 (CAMB), were obtained. The samples (CAB and CAMB) were stored for 10 weeks in desiccators with the a_w adjusted to 0.07, 0.1, and 0.2 using NaOH, LiCl, and CH_3COOK, respectively, at 25°C. NaOH (200 g) was added to 45 ml of water (equivalent to 111.1 M) to achieve an a_w of 0.07; LiCl (200 g) was added to 113 ml of water (equivalent to 41.7 M) to achieve an a_w of 0.11, and CH_3COOK (200 g) was added to 65 ml water (equivalent to 31.4 M) to achieve an a_w of 0.23. CAB and CAMB samples kept in aluminum foil at room temperature (without a_w adjustment) were used as controls.

Storage at low a_ws at room temperature of 25°C. An equilibrium between a_w of the samples and that of the environment (desiccators) was achieved in 2 weeks, which was considered week 0 of storage. The a_w was measured using a water activity meter (CX-2, serial I/O; Decagon, Pullman, WA). Samples were taken at week 10 of storage for measuring changes in cell envelopes and secondary proteins using FTIR spectroscopy.

Sample preparation for FTIR spectroscopy. Infrared absorption measurements were carried out with an FT-IR spectrometer (IRAffinity-1; Shimadzu Corp., Kyoto, Japan) at room temperature (25°C). Spectra were recorded at a resolution of 4 cm^{-1} and 20 scans in a wave number range of 4,000 to 500 cm^{-1}. A reference spectrum was measured prior to each experiment to correct the background effects of all the spectra recorded. The instrument was purged with nitrogen gas to reduce the interference of water vapor and CO_2 in all the FTIR measurements. The sample preparation was according to Izutsu and Kojima (26) and Sharma and Kalonia (47). Briefly, a 10-mg sample of CAB or CAMB was mixed with 100 mg of dried KBr powder and pressed under a vacuum using 10 tons of hydraulic pressure (JC Hydraulics KBr Beta Press 6010 and 6102; Buck Scientific Inc., East Norwalk, CT) with a 13-mm pellet die (model no. 3000; Specac, Orpington, Kent, Great Britain) to obtain a transparent pellet of CAB or CAMB. Spectra of fresh *B. animalis* subsp. *lactis* Bb12 (harvested after 18 h) were used as controls to observe any change in the frequencies of functional groups of cell envelope proteins [PO_2^-, $(CH_3)_3N^+$, and C-H] and secondary proteins (C-N and N-H) of microencapsulated *B. animalis* subsp. *lactis* Bb12. Samples of fresh bacteria were prepared according to the method of Santivarangkna et al. (45). Briefly, washed cell suspensions were spread onto CaF_2 windows and dehydrated at room temperature in vacuum desiccators containing P_2O_5 for 2 days to reduce any interference of H_2O. Spectra were collected from three batches of samples. Smoothing and normalization of second derivatives of deconvoluted spectra were carried out to develop clearer separation of complex bands using IRsolution software (Shimadzu Corp., Kyoto, Japan).

TABLE 1 Assignment of some bands found in CA, CAM, and SA spectra

Assignment of functional group	Frequency (cm^{-1}) in:		
	CA	CAM	SA
OH stretching	3,210.3 ± 0.3	3,207.2 ± 0.2	3,237.2 ± 0.2
OH deformation	1,257.2 ± 0.3	1,251.0 ± 0.1	1,312.2 ± 0.2
COO symmetric stretching	1,444.2 ± 0.2	1,443.5 ± 0.5	1,419.3 ± 0.3
COO asymmetric stretching	1,609.2 ± 0.2	1,609.2 ± 0.3	1,609.4 ± 0.4
C—O—C asymmetric stretching	1,160.3 ± 0.4	1,162.3 ± 0.3	1,166.2 ± 0.3

The freeze-dried Ca-alginate-mannitol without bacteria (CAM) was compared with Ca-alginate without bacteria (CA) to study any chemical interaction between Ca-alginate and mannitol as the microencapsulant. Na-alginate (SA) powder was used as a control. To examine changes in cell envelopes and secondary proteins of microencapsulated *B. animalis* subsp. *lactis* Bb12, the spectra of CAM and CA were subtracted from those of CAMB and CAB (22; A. Mauerer and G. Lee, presented at the Controlled Release Society German Chapter Annual Meeting, Munich, Germany, 2003). The remaining spectra were then compared with those of the freshly harvested *B. animalis* subsp. *lactis* Bb12. All FTIR measurements were repeated three times.

Determination of Ca-alginate-mannitol, cell envelope proteins, and secondary proteins of microencapsulated *B. animalis* subsp. *lactis* Bb12 by FTIR spectroscopy. An interaction between Ca-alginate and mannitol was ascertained by comparing the peaks of CA and CAM at 3,000 to 3,700 cm^{-1} (broad peak of O-H stretching) and at 1,410 to 1,260 (O-H deformation vibration). Sodium alginate (SA) was used as a control (23, 32). An alteration of COO^- stretching symmetric vibration and COO^- stretching asymmetric vibration of alginate can be detected at ~1,420 to 1,300 and ~1,615 to 1,550, respectively (3, 23, 32, 41).

An investigation of cell envelopes of bacteria in CAMB and CAB was carried out in the frequencies in the FTIR spectra of ~1,080 cm^{-1} (P═O of PO_2^- symmetric stretching) and ~1,240 cm^{-1} (P═O of PO_2^- asymmetric stretching) (16, 17); ~2,850 cm^{-1} and ~2,925 cm^{-1} (CH_2 symmetric and asymmetric stretching vibrations, respectively) (45, 59), and ~975 cm^{-1} [$(CH_3)_3N^+$ asymmetric stretching vibration] (42). An observation on secondary proteins was carried out by measuring the frequency of amide II at 1,450 to 1,575 cm^{-1} (CN stretching and NH bending) (33, 35).

ESEM study. The morphology of freeze-dried microcapsules (CAMB and CAB) was observed after freeze-drying using an FEI Quanta 200 environmental scanning electron microscope (ESEM). The working distance was 10.2 mm, beam energy was 20.0 kV, spot size was 5.0, magnification was ×500, and pressure was 0.98 torr. All scale bars presented 200.0 μm of microcapsule size. The microcapsule bead was loaded on a double-sided carbon tape put on multiple studs before being examined by SEM.

RESULTS

Ca-alginate and mannitol interaction in the gel bead system. FTIR analysis of CA and CAM was carried out to observe any shift in spectra representing an interaction between alginate and mannitol; thus, its mechanism as a protectant could be predicted. The presence of mannitol may influence the frequency shift indicating its interaction with alginate. In this study, wave number alteration of some functional groups of CA and CAM was identified by comparing the shift in OH stretching and OH deformation, COO^- symmetric and asymmetric stretching, and C-O-C asymmetric stretching of freeze-dried CA and CAM (Table 1). Sodium alginate (SA) was used as a control. The alteration of the functional

TABLE 2 Assignment of some bands of freshly harvested *B. animalis* subsp. *lactis* Bb12

Assignment of functional group	Frequency in fresh Bb12 (cm^{-1})
P═O symmetric stretching	1,077.8 ± 0.2
P═O asymmetric stretching	1,240.4 ± 0.5
$N^+(CH_3)_3$ asymmetric stretching	969.1 ± 0.2
CH_2 symmetric stretching	2,867.0 ± 0.1
CH_2 asymmetric stretching	2,925.9 ± 0.2
Amide II	1,541.2 ± 0.3

groups of alginate was based on references 55, 32, 59). A decrease in frequency from 3,210 (CA) to 3,207 (CAM) and from 1,257 (CA) to 1,251 (CAM) indicated the presence of OH stretching and OH deformation vibration, respectively; meanwhile, SA in powder form showed a higher frequency of OH stretching and OH deformation vibration. An alteration to a lower frequency indicated an increase in the strength of the hydrogen bond (23), possibly due to the presence of mannitol. In addition, no obvious difference between COO^- of CA and CAM (in both symmetric and asymmetric vibrations) was detected; meanwhile, SA demonstrated a lower frequency of COO^- than CA and CAM (Table 1). The hydrogen bond increase could be due to the presence of hydroxyl groups of mannitol replacing the moisture availability, as suggested by Aranda et al. (2) and Santivarangkna et al. (45).

This suggests that there was no strong interaction between alginate and mannitol due to the stronger ionic bonding of COO^- of alginate with cations. On the other hand, frequencies of C-O-C asymmetric vibration altered from 1,160 (CA) to 1,162 (CAM), while SA showed a slightly higher frequency of C-O-C stretching vibration.

Cell envelopes and secondary proteins of microencapsulated *Bifidobacterium animalis* subsp. *lactis* Bb12 after freeze-drying and after storage at low a_ws at room temperature. Frequencies of some cell envelope components and amide II of freshly harvested *B. animalis* subsp. *lactis* Bb12, CAB, and CAMB after freeze-drying and after 10 week storage were demonstrated in Tables 2, 3 and 4, respectively. P═O and $(CH_3)_3N^+$ represented the polar site of phospholipid bilayers, while CH_2 was the nonpolar site of phospholipid bilayers. The changes in secondary protein structures were indicated by amide II.

A shift of PO_2^- symmetric stretching vibration of CAB after freeze-drying and after 10-week storage at various low a_ws at 25°C is shown in Table 3, and that of CAMB after the same treatments is shown in Table 4. There was an interaction between PO_2^- of cell envelopes and CAMB, as shown by an alteration to lower frequency (1,043.6) (Table 4) from a value of 1,077.8 for freshly harvested *B. animalis* subsp. *lactis* Bb12 (Table 2), while CAB demonstrated a shift to a higher frequency (1,053.4) (Table 3) than CAMB. Both frequencies of freeze-dried CAB and CAMB were below that of the control, indicating an interaction of PO_2^- of the phospholipid site of cell lipids with the microcapsule substances via hydrogen bond, as suggested by several other researchers (2, 33, 40, 45). The similar behaviors were also demonstrated by asymmetric stretching vibration of the P═O of PO_2^- of freeze-dried CAB and CAMB (Tables 3 and 4, respectively) compared to controls (1,240.4 cm^{-1}; Table 2). This indicates that an interaction via hydrogen bond between PO_2^- of phospholipid bilayers and mannitol was maintained during storage at low a_ws. In contrast, there was less interaction between alginate and PO_2^- of phospholipid bilayers of cell envelopes in CAB kept in aluminum foil after 10 weeks of storage, since the frequencies were higher than those of the control (Table 3).

The choline chain terminus of the cell surface could provide additional information to help characterize the microencapsulated bacterial cell envelopes. An alteration of frequency of $(CH_3)_3N^+$ asymmetric stretching vibration of the choline chain terminus of cells within CAB and CAMB after freeze-drying and after 10 week of storage at various low a_ws is shown in Tables 3 and 4, respectively. After 10 weeks of storage, an increase in frequencies occurred in CAB kept at low a_ws and in aluminum foil. This may be due to the effect of OH of alginate or OH of residual moisture on choline. Loss in the frequency area of $(CH_3)_3N^+$ of choline occurred in CAB kept in aluminum foil. This could be due to an increase in the molecular mobility and chemical reactions along with an increase in a_w during storage, as suggested by Bell and Labuza (4); therefore, an adverse effect on some substances such as choline could not be avoided in freeze-dried bacteria kept in aluminum foil. This may be the reason why our previous study (12) demonstrated low survival of bacteria in aluminum foil after 10 weeks of storage.

CH_2 symmetric and asymmetric stretching vibration of fatty acids of cell envelopes of *B. animalis* subsp. *lactis* Bb12 encapsulated within CAB and CAMB after freeze-drying and after storage is also shown in Tables 3 and 4, respectively. The spectrum of CH_2 of fatty acids, an apolar site of phospholipid bilayers of the bacterium, was detected at ~2,867 and 2,926 cm^{-1}. A frequency increase occurred in CAB during storage at various a_ws and in aluminum foil; a further frequency shift occurred in CAB with a_w of 0.07 and CAB kept in aluminum foil. On the other hand, no increase in frequency was observed in fatty acids of cells kept in CAMB with various a_ws during storage; instead a slight frequency decrease occurred. In addition, frequencies of CH_2 asymmetric

TABLE 3 Assignment of some bands of CAB after freeze drying and after storage

Assignment of functional group	Frequency (cm^{-1}) in: CAB after FD	FD CAB (a_w, 0.07)	FD CAB (a_w, 0.1)	FD CAB (a_w, 0.2)	FD CAB (in aluminum foil)
P═O symmetric stretching	1,053.4 ± 0.4	1,055.5 ± 0.3	1,055.9 ± 0.3	1,057.7 ± 0.3	1,081.6 ± 0.5
P═O asymmetric stretching	1,222.8 ± 0.3	1,231.8 ± 0.3	1,235.9 ± 0.2	1,241.5 ± 0.5	1,244.0 ± 0.5
$N^+(CH_3)_3$ asymmetric stretching	980.2 ± 0.3	996.8 ± 0.3	998.8 ± 0.3	1,002.8 ± 0.3	Undetectable
CH_2 symmetric stretching	2,866.8 ± 0.3	2,902.3 ± 0.3	2,870.6 ± 0.5	2,875.9 ± 0.2	2,906.6 ± 0.5
CH_2 asymmetric stretching	2,924.9 ± 0.3	2,947.5 ± 0.5	2,944.5 ± 0.5	2,946.5 ± 0.5	2,963.6 ± 0.5
Amide II	1,545.8 ± 0.3	1,568.5 ± 0.5	1,569.8 ± 0.8	1,569.8 ± 0.3	1,569.3 ± 0.6

TABLE 4 Assignment of some bands of CAMB after freeze drying and after storage (cm^{-1})

Assignment of functional group	Frequency (cm^{-1}) in: CAMB after FD	CAMB (a_w, 0.07)	CAMB (a_w, 0.1)	CAMB (a_w, 0.2)	CAMB in aluminum foil
P═O symmetric stretching	1,043.6 ± 0.5	1,043.8 ± 0.2	1,042.3 ± 0.3	1,043.2 ± 0.3	1,047.4 ± 0.4
P═O asymmetric stretching	1,218.5 ± 0.5	1,226.5 ± 0.5	1,225.2 ± 0.3	1,226.2 ± 0.3	1,227.2 ± 0.3
$N^+(CH_3)_3$ asymmetric stretching	978.2 ± 0.3	979.8 ± 0.3	983.8 ± 0.3	994.7 ± 0.3	995.9 ± 0.2
CH_2 symmetric stretching	2,867.4 ± 0.1	2,854.3 ± 0.6	2,855.5 ± 0.5	2,853.2 ± 0.3	2,849.0 ± 0.0
CH_2 asymmetric stretching	2,923.3 ± 0.2	2,920.7 ± 0.6	2,921.5 ± 0.6	2,920.7 ± 0.6	2,931.5 ± 0.5
Amide II	1,536.4 ± 0.4	1,534.5 ± 0.5	1,537.8 ± 0.3	1,536.5 ± 0.5	1,529.8 ± 0.3 and 1,515 ± 0.1

stretching vibration of fatty acids of CAMB increased after storage in aluminum foil, while those of CAB increased due to storage regardless the presence or an absence of desiccants.

The results of frequency alteration of the secondary protein amide II of *B. animalis* subsp. *lactis* Bb12 within CAB or CAMB after freeze-drying and that after storage at various a_ws at room temperature are shown in Tables 3 and 4, respectively. The control showed a native amide II peak at 1,541 cm^{-1}. Amide II of CAMB after freeze-drying showed lower frequency (1,536 cm^{-1}) (Table 4) than that of the control, while that of CAB after freeze-drying altered to a higher frequency (1,546 cm^{-1}) (Table 3). A further obvious shift of freeze-dried CAB after 10-week storage occurred; the peaks were altered to ~1,569. On the other hand, the bands of CAMB kept at low a_ws were relatively unaltered compared to bands of CAMB after freeze-drying, i.e., at 1,534 to 1,538 cm^{-1}, but frequency decreased in CAMB kept in aluminum foil along with a shoulder formation at 1,515. This alteration to ~1,510 indicates a partially change in secondary proteins from α-helices to β-sheets (50).

Microstructure of microcapsules. The microstructures of CAB and CAMB microcapsules containing *B. animalis* subsp. *lactis* Bb12 after freeze-drying are shown in Fig. 1a and Fig. 2a, respectively, while freeze-dried CAB and CAMB after 10 weeks of storage at an a_w of 0.07 are shown in Fig. 2a and 2b. The surfaces of CAB and CAMB microcapsules appeared dense and relatively rough after freeze-drying (Fig. 1a and 2a), with few wrinkles. After 10 weeks of storage, the wrinkles were more obvious (Fig. 1b and 2b), which could be due to residual moisture removal during storage at a_w of 0.07. Incorporation of mannitol into alginate gel might soften the bead surface (Fig. 1b and 2b). The bacteria did not appear on the microcapsule surface, indicating that they were trapped within the matrices. The microstructure of our alginate microcapsules was similar to that of Muthukumarasamy et al. (39) and Chen et al. (7). Gbassi et al. (19) found that probiotic bacteria were randomly distributed in the alginate matrices.

DISCUSSION

FTIR analysis of the CA and CAM was carried out to observe any shift in spectra representing an interaction between alginate and mannitol; thus, its mechanism as a protectant could be predicted. The presence of mannitol may influence the frequency shift indicating its interaction with alginate. Some functional groups, such as OH stretching, OH deformation, COO^- symmetric and asymmetric stretching, and C-O-C asymmetric stretching, have been used to characterize the Ca-alginate interaction with chitosan or xanthan (32, 41).

The formation of a matrix of alginate and divalent cations such as Ca^{2+} has been widely studied (36, 41, 48). Such a matrix is known as an "egg-box" formation of alginate-Ca. This matrix formation is mainly due to the interaction between COO^- of β-D-mannuronic acid and α-L-guluronic acid of alginate and Ca^{2+} via an ionic bond. Our result is in agreement with that of Pongjanyakul and Puttipipatkhachorn (41), who stated that a cross-linking matrix of alginate with calcium ions resulted in an alteration to higher frequencies of COO^- of alginate than a matrix bound with sodium ions. Besides the ionic bond, a partial covalent bond between calcium and oxygen atom of C-O-C groups of alginate occurs (41); this interaction might cause the difference in C-O-C frequencies of CA and CAM due to the presence of mannitol. In addition, an alteration of the COO^- stretching peak to lower frequencies was observed owing to xanthan gum incorporation into CA (41). However, our result demonstrated no obvious difference in frequency alteration of COO^- of CA and CAM, indicating that OH of mannitol has a less important role in interacting with COO^-. Lack of COO^- interaction between CA and mannitol could be due to the effect of an ionic bond between COO^- of alginate and Ca^{2+}, forming a strong cross-link (36). A decrease in OH vibration and OH deformation frequencies due to mannitol inclusion is in agreement with the work of Hesse and others (23) and Santivarangkna and others (46), who stated that a stronger hydrogen bond was indicated by a shift to lower vibrational frequencies. However, the presence of bacteria influenced the interaction between functional groups of Ca-alginate and mannitol.

FTIR spectroscopy has been used to determine the chemical components such as lipids, proteins, and polysaccharides of microorganisms (51). FTIR spectroscopy permits us to study the molecular structures of colonies or even single cells *in situ* without any additional reagents or stains (3, 40, 46). A second derivative method based on mathematical analysis has been applied to improve the level of separation of molecular spectra; thus, specific peaks, such as P═O of PO_2^- of phospholipid bilayers, C-H of fatty acids, $(CH_3)_3N^+$ of choline, and secondary proteins, can be more easily recognized (29).

PO_2^- of phospholipid bilayers has been commonly used to recognize any interaction with other substances through hydrogen bonds (2, 45). Variability in frequencies after storage at low water activities could be due to the influence of OH of residual unbound water in CAMB beads, besides OH from mannitol; thus, interaction with PO_2^- can be varied. However, all PO_2^- frequencies of cell envelopes of CAMB showed lower frequencies than the control. Frequency changes of PO_2^- bands to a lower wave number suggested an increase in hydrogen bonds due to the presence of sugars (45). In regard to the lack of interaction between alginate and phospholipid bilayers of CAB, our result is in agreement with

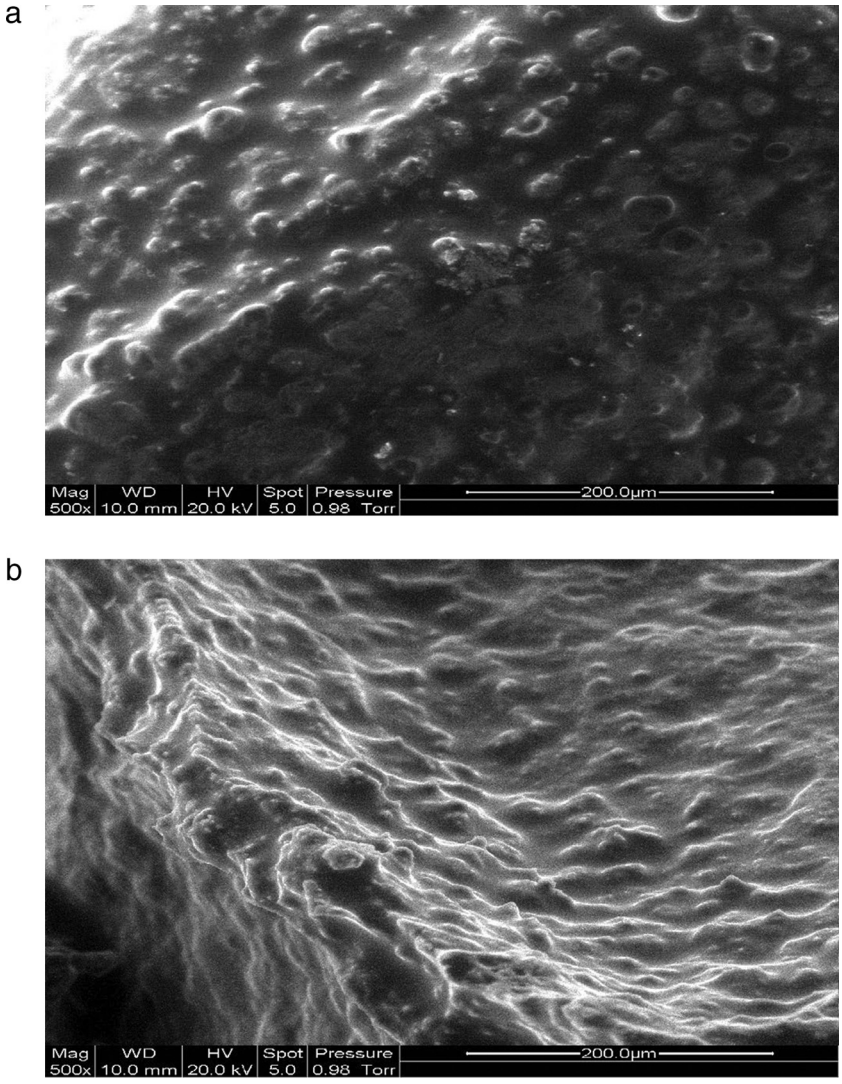

FIG 1 (a) CAB after freeze-drying; (b) freeze-dried CAB after 10 weeks of storage at an a_w of 0.07.

that of Oldenhof and others (40). Those authors suggested that high-molecular-weight polysaccharides such as maltodextrin (or alginate) were unable to interact with P═O of PO_2^- of phospholipid bilayers. Alginate interacted with mannitol through hydrogen bonds in the absence of probiotic bacteria (Table 1). However, in the presence of bacteria, mannitol interacted with PO_2^- of cell envelopes instead of Ca-alginate, as shown by the difference in frequencies between CAB and CAMB (Tables 3 and 4).

An interaction of choline of cell envelopes with OH groups was indicated by an increase in frequencies of $N^+(CH_3)_3$ (5). A peak at ~970 cm^{-1} has been identified as asymmetric stretching for $(CH_3)_3N^+$ of lipids (42, 51). Grdadolnik and Hadzi (20) found a similar trend in the alteration between hydration and sugar incorporation on choline's trimethylammonium group. They found that sugars, including mannitol, could replace water molecules during dehydration and stabilize the polar head region during storage at room temperature at a controlled a_w. CAMB kept at low a_ws appears to be effective in stabilizing $(CH_3)_3N^+$ of phospholipid bilayers, while after storage in aluminum foil, an increase in frequency along with a broader peak was observed, likely due to a strong effect of unbound water. The interaction of surface-exposed choline with water molecules or sugars caused a shift to higher frequencies due to the sensitivity of $(CH_3)_3N^+$ asymmetric stretching to dipolar interaction (42). Mannitol kept at a higher a_w (such as in aluminum foil) might contribute to the plasticizing effect and cause conformational changes in polar site of phospholipids due to an increased level of OH (53).

Fatty acids of phospholipid bilayers have also been used to recognize changes in cell envelope characteristics (10). Vibration of CH_2 of fatty acids of phospholipid bilayers can be determined from the frequency around 2,850 cm^{-1} and 2,930 cm^{-1} (51); this can be different due to differences between bacterial strains. Freeze-drying showed no apparent effect on stability of fatty acids of freeze-dried cells within either CAB or CAMB (Tables 3 and 4, respectively). This indicated that CAB or CAMB was effective in maintaining apolar site of lipids due to a protection effect of CAB or CAMB on the surface site of phospholipid bilayers, as shown by PO_2^- and choline frequency alteration. However, long-term storage of CAB at room temperature at low a_ws or in aluminum foil demonstrated an alteration to higher frequencies. The peak alter-

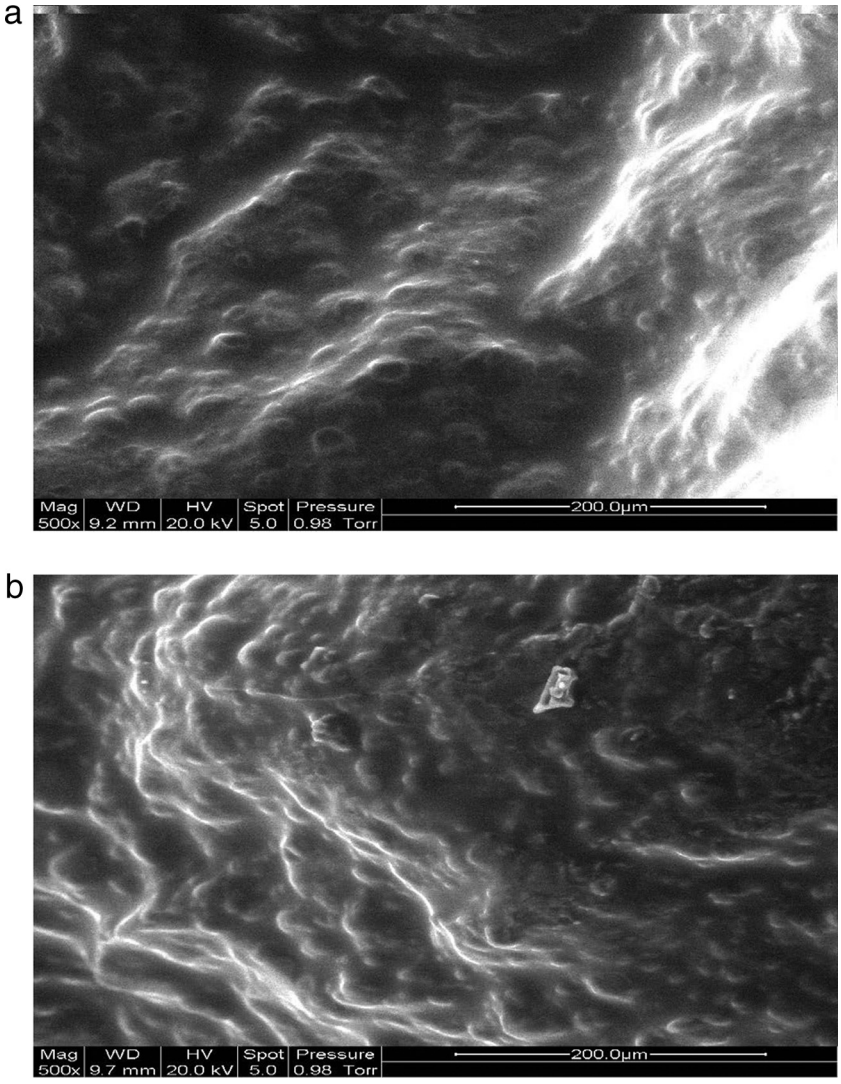

FIG 2 (a) CAMB after freeze-drying; (b) freeze-dried CAMB after 10 weeks of storage at an a_w of 0.07.

ation to higher frequencies suggests a melting of lipid acyl chains along with a gel-liquid crystalline transition (42). It appears that Ca-alginate was not able to preserve the fatty acid site of phospholipid bilayers of freeze-dried bacterial cell envelopes during storage at room temperature, even at low a_ws. Conversely, storage of CAMB at low a_ws and in aluminum foil resulted in lower frequencies than that of the control. The presence of sugars (including mannitol) which interact with polar site of lipids during dehydration inhibits the interior apolar site of lipid changes, such as lipid phase transition and fusion (43), whereas storage at low a_ws maintained the glassy state of sugars (31). Hence, lipid stability could be preserved. The alteration to a lower frequency (compared to the control frequency of 2,867) could be due to the presence of the membrane proteins and glycolipids as a constituent of cell envelopes (45), which became more obvious on water removal.

This study used amide II band to examine the changes in secondary proteins instead of amide I, which is commonly examined by FTIR (18, 40). The use of amide I is unreliable compared to amide II, as the C═O stretching vibration of alginate interferes with amide I bands (35). Amide II bands represent 60% N-H bending and 40% C-N stretching (51). Any changes in amide II bands represent changes in secondary proteins, as reported by Carpenter and Crowe (6), Leslie and others (33), and Marcotte and others (35). The amide II band alteration indicated a change in secondary protein structures, such as a decrease in the number of native α-helices and an increase in the number of β-sheets (50). Encapsulation of the cells within alginate fortified with mannitol was able to preserve the native conformation of proteins of the cells during freeze-drying and during storage at room temperature at low a_ws. This result was in agreement with that of Leslie and others (33), Garzon-Rodriguez and others, (18) and Thomas and others (53). In contrast, CAB might undergo a failure in protecting secondary proteins of freeze-dried bacteria during long-term storage at room temperature, as indicated by an alteration to higher frequencies (from 1,538 cm^{-1}); this phenomenon appeared to be independent of a_w. High-molecular-weight carbohydrates have been found to be ineffective in retarding protein unfolding during lyophilization (18). This result showed the importance of mannitol incorporation in preserving secondary protein conformation of probiotic bacteria during storage at

room temperature at low a_ws. This may be the reason why the survival of *B. animalis* subsp. *lactis* Bb12 in CAMB was higher than that in CAB after 10 weeks of storage at a_ws of 0.1 and 0.2 (12). Our previous study showed that the survival of bacteria in alginate-mannitol microcapsules was 82.6% and 82.0% after 10 weeks of storage at a_ws of 0.1 and 0.2, respectively, while bacterial survival in alginate microcapsules without mannitol incorporation was 81.1% and 80.2% after storage under the same conditions.

Conclusions. FTIR study showed an interaction between Ca-alginate and mannitol, mainly between OH of mannitol and C-O-C groups of alginate via a hydrogen bond. However, mannitol tended to interact with cell components when *B. animalis* subsp. *lactis* Bb12 was incorporated; hence, mannitol might act as a protectant instead of an inert bulking substance, like alginate. Mannitol in the alginate system was able to interact with head groups of lipids of cell envelopes of *B. animalis* subsp. *lactis* Bb12. CAMB interacted with P═O of PO_2^- of phospholipid bilayers after freeze-drying and after storage at low a_ws, while CAB was able to protect this functional group only after freeze-drying, not after 10 weeks of storage at low a_ws. CAMB also showed an interaction with the choline head group of lipids and prevented the fatty acids (apolar site of phospholipid bilayers) from gel-liquid crystalline transition. Similarly, CAMB was effective in protecting secondary proteins of the bacteria during freeze-drying and during storage at low a_ws, while CAB failed to protect the cells. In general, Ca-alginate was not effective in protecting cell envelopes and secondary proteins of the probiotic bacteria during freeze-drying and during storage at low a_ws or in aluminum foil. Incorporation of mannitol was required to improve stability of cell envelopes and secondary proteins of the cells.

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Chapter 7: Stability of *Bifidobacterium animalis* ssp. *lactis* Bb 12 encapsulated with casein-based system after spray drying and after storage at room temperature at low a_w

Introduction

The aims of this study were to investigate survival, acid tolerance, bile tolerance, surface hydrophobicity and retention of selected enzymes of *B. animalis* ssp. *lactis* Bb12 coated with casein-based formulation containing glucose and mannitol after spray drying and after storage at 25 °C at low a_w for 10 weeks. Changes in bacterial cell envelopes, secondary protein structures and microstructures of microcapsules were also determined to understand the protection mechanism.

The chapter entitled "Stability of *Bifidobacterium animalis* ssp. *lactis* Bb 12 encapsulated with casein-based system after spray drying and after storage at room temperature at low a_w" by D. Dianawati, V. Mishra and N. P. Shah has been submitted to Food Research International and is being reviewed.

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PART B: DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by (candidate name): DIANAWATI DIANAWATI **Signature:** [redacted] **Date:** 11 March 2013

Paper Title

Stability of *Bifidobacterium animalis* ssp. *lactis* Bb 12 encapsulated with casein-based system after spray drying and after storage at room temperature at low a_w

In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Dianawati Dianawati	70	Designed the experiment Perform the sample analyses Performed statistical analysis using MINITAB 16 Prepared major part of the manuscript
Nagendra P. Shah	25	Contribution to writing of paper and journal submission
Vijay Mishra	5	Contribution to writing of paper

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

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2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to **a)** granting bodies, **b)** the editor or publisher of journals or other publications, and **c)** the head of the responsible academic unit; and
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Stability of *Bifidobacterium animalis* ssp. *lactis* Bb 12 encapsulated with casein-based system after spray drying and after storage at room temperature at low a_w

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Abstract: The aim of this study was to observe some selected properties of *Bifidobacterium animalis* ssp. *lactis* Bb12 coated with emulsion-based formulation after spray drying and after 10-week storage at 25^{o}C at low water activity (a_w) of 0.07, 0.1 and 0.2; storage in an aluminium foil without a_w adjustment was used as control. Mechanism of protection of microcapsules and its microstructure were determined by FTIR and ESEM, respectively. Two types of casein-based systems containing glucose were prepared without mannitol (G) or with mannitol (GM). Survival, acid and bile tolerances, surface hydrophobicity, retention of enzymes such as β-galactosidase, β-glucosidase, hexokinase, pyruvate kinase, lactate dehydrogenase and adenosine triphosphatase were examined. GM provided better protection than G to *Bifidobacterium* after spray drying. Survival, acid and bile tolerance, and SHb retention of spray dried bacteria encapsulated with GM kept at low a_w (0.07) after 10 weeks were 85.2%; 67.4%; 86.7% and 78.6%, respectively. These conditions of storage were effective in improving enzyme stability (from 82.8 to 97.8% depending upon enzymes). The protection effect was due to structural interactions of mannitol with the head-group of phospholipid bilayers protecting fatty acids of cell envelopes and secondary proteins of the bacteria. Presence of mannitol in the protectant showed large size of globular microcapsules; but storage at a_w of 0.07 reduced the size and resulted in the microcapsules becoming more compact suggesting the effect of dehydration.

Keywords: caseins, mannitol, probiotic, low a_w, survival

1 Introduction

Microencapsulation has been recognized as an effective method to improve survival of probiotics (Mortazavian et al., 2007). Among microencapsulating materials, alginate in combination with proteins, sugars and antioxidants is widely used to protect probiotic bacteria (Cui et al., 2006; Gbassi et al., 2009; Xiaoyan & Xiguang, 2009). In spite of its common use, some studies showed ineffectiveness of alginate in maintaining probiotic bacterial viability (Champagne & Gardner, 2001; Champagne et al., 1992; Dianawati and Shah, 2011b). Some other carbohydrates such as maltodextrin and modified starch also provided less protection (O'Riordan et al., 2001; Oldenhof et al., 2005). On the other hand, milk and milk proteins provide better protection as increased survival of probiotics has been reported (Ananta et al., 2005; Corcoran et al., 2004; Zamora et al., 2006). Caseins protected probiotic effectively during spray- and freeze-drying and during storage at room temperature (Crittenden et al., 2006; Heidebach et al., 2010). However, due to the lower costs of spray drying than freeze drying (Peighambardoust et al., 2011), the former is preferred.

Incorporation of mannitol into alginate-based microcapsule was capable of increasing survival of *Bifidobacterium* (Dianawati and Shah, 2011b) due to interaction of sugar alcohols with the polar site of phospholipid bilayers of bacterial cell envelopes (Dianawati et al., 2012; Santivarangkna et al., 2010). Mannitol in probiotic microencapsulation contributes to stability in extreme pH conditions (Telang et al., 2003) protecting probiotic during exposure to high acidity of gastrointestinal tract (Ding & Shah, 2009). However, low survival of *Bifidobacterium* in alginate-mannitol (6.61 log CFU/g) due to freeze drying was inevitable (Dianawati & Shah, 2011b). We hypothesized that incorporation of mannitol into casein-based system would

improve protective effect on *Bifidobacterium* and preserve β-glucosidase, β-galactosidase LDH, PK, HK and ATPase, key enzymes in its metabolic pathways.

The type of encapsulating materials and their interactions with cell envelopes of bacteria are keys to understand the protection mechanism resulting into increase in bacterial survival (Leslie et al, 1995). FTIR spectroscopy is an effective technique to gain precise information about secondary structure of polypeptides and proteins (Grdadolnik, 2002; Kong & Yu, 2007), phospholipid bilayers (Gauger et al., 2002) and therefore, has been used to study interactions between protectant and cell envelopes (Oldenhof et al., 2005; Santivarangkna et al., 2010). Effect of mannitol on stabilization of proteins during drying has been carried out by Izutsu et al. (2002) and Sharma and Kalonia (2004), and during long term storage at room temperature by Han et al. (2007). In addition, the microstructure of microcapsules could be additional information to observe the external appearance of microcapsules. Most studies reported microstructure of microencapsules containing bacteria after freeze drying (Heidebach et al., 2010; Xiaoyan & Xiguang, 2009; Zohar-Perez et al., 2004) and spray drying (Crittenden et al., 2006; Gardiner et al., 2000; O'Riordan et al., 2001), but not after storage in low a_w.

The purposes of this study were to ascertain the effect of a casein-based system containing mannitol on survival, acid and bile tolerances, surface hydrophobicity, and retention of enzymes of *Bifidobacterium animalis* ssp. *lactis* Bb12 after spray drying and after 10-week storage at low a_ws at 25^{o}C. The protective mechanism of microencapsulant and microstructures of microcapsules were determined using FTIR and ESEM, respectively.

2 Materials and Methods

2.1 *Bifidobacterium animalis* ssp. *lactis* Bb12 and their cultivation

Freeze dried pure culture of *B. animalis* ssp. *lactis* Bb12 was received from Chr. Hansen (Hørsholm, Denmark). Cultivation, proliferation and concentration of *B. animalis* ssp. *lactis* Bb12 were carried out as per method of Dianawati and Shah (2011b). The initial population of concentrated bacteria in the suspension was 2.5 x 10^{10} CFU/mL.

2.2 Microencapsulation of *Bifidobacterium animalis* ssp. *lactis* Bb12 and storage at low a_w

Microencapsulation was based on oil-in-water (O/W) emulsion system containing sodium caseinate (6% w/v), vegetable oil (10% w/v) and fructooligosaccharides (FOS) (2% w/v), and supplemented with either D-glucose (6% w/v) (G) or D-glucose (3% w/v) and mannitol (3% w/v) (GM). Chemicals used in the formulation were from Sigma Aldrich Corp. except vegetable oil which was purchased from the local market. The emulsion was prepared by mixing the formulation ingredients using magnetic stirrer (300 rpm) for 1h. The temperature was kept constant at 40^{o}C. For this arrangement approximately 75.96 kJ of energy was used. Each emulsion was heated to 95^{o}C for 30 minutes to develop Maillard substances and cooled to 10^{o}C. The expected emulsion stability was about 81% as reported for casein based systems (Shepherd et al, 2000). One fifth of concentrated *B. animalis* ssp. *lactis* Bb12 cells were added directly to the emulsion system prior to spray drying. Spray drying was conducted using NIRO atomizer (serial no. 1902, Copenhagen, Denmark) with T_i/T_o = 120/50$\pm$5^{o}C. The flow rate was 300 mL/h adjusted by a peristaltic pump (Masterflex Model 7518-00). The GM or G formulation containing the bacteria was continuously stirred using magnetic stirrer immediately before feeding into spray drier to maintain homogeneity, uniform bacterial dispersion and emulsion stability. After completion of spray drying, the powder was directly transferred in desiccators

containing saturated NaOH, LiCl or CH_3COOK to achieve a_w of 0.07, 0.1 and 0.2, respectively. For determination of the selected functional properties after spray drying, samples were taken immediately after spray drying for analysis. The equilibrium reached after 2 weeks was considered as week 0 of the storage; storage was carried out for 10 weeks at 25^oC (Dianawati and Shah, 2011b). Bacteria kept inside aluminum foil without a_w adjustment were used as control.

2.3 Survival after storage, survival in acid and bile environment, and retention of SHb of microencapsulated bacteria

The initial population of *B. animalis* ssp. *lactis* Bb12 in G or GM formulation prior to spray drying was 9.44 log CFU/g and 9.46 log CFU/g, respectively. Survival after storage, in acid and bile environments, and retention of SHb of microencapsulated bacteria immediately after spray drying and storage at different a_ws was carried out according to the method of Dianawati and Shah (2011b); all results are presented in percentage. For example, % survival was calculated as: 100 x [bacterial count after treatment/initial bacterial count].

2.4 Retention of β-glu, β-gal, LDH, PK, HK and ATPase of microencapsulated bacteria

β-glu and β-gal activities were measured as per method of Otieno and Shah (2007). LDH, PK and HK assays were carried out as per method of Anonymous (2010). ATPase assay was carried out according to manufacturer's instruction (Innova-Biosciences, 2005). Retention of enzymes of microencapsulated bacteria after spray drying and after storage was expressed in percentage and calculated as per method of Dianawati and Shah (2011a).

2.5 FTIR spectroscopy

Alteration of wave numbers of some functional groups of cell envelopes and secondary proteins of the bacteria after spray drying and after the storage was observed by FTIR spectroscopy. Measurements were carried out at room temperature (25^{o}C) using an FTIR spectrometer (IRAffinity- 1; Shimadzu Corp., Kyoto, Japan). Solid sample preparation was carried out according to method of Oldenhof et al. (2005) and Dianawati et al. (2012) to produce a transparent pellet of microcapsule containing bacteria. Pellet of GM or G without bacteria was also prepared. Spectra of fresh *B. animalis* ssp. *lactis* Bb12 as a control were collected as per Dianawati et al. (2012). All measurements were repeated three times.

Interaction between casein and mannitol in GM formulation (without bacteria) was determined by observing alteration of wavenumbers in region of 3450-3200 cm^{-1} (-OH stretching vibration) and ~1160-1000 cm^{-1} (C—O—C asymmetric stretching, combination of νC-O and δC-O-H) (Williams & Fleming, 1989; Wolkers et al., 2004). Changes in cell envelopes were observed in the frequencies of ~1240 cm^{-1} (P=O asymmetric stretching of PO_2^-) (Williams and Fleming, 1989; Erukhimovitch et al., 2005; Filip et al., 2008; Davis and Mauer, 2010); 1715-1740 cm^{-1} (carbonyl esters C = O from lipids) (Lewis & McElhaney, 1998; Santivarangkna et al., 2010), 2950-2990 cm^{-1} and ~2925 cm^{-1} (C-H asymmetric stretching of CH_3 and C-H asymmetric stretching of CH_2 of fatty acids), respectively (Davis & Mauer, 2010; Santivarangkna et al., 2010; Williams and Fleming, 1989). Changes in secondary proteins of intact cells were recorded as per the method of Mobili et al. (2009) for determination of amide I of α-helix, β-sheet, β-turn and unordered structures at frequencies of 1620-1700 cm^{-1}; amide II was also observed at 1450-1575 cm^{-1} (Gallagher, 2011; Kong & Yu, 2007; Leslie et al., 1995).

2.6 Morphology of microcapsules by ESEM

Morphology of microcapsules (GM and G) taken immediately after spray-drying and after 10 weeks of storage at low a_w was observed using FEI Quanta 200 environmental scanning electron microscope (ESEM) as per Dianawati et al. (2012). The GM or G powder was loaded on a double side carbon tape put on multiple studs. The number of field viewed was three for each sample to observe the whole matrix as such.

2.7 Statistical Analysis

Results from at least three replications were used to calculate the means. Independent variables were the type of formulation and a_w of storage, whereas dependent variables were survival, acid and bile tolerance; surface hydrophobicity, and retention of observed enzymes of microencapsulated bacteria. Two-way ANOVA was used to analyse any significant difference among the treatments. Multiple comparision tests were perform using Tukey tests to separate out means with significant difference (i.e $P< 0.05$). Pearson correlation was used for testing correlation between survival and enzyme retention. Level of significance was set at 5%. Statistical analysis was carried out using Minitab 16 released in 2010 (Minitab Pty Ltd., Sydney NSW, Australia).

3 Results

3.1 Selected functional properties and retention of some enzymes of *B. animalis* ssp. *lactis* Bb12 after spray drying and after storage

GM was more effective in protecting *Bifidobacterium* after spray drying than G formulation, as indicated by higher survival ($P<0.05$), acid tolerance ($P<0.05$), bile tolerance ($P<0.05$) as well as SHb ($P<0.05$) (Table 1). Decrease in *B. animalis* ssp. *lactis* Bb12 population encapsulated with GM or G formulation after spray drying was less than 1 log CFU/g. Residual

moisture content of spray dried GM and G was 4.9 and 5.4%, respectively; with a_w of 0.26 and 0.30, respectively.

Retention of activity of β-glu, β-gal, HK, LDH, PK and ATPase before and after spray-drying are shown in Table 2. Retention of β-glu after spray drying in GM or G formulation showed no difference; meanwhile GM was superior to G in retaining β-gal of the bacteria after spray drying ($P<0.05$). There was a difference in retention of three glycolytic enzymes after spray drying. The activities of LDH and HK were reduced more than that of PK by shear induced deterioration during spray drying. ATPase retention within GM and G formulation was 97.4% and 91.9%, respectively ($P<0.05$).

Selected functional properties such as survival, acid and bile tolerance, surface hydrophobicity of microencapsulated *Bifidobacterium* after week 10 storage at a_w of 0.07, 0.1 and 0.2 are shown in Table 3. Survival of the bacteria in GM after storage at a_w of 0.07 was the highest (85.2%); whereas that in G formulation kept under the same conditions was 59.0%. Microencapsulated *B. animalis* ssp. *lactis* Bb12 using GM showed higher acid and bile tolerances after 10-week storage compared to G (Table 3). Effect of a_w was also significant at $\alpha=5\%$. At storage a_w of 0.07 and 0.1, bacterial survival in GM formulation increased during exposure to acid or bile environment. GM formulation and storage at a_w of 0.07 also maintained SHb of the cells.

Retention of β-glu, β-gal, LDH, PK, HK and ATPase activities after week 10 storage at 25^{o}C at a_w of 0.07-0.2 is shown in Table 4. Storage of spray dried bacteria encapsulated with GM at 0.07 – 0.2 provided a significant protection on β-glu and β-gal ($P<0.05$). GM or G formulation and a_w of 0.07 to 0.2 showed different effect on retention of LDH and PK; and retention of HK was relatively high regardless of the difference in the formulation and a_w. On the other hand,

different a_w exhibited significant retention of ATPase activity; but the formulation (GM or G) had less effect on the retention. The lowest ATPase retention occurred in the control. In addition, survival of microencapsulated bacteria after spray drying and after the storage was correlated with activities for β-gal (r=0.817; P=0.004); PK (r=0.649; P=0.042) and ATPase (r=0.746; P=0.013), but not with β-glu, LDH, HK (r=0.605, 0.394, 0.289, respectively; all P>0.05).

3.2 Protective mechanism of casein-glucose-mannitol

An interaction between mannitol and casein in GM formulation (without bacteria) observed after spray drying is shown in Table 5. A decrease in –OH stretching vibration of spray dried GM (into 3332.0 and 3231.9) occurred as compared to that of casein (3368.2 and 3250.4) and mannitol (3441.2 and 3292.4). Bands obtained between 1160 and 1000 cm^{-1} represent some functional groups of sugars. The position C—O—C bands of GM formulation is lower (1163.2; wide) than that of mannitol (1165.1; sharp). Similarly, spray dried GM showed lowest band (1026.4) as compared to that of mannitol (1058.8) and casein (1045.4); a decrease from 1123.4 (casein) to 1107.2 (spray dried GM) was also observed. This shifting indicated an interaction between mannitol and casein, which is consistent with decrease in –OH functional groups in the region of 3400-3200.

Freshly harvested *B. animalis* ssp. *lactis* Bb12 (Table 6) was used as a control to recognize any changes of wavenumbers of some of the functional groups of phospholipid bilayers of cell envelopes and secondary proteins of microencapsulated *B. animalis* ssp. *lactis* Bb12 after spray drying and after week 10 of storage at different a_ws (Table 7) compared to those of the fresh cells. Frequency of C = O stretching vibration of freshly harvested *B. animalis* ssp. *lactis* Bb12 was at 1777.7 cm^{-1}; whereas that of P=O of PO_2^- of the bacteria was 1240.4 cm^{-1} (Table 6). Band of C = O of spray dried bacteria changed to ~1745 (Table 7). No obvious C = O

frequency difference between GM and G formulations after storage at low a_ws was noticed. Alteration of asymmetric P=O of either GM or G microcapsules into lower frequencies was also detected after spray drying and after storage (Table 7). Frequencies of asymmetric P=O of spray dried bacterial cell envelopes within GM formulation after storage at 0.07 and 0.1 water activities were lower than those within G formulation. An increase in P=O frequencies occurred when spray dried bacteria was kept at a_w of 0.2 or without controlled a_w regardless of the type of microcapsules. CH_2 asymmetric functional group of fatty acids of spray dried bacteria kept in GM formulation were relatively stable after storage at a_ws of 0.07 and 0.1; whereas that of spray dried bacteria in G formulation changed into higher wave numbers due to storage at a_w of 0.1 or higher (Table 7).

The peaks of amide I and amide II of GM and G microcapsules after spray drying and after storage are shown in Table 7. Amide I bands demonstrated no obvious changes in secondary protein structures of bacteria microencapsulated by GM or G formulation after spray drying. However, wavenumber of amide II of the bacteria in G formulation after spray drying shifted from 1541.2 (Table 6) to 1537.6 (Table 7). Only storage at 0.07 was capable of maintaining the proteins of spray dried bacteria in GM formulation. Conformational changes in secondary protein structures of the bacteria in G formulation were observed after 10 weeks of storage at a_w of 0.1, 0.2 or without controlled a_w.

3.3 Microstructure of microcapsules after drying and storage

ESEM images for powders containing *B. animalis* ssp. *lactis* Bb12 after spray drying are shown in Figures 1(a) – 1(b). Spray dried powders (GM and G) after 10 week of storage at a_w of 0.07 and of the control are shown in Figures 2(a) – 2(d). The clumps of GM microspheres appeared relatively larger (Figure 1a) than those of G microspheres (Figure 1b). After storage at

a_w of 0.07, the clusters of spray dried GM and G powders appeared more prominent (Figure 2a and 2b, respectively). More clumped microspheres appeared in spray dried GM kept at a_w of 0.07 (Figure 2a) than those kept in aluminium foil as shown by an arrow (Figure 2c). Similar behaviour was found in G microcapsules after spray drying (Figure 1b), after storage at a_w of 0.07 (Figure 2b) or in aluminium foil (Figure 2d).

4 Discussion

Survival of spray dried bacteria coated with casein-based system in this study concur with those of Crittenden et al. (2006) and Boza et al. (2004); whereas the effectiveness of mannitol in improving survival of bacteria during the storage was in agreement with Mugnier and Jung (1985). Therefore, casein-based microencapsulation combined with glucose and mannitol was superior to alginate-based system followed by freeze drying (Dianawati & Shah, 2011b). Survival of spray dried *B. animalis* in GM and G formulation was 93.7 and 92.8%, respectively; whereas that of freeze dried *B. animalis* in alginate and alginate-mannitol was only 63.4 and 65.5%, respectively.

Our results of acid tolerance concurred with those of Crittenden et al. (2006); whereas O'Riordan et al. (2001) stated that there was no survival of *Bifidobacterium* after being exposed to pH 2.8 for 3 h using starch as microencapsulating material. This demonstrated that casein-based formulation provided more protection to *Bifidobacterium* than starch. Mannitol might protect the bacteria during exposure to simulated GIT since it does not experience hydrolysis at low pH (Telang et al., 2003), although it can be protonated by strong acid (Boikess et al., 1986). Bile tolerance of microencapsulated bacteria in this study was higher than that reported by Picot and Lacroix (2004) using whey proteins. It could be due to difference in drying temperatures, microcapsules, bifidobacterial strains, and bile conditions. SHb of spray dried *B. animalis*

encapsulated with GM and G was higher than those of Pan et al. (2006); however, it is strain-dependent (Canzi et al., 2005).

Microencapsulation technology developed in our study was effective in retaining β-gal similar to those previously reported for other systems (Vasiljevic and Jelen, 2003; Okamoto et al., 2002); however, those studies used pure enzymes or peptides instead of the enzymes within bacterial cells. Retention of β-gal, PK and ATPase was correlated with survival of *B. animalis* ssp *lactis* Bb12 in both GM and G formulations, but β-glu, LDH and HK were not. A positive correlation between survival of *L. acidophilus* and *B. bifidum* and the activity of β-gal has also been reported by Hekmat and McMahon (1992). Nevertheless, that study observed stability of both bacteria in ice cream at -29^{o}C; the effect of spray drying and storage at room temperature at low a_w on β-gal activity has never been studied. Retention of LDH in trehalose has been observed by Adler and Lee (1999). Li et al. (2011) found that the activities of LDH and ATPase of *Lactobacillus reuteri* after freeze drying were affected by the presence or absence of cryoprotectants (trehalose, sucrose or skim milk), but not for HK and PK. The authors stated that LDH and ATPase could be the "main factor for freeze drying injury" of *L. reuteri*. Our study in regards to the correlation between ATPase and survival of microencapsulated bacteria is in agreement with the result of Li et al. (2011). It suggests the ability of sugars and RSM to preserve the function of cell envelopes and to retain the activity of membrane-bound ATPase. However, it should be underlined that Li et al. (2011) used a different type of microcapsule, drying method, and different species of probiotics; thus the responses of HK, PK and LDH activities could not be expected. Enzyme inactivation is known to be triggered by an increase in water content, a_w or mobility (Yoshioka et al., 1993); it explained why storage without a_w adjustment showed significant decrease in enzyme retention.

Our study applied casein, glucose and mannitol as microencapsulating material. Casein combined with glucose was heated at 90^{o}C for 30 min to produce the high molecular weight melanoproteins known as Maillard reaction products (MRPs) (Hofmann, 1998), which improved the film forming and antioxidant activities (Crittenden et al., 2006; Gu et al., 2010). Due to their high MW (> 50 kDa) (Gu et al., 2010), interactions between the polymers and polar site of phospholipid bilayers of bacteria should be unlikely; it is in agreement with Oldenhof et al. (2005). A complex mechanism initiated by an interaction between carbonyl group of glucose and free amino group of caseins to form MRPs (Lea and Hannan, 1949; Huppertz and Patel, 2013) might reduce chance of glucose to interact with other polar materials. As a result, there might be more chance for mannitol to interact with residual casein through hydrogen bonding.

Decrease in wave numbers of –OH functional group of spray dried GM (Table 1) is in agreement with Wolkers et al. (2004). In conjunction with band alteration of –OH, decrease in wave numbers of some functional groups of sugars of spray dried GM (no bacteria) also occurred as compared to that of mannitol and casein (Table 1) suggesting the presence of interaction between mannitol and polar site of caseins *via* hydrogen bonding (Khwaldia et al., 2004). Decrease in bands of carbohydrate's functional groups due to alteration of hydrogen bonding strength of C-O-H has been elucidated by Wolkers (2004). In fact, high number of proline residues and lack of tertiary structures of caseins results in its tendency to be hydrophobic (Anonymous, 2012). This might prohibit mannitol molecules to fully interact with caseins *via* H-bond. Hence, we hypothesize that mannitol can interact with the polar site of phospholipid bilayers in the presence of bacteria.

Protective mechanism of microencapsulation depends on interaction of sugars with polar site of phospholipid bilayers of cell envelopes of bacteria (Oldenhof et al., 2005; Santivarangkna

et al., 2010). Decrease in wavenumbers of C=O and P=O of cell envelopes of dehydrated probiotic protected with sugar alcohols suggests prevalence of these interactions (Santivarangkna et al., 2010). Spray drying had no effect on bands of P=O present in both GM and G formulations. However, P=O of bacterial phospholipids in GM kept at a_w of 0.07 and 0.1 produced lower wavenumbers compared to G kept at these a_ws suggesting interactions between polar head group of phospholipid bilayers and GM. Residual water removal during a_w equilibrium process to achieve a_w of 0.07 or 0.1 might increased a chance of stronger hydrogen-bonding donors, such as mannitol, to interact with polar site of phospholipid bilayers (Oldenhof et al., 2005).

Decrease in viability of bacteria during dehydration is related to structural changes occurring in protein structures (Leslie et al., 1995) and cell envelopes (Santivarangkna et al., 2007). Band of amide II and CH_2 asymmetric of spray dried *B. animalis* ssp. *lactis* in GM kept at a_w of 0.07 after 10 week of storage were 1543.3 and 2925.5 cm^{-1}, respectively (Table 3), while those of freeze dried *Bifidobacterium* in alginate-mannitol after the same storage conditions were 1534.5 cm^{-1} and 2920.7, respectively (Dianawati, Mishra & Shah, 2012); as a comparison, amide II and CH_2 asymmetric of fresh *B. animalis* ssp. *lactis* was 1541.2 and 2925.9 cm^{-1}, respectively (Table 2). This might explain why survival of spray dried *B. animalis* ssp. *lactis* was higher (85.2% = 7.6 log CFU/g) in GM than that of freeze dried *Bifidobacterium* in alginate-mannitol under a similar storage conditions (77.9% = 4.9 log CFU/g) (Dianawati and Shah, 2011b).

Microstructures of microcapsules prepared with G or GM formulation and storage at 25^{o}C (at a_w of 0.07 and in aluminium foil without a_w adjustment) showed differences in the structures which were similar to spray dried microcapsules containing *Lactobacillus rhamnosus* GG in whey protein and resistant starch as reported by Ying et al. (2010).

5 Conclusion

Casein-based system combined with mannitol protected *B. animalis* ssp *lactis* Bb12 during spray drying and during storage at low a_w for 10 weeks. Storage of microencapsulated *B. animalis* Bb12 using a system containing mannitol at a_w of 0.07 maintained highest viability of cells, increased their acid and bile tolerance as well as maintained the surface hydrophobicity and activities of β-glu, β-gal, LDH, PK, HK, ATPase during 10 week storage. Interactions between mannitol of GM formulation and P=O of phospholipid bilayers protected fatty acids and secondary proteins of *Bifidobacterium* after spray drying and after 10-week storage at a_w of 0.07 (25^{o}C) as demonstrated by FTIR suggesting this role in the protection mechanism. Damage of cell envelope and secondary proteins of *B. animalis* Bb12 was detected when GM or G microcapsules was kept at a_w of 0.2 or in an aluminium foil. GM formulation provided larger clumps of microcapsule than G formulation as shown by ESEM. Storage of microcapsule at a_w of 0.07 resulted in more compact structure of microspheres; the compactness decreased when spray dried GM was kept in an aluminium foil without a_w adjustment. In general, this study demonstrated that GM formulation and a_w of 0.07 ensures high stability of *B. animalis* ssp *lactis* Bb12 observed immediately after spray drying and after storage particularly at low a_w.

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Table 1. Selected functional properties of microencapsulated *Bifidobacterium animalis* ssp. *lactis* Bb12 after spray drying

	Survival	Acid tolerance	Bile tolerance	SHb
GM	93.7±0.032[a]	69.6±0.049 [a]	93.8±0.039 [a]	82.6±0.0 [a]
G	92.8±0.038[b]	68.2±0.033 [b]	86.3±0.046 [b]	75.0±0.024 [b]

GM = Casein-based formulation containing glucose and mannitol
G = Casein-based formulation containing glucose
Means±Standard Deviation with different lowercase superscripts in the same column are significantly different ($P < 0.05$)

Table 2. The effect of GM and G as coating agents on enzyme retention after spray drying

	β-glu	β -gal	LDH	PK	HK	ATPase
GM	94.59±0.099[a]	93.447±0.027[a]	83.848±0.145[a]	92.344±0.048[a]	79.251±0.074[a]	97.426±0.365[a]
G	94.66±0.130[a]	90.465+0.034[b]	83.204±0.255[a]	86.567±0.048[b]	69.221±0.174[b]	91.942±0.221[b]

GM = Casein-based formulation containing glucose and mannitol
G = Casein-based formulation containing glucose
Means±Standard Deviation with different lowercase superscripts in the same column are significantly different ($P < 0.05$)

Table 3. Selected functional properties of microencapsulated *Bifidobacterium animalis* ssp. *lactis* Bb12 after 10-week of storage at low a_ws (25^oC)

		Survival	Acid tolerance	Bile tolerance	Retention of SHb
	0.07	85.192±0.010^a	67.361±0.006^b	86.655±0.008^a	78.591±0.035^a
	0.1	67.994±0.005^b	70.812±0.008^a	79.306±0.006^b	71.249±0.033^b
	0.2	62.962±0.010^c	40.479±0.008^c	66.788±0.006^c	58.437±0.035^{cd}
GM	Control	50.049±0.015^e	20.831±0.047^f	51.93±0.011^e	52.091±0.037^e
	0.07	59.04±0.008^d	38.923±0.013^{cd}	59.749±0.004^d	57.53±0.024^d
	0.1	47.754±0.005^f	34.24±0.005^d	53.157±0.011^e	59.861±0.021^c
	0.2	46.916±0.006^f	23.238±0.004^e	46.342±0.005^f	55.002±0.029^{de}
G	Control	46.008±0.005^f	24.141±0.013^e	46.535±0.008^f	38.603±0.044^f

Control = storage in aluminium foil without a_w adjustment
GM = Casein-based formulation containing glucose and mannitol
G = Casein-based formulation containing glucose
Means±Standard Deviation with different lowercase superscripts in the same column are significantly different ($P < 0.05$)

Table 4. The effect of GM and G as coating agents on enzyme retention after 10-week of storage (25^{o}C)

Microcapsules	a_w	β-glu	β-gal	LDH	PK	HK	ATPase
GM	0.07	97.220±0.099^{a}	87.920±0.012^{a}	95.951±0.434^{a}	91.142±0.071^{a}	97.847±0.197^{a}	94.730±0.732^{a}
	0.1	96.438±0.081^{a}	85.086±0.019^{ab}	88.626±0.321^{b}	87.698±0.098^{ab}	95.897±0.118^{a}	80.149±0.537^{bc}
	0.2	95.520±0.060^{ab}	82.442±0.019^{b}	88.231±0.818^{b}	86.21±0.050^{b}	92.661±0.182^{ab}	84.097±0.234^{b}
	Control	90.447±0.067^{b}	74.723±0.014^{c}	71.824±0.735^{c}	79.101±0.036^{c}	88.802±0.112^{b}	69.264±0.277^{d}
G	0.07	94.803±0.149^{ab}	82.798±0.011^{b}	92.541±0.274^{ab}	88.823±0.068^{ab}	93.178±0.095^{ab}	91.874±0.337^{ab}
	0.1	93.304±0.104^{ab}	82.438±0.007^{b}	87.996±0.734^{b}	85.714±0.058^{b}	92.200±0.118^{ab}	89.222±0.352^{ab}
	0.2	94.615±0.045^{ab}	84.169±0.010^{b}	90.294±0.655^{ab}	87.665±0.060^{ab}	93.849±0.117^{a}	78.005±0.160^{c}
	Control	91.715±0.038^{b}	76.920±0.009^{c}	74.010±0.824^{c}	81.647±0.056^{c}	90.814±0.184^{b}	69.894±0.236^{d}

Control = storage in aluminium foil without a_w adjustment
GM = Casein-based formulation containing glucose and mannitol
G = Casein-based formulation containing glucose
Means±Standard Deviation with different lowercase superscripts in the same column are significantly different ($P < 0.05$)

Table 5. Assignment of some bands of Na-caseinate, mannitol and spray dried microcapsules containing glucose-mannitol (without bacteria)

Substances	Wave numbers (cm^{-1}) (means±SD)		
	-OH	C—O—C asymmetric stretching	Combination of νC-O and δC-O-H
Na-caseinate	3368.20±0.56	-	1123.43±0.81
	3250.37±0.47		1045.40±0.56
mannitol	3441.20±0.66	1165.10±0.19 (sharp)	1058.77±0.42
	3292.40±0.85		
Spray dried GM (no bacteria)	3332.00±0.75	1163.21±0.48 (wide)	1107.23±0.81
	3231.87±0.32		1026.37±0.38

GM = Casein-based microcapsule containing glucose and mannitol

Table 6. Assignment of bands of cell envelopes and secondary proteins of *B. animalis* ssp. *lactis* Bb12 (control)

Bacterial components	Functional groups	Fresh *B. animalis* ssp. *lactis* Bb12
hydrophobic site of cell envelopes	CH_3 asym	2959.67±0.15
	CH_2 asym	2925.90±0.21
hydrophilic site of cell envelopes	P=O asym	1240.37±0.48
Polar/apolar site of cell envelopes	C=O	1777.70±0.30
secondary proteins	α-helix	1651.20±0.19
	β-sheet, turn, unordered	-
	Amide II	1541.17±0.27

*The ± values are the standard deviations of three individual samples

Table 7. Assignment of cell envelopes and secondary proteins of microencapsulated *B. animalis* ssp. *lactis* Bb12 after spray drying and after week 10 of storage (25°C)

Bacterial components	Group	G after SD	G SD 0.07	G SD 0.1	G SD 0.2	G SD foil	GM after SD	GM SD 0.07	GM SD 0.1	GM SD 0.2	GM SD foil
Hydrophobic site of cell envelopes	CH_3 asym	2958.30±0.20	2960.47±0.16	2960.33±0.16	2960.33±0.21	2963.70±0.17	2957.57±0.12	2960.67±0.15	2960.27±0.15	2960.40±0.26	2962.43±0.23
	CH_2 asym	2925.40±0.10	2926.10±0.10	2926.40±0.1	2926.57±0.16	2927.40±0.10	2925.03±0.35	2925.47±0.21	2925.53±0.15	2925.60±0.26	2926.07±0.12
Hydrophilic site of cell envelopes	P=O asym	1237.40±0.30	1237.04±0.21	1237.67±0.21	1238.83±0.35	1239.43±0.26	1237.23±0.12	1236.10±0.10	1236.63±0.12	1238.73±0.25	1238.93±0.31
Polar/apolar site of cell envelopes	C=O	1745.33±0.21	1745.33±0.29	1745.57±0.23	1745.13±0.25	1745.23±0.15	1745.47±0.21	1745.53±0.15	1745.23±0.06	1745.50±0.26	1745.80±0.20
Amide I	α-helix	1655.53±0.38	1654.97±0.25	1657.40±0.2			1653.37±0.15	1650.40±0.26	1654.50±0.30	1659.93±0.31	1654.83±0.25
	β-sheet, turn, unordered				1646.53±0.31	1641.40±0.17					1679.63±0.21
					1669.27±0.15	1687.23±0.35					
Amide II		1537.60±0.22	1542.53±0.25	1536.43±0.32	1530.21±0.10	1549.57±0.21	1542.67±0.25	1543.33±0.25	1535.17±0.40	1532.47±0.32	1528.05±0.20

SD = Spray drying
GM = Casein-based microcapsule containing glucose and mannitol
G = Casein-based microcapsule containing glucose

Microcapsules containing *B. animalis ssp. lactis* Bb12 after spray drying

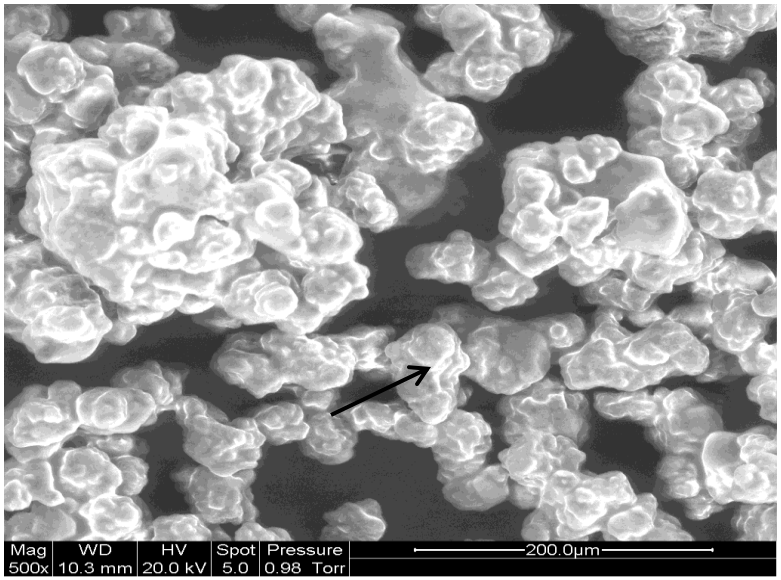

Figure 1a. GM formulation after spray drying

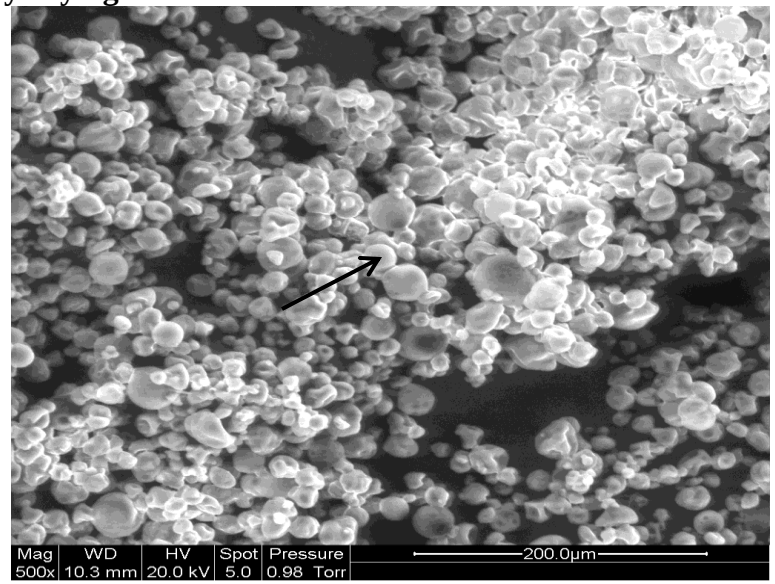

Figure 1b. G formulation after spray drying

Microcapsules containing *B. animalis ssp. lactis* Bb12 after 10 week of storage at room temperature of 25°C

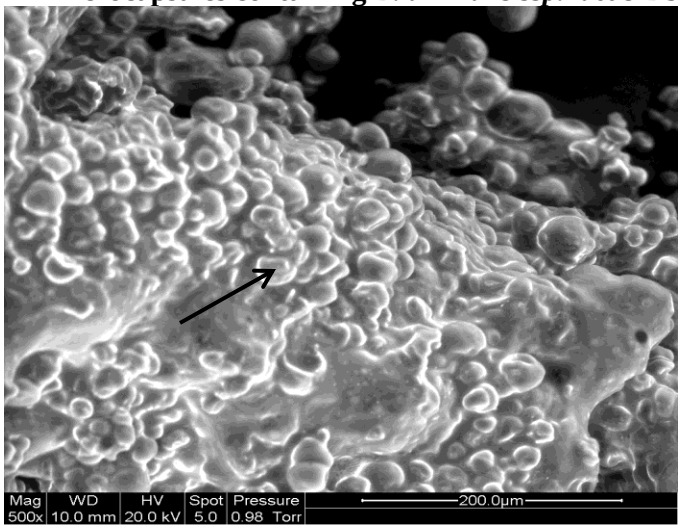

Figure 2a. GM formulation kept at a_w of 0.07

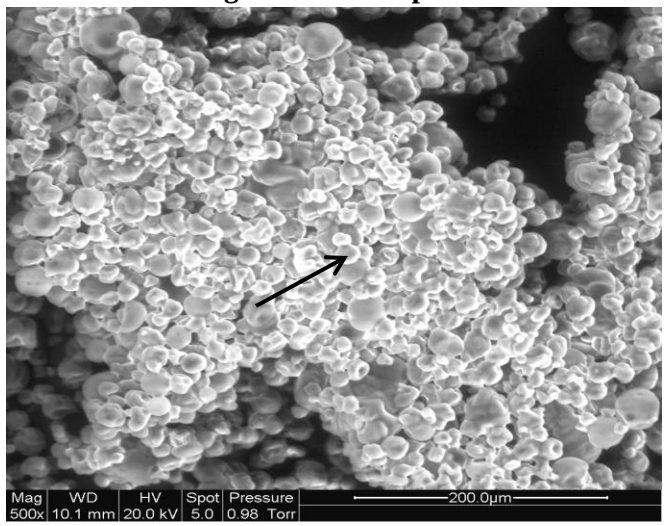

Figure 2b. G formulation kept at a_w of 0.07

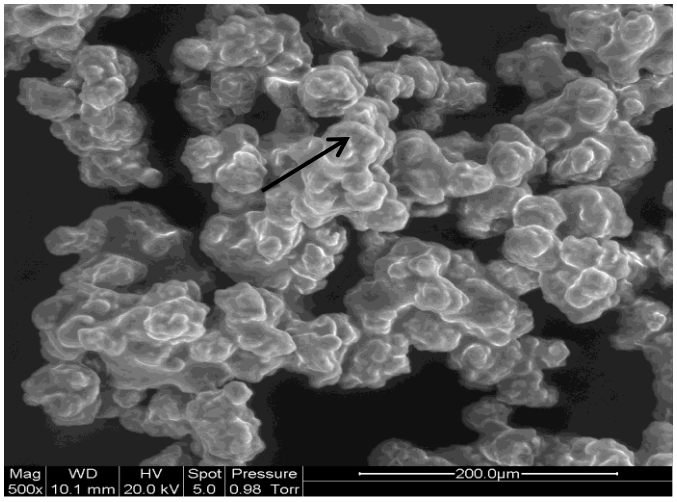

Figure 2c. GM formulation kept in aluminium foil

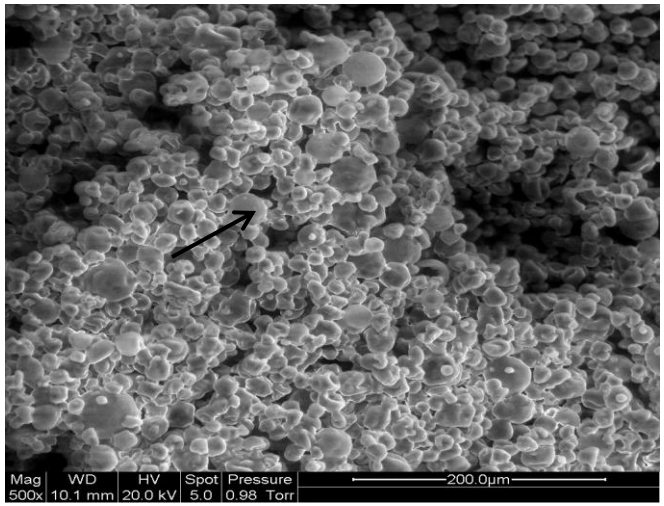

Figure 2d. G formulation kept in aluminium foil

Chapter 8 : Survival of *Bifidobacterium longum* 1941 microencapsulated with proteins and sugars after freezing and freeze drying

Introduction

This experiment examined the effectiveness of sodium caseinate (CAS), whey protein concentrate (WPC), skim milk (SM) or SPI combined with glycerol (GLY), mannitol (MAN) or maltodextrin (MD) in protecting *Bifidobacterium longum* 1941 after freeze drying. Survival, acid and bile tolerance, retention of surface hydrophobicity and of β-glucosidase, of lactate dehydrogenase and of ATPase were determined.

The following paper entitled "Survival of *Bifidobacterium longum* 1941 microencapsulated with proteins and sugars after freezing and freeze drying" by D. Dianawati, V. Mishra and N. P. Shah has been published in The following paper entitled "Survival of *Bifidobacterium longum* 1941 microencapsulated with proteins and sugars after freezing and freeze drying" by D. Dianawati, V. Mishra and N. P. Shah has been published in *Food Research International*, 51, 503–509 (http://dx.doi.org/10.1016/j.foodres.2013.01.022).

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PART B: DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by (candidate name): DIANAWATI DIANAWATI

Signature: [redacted]

Date: 11/3/2013

Paper Title

Survival of *Bifidobacterium longum* 1941 microencapsulated with proteins and sugars after freezing and freeze drying

In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Dianawati Dianawati	70	Designed the experiment Perform the sample analysis Performed statistical analysis using MINITAB 16 Prepared major part of the manuscript
Nagendra P. Shah	20	Contribution to writing of paper and journal submission
Vijay Mishra	10	Contribution to writing of paper

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to **a)** granting bodies, **b)** the editor or publisher of journals or other publications, and **c)** the head of the responsible academic unit; and
5. The original data is stored at the following location(s):

Location(s): College of Health and Biomedicine, Victoria University, Werribee campus, Victoria, Australia

and will be held for at least five years from the date indicated below:

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Signature 3	[redacted]	19/2/2013

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Survival of *Bifidobacterium longum* 1941 microencapsulated with proteins and sugars after freezing and freeze drying

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ABSTRACT

Five types of proteins and three types of sugars were examined for their effectiveness in protecting *B. longum* after freeze drying, including their acid and bile tolerance, surface hydrophobicity, retention of β-glucosidase, lactate dehydrogenase and adenosine triphosphatase. Sodium caseinate 12%, whey protein concentrate 12%, sodium caseinate:whey protein concentrate 6%:6%, skim milk 12%, or soy protein isolate 12% was combined with glycerol (3% w/v), mannitol (3% w/v) or maltodextrin (3% w/v). Fifteen emulsion systems containing sugars were obtained. Concentrated *B. longum* 1941 was incorporated into each emulsion system at a ratio of 1:4 (bacteria:emulsion). All the mixtures were then freeze dried. Water activity (a_w) of freeze dried microcapsules was in the range of 0.30 to 0.35. WPC–CAS GLY provided high stability of bacteria (99.2%) during freezing, while high stability of cells after freeze drying and during exposure to acid and bile environment was achieved when CAS–MAN was applied (97.4%, 81.6% and 99.3%, respectively). High retention of β-glu of freeze-dried bacteria was achieved using SM–MAN as protectant (94.6%). ATPase and LDH were successfully retained by SM–GLY (94.9 and 83.6%, respectively) but there was no significant difference in protection effect using CAS–MAN (93.8 and 82.6%, respectively). Overall, milk proteins were superior to SPI and sugar alcohols provided more protection than MD.

1. Introduction

Microencapsulation of bioactive materials for functional foods has been widely studied. The role of microencapsulation is to protect the bioactive materials such as vitamins, minerals, fatty acids and probiotic bacteria during processing, during storage, during exposure to gastrointestinal tract and to prevent undesirable interaction with food substances in which bioactive is incorporated (Champagne and Fustier, 2007). Depending upon the desired barrier properties and the purposes of microencapsulation (for protection or time-release), water-soluble microencapsulant materials such as polysaccharides have been commonly used (Kuang, Oliveira, & Crean, 2010). On the other hand, microemulsion has been considered as a promising "delivery systems" for distributing bioactive materials and for maintaining beneficial bioactive characteristics (Flanagan and Singh, 2006). The application of both types of materials as probiotic microencapsulants has been reviewed (Anal & Singh, 2007; Chávarri, Marañón, & Villarán, 2012).

Gel-matrix formation of polysaccharides such as Ca-alginate is easy to prepare on a laboratory scale; but scaling it up needs very high processing cost (Anal & Singh, 2007). Alginate-based microencapsulation has been widely studied and proven effective to improve bacterial survival in acidic and bile conditions (Chávarri et al., 2010; Goderska, Zyba, & Czarnecki, 2003; Kim et al., 2008), but other studies revealed that alginate was not always successful as a protectant of probiotic bacteria (Dianawati & Shah, 2011b; Krasaekoopt, Bhandari, & Deeth, 2004; Lee, Cha, and Park, 2004). The use of carbohydrates, such as guar gum, locust bean gum (Ding & Shah, 2009a) or waxy maize starch (O'Riordan, Andrews, Buckle, & Conway, 2001) was also not effective in protecting probiotic bacteria upon exposure to very low pH (2.0–3.0). On the other hand, emulsion system is easy to produce and is designed to improve probiotic survival during exposure to the stomach (Chávarri et al., 2012).

There has been an increasing interest in using emulsion system or its combination with a cryoprotectant due to its effectiveness in protecting probiotic bacteria. For instance, casein improved viability of probiotic after freeze drying and during storage (Heidebach, Forst, & Kulozik, 2010). Encapsulation with whey protein has been found effective in improving survival of *Lactobacillus* sp. and *Bifidobacterium* sp. (Picot & Lacroix, 2004; Weinbreck, Bodnár, & Marco, 2010; Ying et al., 2010) during drying and exposure to acid or bile conditions. High survival of probiotic bacteria in skim milk as a protectant was demonstrated by Heidebach, Forst, and Kulozik (2009), Hsiao, Lian, and Chou (2004), Miao et al. (2008), Ming, Rahim, Wan, and Ariff (2009) and Nanasombat and Sriwong (2007). Incorporation of soy protein isolate (SPI) to alginate to protect probiotic bacteria has also been carried out; however,

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SPI showed no beneficial effect in improving bacterial survival (Andrade, Ferreira, Cardoso, & Cardoso, 2010). Glycerol, mannitol, sorbitol, sucrose, glucose, trehalose, lactose, maltose, and maltodextrin have been applied as bacterial cryoprotectants to protect probiotic bacteria (Coulibaly et al., 2010; Leslie, Israeli, Lighthart, Crowe, & Crowe, 1995; Miao et al., 2008; Nanasombat & Sriwong, 2007; Savini et al., 2010).

It has been stated that survival freeze dried probiotic bacteria would be stable a_w of between 0.07 and 0.1 (Higl et al., 2007; Kurtmann, Carlsen, Skibted, & Risbo, 2009) for long term storage. Our previous study demonstrated that storage for 10 weeks at 25 °C with a_w adjusted to 0.07–0.1 was capable of maintaining high survival of freeze dried or spray dried *L. acidophilus* and *L. lactis* ssp. *cremoris* encapsulated with casein-based emulsion added with glucose and mannitol (Dianawati, Mishra, & Shah, 2013); which was in agreement with the result of Dianawati and Shah (2011b), Higl et al. (2007), Kurtmann et al. (2009), Miao et al. (2008) and Mugnier and Jung (1985). Therefore, a_w of the encapsulated probiotic bacteria after freeze- or spray-drying was not a concern of the present study since low a_w of microencapsulated bacteria could be easily achieved using saturated salt solutions (Rahman, 1995) for storage purposes. Instead, study on the effectiveness of various proteins combined with sugars in protecting some functional properties of probiotic bacteria during freeze drying was still few. Hence, the aim of this study was to evaluate the capability of sodium caseinate (CAS), whey protein concentrate (WPC), SPI, skim milk (SM), and their combination with glycerol (GLY), mannitol (MAN) or maltodextrin (MD) in improving survival, acid and bile tolerance, retention of surface hydrophobicity (SHb), β-glucosidase (β-glu), lactate dehydrogenase (LDH) and adenosine triphosphatase (ATPase) activities of *Bifidobacterium longum* 1941 after freeze drying.

2. Materials and methods

2.1. B. longum 1941 and their cultivation

Pure culture of *B. longum* 1941 received from Victoria University stock culture was grown in MRS broth (Oxoid Ltd., Hampshire, U.K.) supplemented with filter sterilized 0.05% w/v L-cysteine.hydrochloride (Sigma Chemical Co., Castle Hill, Australia) at 37 °C using 1% inoculum. The organism was activated 3 times successively, and its purity was confirmed using gram staining (Ding & Shah, 2009b).

The cells of *B. longum* 1941 were concentrated by centrifugation of the broth at 14,000 ×*g* for 15 min at 4 °C (Vinderola & Reinheimer, 2003), and the cell pellet was washed twice with 0.85% of sterilized saline solution (Ding & Shah, 2009b). The cell pellet was then re-suspended in the same solution (10 mL cell pellet was added to 10 mL of saline solution). The population of bacteria in the suspension was 8×10^{10} CFU/mL. Final concentration of *B. longum* 1941 was 8×10^{10} CFU/mL.

2.2. Preparation of freeze dried microcapsules

Preparation of freeze-dried microcapsules comprised the preparation of O/W emulsion followed by pasteurization, incorporation of bacteria and freeze drying. Sodium caseinate (CAS) and mannitol (MAN) (Sigma Aldrich), glycerol (GLY) (Merck), whey protein concentrate (WPC) (UMT, Spreyton, Tasmania), skim milk (SM) (Murray Goulburn, Victoria), maltodextrin (MD) (GPC, Muscatine, Iowa) and soy protein isolate (SPI) (Pure Supplement Powders Inc., Bentleigh, Victoria) were used as encapsulating materials. CAS (12%), WPC (12%), WPC:CAS (6%:6%), SM (12%), and SPI (12%) were diluted into ultra-pure water (miliQ) (all in w/v). Glycerol (3% w/v), MAN (3% w/v) or MD (3% w/v) was added into each protein. Fifteen combinations between protein and sugar were obtained. Inulin 2% (w/v) was incorporated into each combination; it functioned as prebiotic. Canola oil 10% (w/v) was added to develop an emulsion system. Each formulation was then mixed using magnetic stirrer to achieve the emulsion (300 rpm, 1 h) followed by pasteurization (70 °C, 30 min, under agitation). The formulation was then cooled in ice bath to reach 10 °C and one fifth of concentrated *B. longum* 1941 cells were incorporated (25 mL into 100 mL of emulsion system). Concentration of bacteria in each emulsion was 1.6×10^{10} CFU/mL.

Emulsions containing probiotic bacteria were then poured onto petri dishes and frozen at −18 °C overnight. Viability of *B. longum* within various emulsion systems supplied with sugars after freezing was determined. Freeze drying was carried out as per Dianawati et al. (2013). A control (C) was prepared by dehydrating *B. longum* 1941 using a freeze drier having the same set up and same conditions but without any protectant. The freeze dried cakes were ground manually under aseptic conditions and transferred into sterilized dark bottles. Samples were kept in −80 °C within 7 days for further analysis to minimize the viability changes. Water activity of powder, survival, acid tolerance, bile tolerance, SHb, β-glu, LDH and ATPase of freeze dried bacteria within microcapsules were determined and were compared to those of control.

2.3. Water activity (a_w) measurement

The a_w of freeze dried microcapsules was measured at ambient temperature (20 °C) using a water activity meter (Decagon CX-2 Serial I/O, Washington, USA).

2.4. Survival of microencapsulated bacteria

Survival of the microencapsulated bacteria after freeze drying was carried as per method of Heidebach et al. (2010); detailed as per Dianawati et al. (2013). The percent survival of microencapsulated bacteria after freeze drying ($Sur_{aft\text{-}FD}$) was expressed as below (Higl et al., 2007).

$Sur_{aft\text{-}FD}(\%) = [(CFU_{aft\text{-}FD})/(CFU_{bef\text{-}FD})]\times100$; where $CFU_{aft\text{-}FD}$ and $CFU_{bef\text{-}FD}$ were CFU after freeze drying and CFU/g in the formulation before freeze drying, respectively.

2.5. Acid tolerance

Acid tolerance of microencapsulated bacteria after freeze drying was carried out as per method of Ding and Shah (2009b) and Liong and Shah (2005a); detailed as per Dianawati et al. (2013). The percent survival in the acidic environment of the microencapsulated bacteria after freeze drying ($Sur_{aft\text{-}FD(acid)}\%$) was determined as below:

$(Sur_{acid}\%) = [(CFU_{aft\text{-}FD(acid)})/(CFU_{bef\text{-}FD})]\times100$; where $CFU_{aft\text{-}FD(acid)}$ and $(CFU_{bef\text{-}FD})$ were CFU/g of freeze dried bacteria after exposure to MRS-broth pH 2.0 (2 h) and CFU in emulsion system before freeze drying, respectively. Control was prepared by inoculating 1 g of freeze dried bacteria (un-encapsulated) into 9 mL of MRS-broth pH 2.0 for 2 h.

2.6. Bile tolerance

Bile tolerance of microencapsulated bacteria after freeze drying was carried out according to the method of Ding and Shah (2009b), detailed as per Dianawati et al. (2013). The percent survival in bile environment of microencapsulated bacteria after freeze drying ($Sur_{bile}\%$) was determined as below:

$(Sur_{bile}\%) = [(CFU_{aft\text{-}FD(bile)})/(CFU_{bef\text{-}FD})]\times100$; where $CFU_{aft\text{-}FD(bile)}$ and $(CFU_{bef\text{-}FD})$ were CFU/g of freeze dried bacteria after exposure to MRS-bile pH 5.8 (8 h) and CFU in emulsion system before freeze drying, respectively.

2.7. Surface hydrophobicity

The SHb measurement was carried out according to the method of Vinderola and Reinheimer (2003) and Riveros, Ferrer, and Borquez (2009).

The SHb retention was measured by comparing the SHb after freeze ($SHb_{(aft\text{-}FD)}$) with SHb before freeze drying ($SHb_{(bef\text{-}FD)}$), the result was expressed in percent. All SHb experiments were repeated 3 times for statistical analysis.

2.8. Cell extraction for enzyme assays

0.4 M of sodium phosphate buffer was used to release 1 g of microcapsules followed by centrifugation (14,000×*g*, 30 min, 4 °C). The released bacteria were then dissolved in 9 mL of 100 mmol/L of sodium phosphate buffer (pH 7.0) for preparation of β-glu assay; 0.2 M Tris–HCl (pH 7.3) for preparation of LDH assay; or 0.5 M Tris (pH 7.5) for preparation of ATPase assay. Cell disruption was carried out using a sonicator (2×2 min, 50% duty cycle, output 5). Centrifugation was carried out at 38,000 ×*g* (20 min) (Germain, Toukourou, & Donaduzzi, 1986). The supernatant was collected and filtered (0.45-μm millipore filter) (Izutsu, Yoshioka, & Terao, 1993) for enzyme analysis. All were performed at 4 °C.

2.9. β-Glucosidase assay

β-Glu was determined as per Otieno and Shah (2007). Retention of β-glu activity after freeze drying was calculated as = {($E_{aft\ FD}$)/($E_{bef\ FD}$)}×100%; where $E_{aft\ FD}$ = enzyme activity after freeze drying; $E_{bef\ FD}$ = enzyme activity before freeze drying.

2.10. Lactate dehydrogenase assay

LDH was determined as per Germain et al. (1986). Retention of LDH activity after freeze drying was calculated as described above (Section 2.9).

2.11. ATPase assay

ATPase assay was carried out according to manufacturer's instruction (Innova-Biosciences, 2005). Retention of ATPase activity after freeze drying was calculated as described above (Section 2.9).

2.12. Statistical analysis

A randomized full factorial design was applied. Survival, acid tolerance and bile tolerance were repeated 3 times in duplicate (n = 6) (Ding & Shah, 2009b; Otieno, Shah, & Ashton, 2007); while SHb and enzyme activities were repeated three times. A two-way analysis of variance (ANOVA) was used to ascertain which factor had a significant effect and to examine whether there was an interaction between factors. Difference between means was determined by Tukey's HSD test. All statistical analyses were carried out using Minitab 16.

3. Results

Combination of proteins and sugars showed varying effect on percent survival of bacteria after freezing (between 88% for CAS–MD and 99.2% for WPC–CAS–GLY) (Fig. 1). SM resulted in similar survival after freezing (96.1–96.6%). Combined with various proteins, a less protection effect during freezing was shown by MD as compared to that of GLY and MAN. CAS–MAN, SPI–MAN, WPC–CAS–GLY and WPC–CAS–MAN contributed to significant protective effects on bacterial survival after freezing (97.5–99.2%). SPI provided good protection on freezing when it was combined with MAN and MD.

Protective effect of various proteins and sugars on survival after freeze drying varied (between 73.4% for SPI–GLY and 97.4% for CAS_MAN) (Fig. 2). Survival of *B. longum* 1941 after freeze drying protected with CAS–MAN and WP–CAS–GLY was similarly high (97.4 and 97.2%, respectively; Fig. 2). SM showed almost similar effect on bacterial protection regardless of the type of sugars; these results were also not

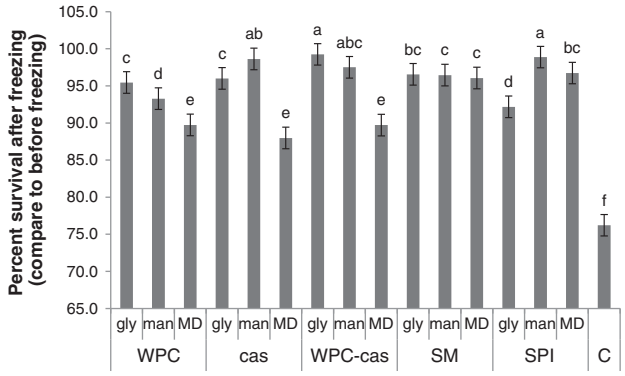

Fig. 1. Viability of *B. longum* 1941 after freezing; CAS = sodium caseinate; WPC = whey protein concentrate; SPI = soy protein isolate; SM = skim milk; GLY = glycerol; MAN = mannitol; MD = maltodextrin.

statistically different with that of using WPC–CAS–MAN and CAS–GLY. CAS, WPC–CAS and SM were more effective in protecting bacteria during freeze drying than WPC and SPI, however, WPC–GLY showed high survival (Fig. 2). The effect of sugars varied. In general, mannitol and glycerol appeared more effective than MD.

Water activity of freeze-dried cakes containing *B. longum* 1941 varied depending on the type of proteins and sugars (Fig. 3). Combination between proteins and sugars had a significant effect on a_w of powder (P = 0.001). The application of sugars on each protein showed no significant difference, but glycerol had a tendency to increase a_w compared to MAN and MD. In general, all a_w of microcapsules was above 0.3.

Freeze-dried bacterial survival in acidic environment (MRS broth pH 2.0, 2 h exposure) was significantly affected by proteins and sugars (P<0.0001). Acid tolerance of freeze-dried *B. longum* 1941 in CAS–GLY, CAS–MAN, WPC–CAS–MAN and SM–MAN was relatively high (81.4, 81.6, 80.0 and 79.4%, respectively) (Fig. 4). Effectiveness of SPI in protecting bacteria during exposure to low pH was low as compared to milk proteins; similarly, MD was also less effective than MAN and GLY. Survival of control (free bacteria) after 2 h exposure to acid environment at pH 2.0 was only 10.8% (viability = 1.2 log CFU/g). This demonstrated the importance of microencapsulation of bifidobacteria.

Survival of freeze-dried *B. longum* 1941 protected by milk proteins combined with GLY or MAN was relatively higher during 8 h exposure to MRS broth containing 0.3% taurocholic acid than that protected by SPI–GLY (Fig. 5). CAS–MAN and WPC–CAS–GLY provided a remarkable effect; whereas WPC–GLY contributed to a similar protective effect with WPC–CAS–GLY but was significantly different from CAS–MAN (P = 0.006). WPC–MAN and WPC–MD appeared less effective in protecting bacteria from bile environment compared to CAS–MAN,

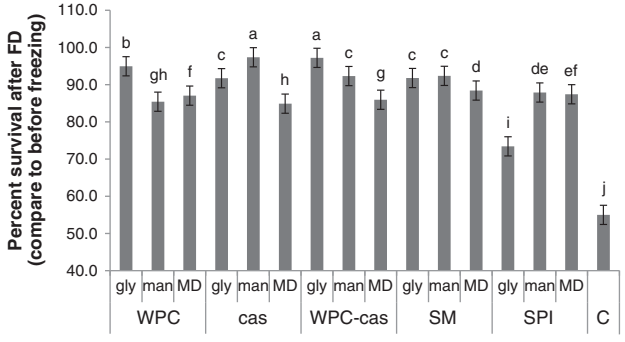

Fig. 2. Survival of freeze dried *B. longum* 1941 (%).

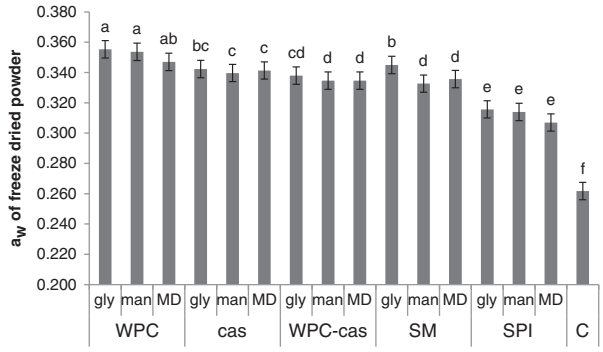

Fig. 3. a_w of freeze dried powder containing *B. longum* 1941.

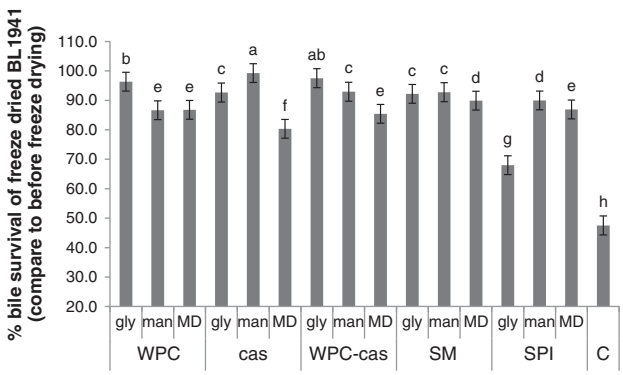

Fig. 5. Retention of freeze dried *B. longum* 1941 in bile environment (taurocholic acid 0.3%; pH 5.8; 8 h).

CAS–GLY or WPC–CAS–MAN and WPC–CAS–GLY. SPI–GLY was the least effective encapsulating materials as compared to other combinations.

Type of proteins, sugars and their combinations showed no significant effect on SHb retention of freeze-dried *B. longum* 1941. The range of SHb retention was relatively high i.e. between 82.8% (WPC–MD) to 94.9% (SM–MD and SM–MAN) (Fig. 6) and was not significantly different with that of control. It demonstrated that SHb of the bacteria was not significantly affected by freeze drying process.

Freeze dried *B. longum* 1941 showed varying levels of enzyme retention due to the use of proteins and sugars as cryoprotectans. β-Glu retention was significantly influenced by the type of proteins ($P<0.0001$), the type of sugars ($P<0.0001$) and their combinations ($P=0.026$). SM, regardless of the type of sugars, had an excellent effect in protecting β-glu, LDH and ATPase of *B. longum* 1941 during freeze drying (Figs. 7, 8 and 9, respectively). SPI and WPC contributed low protection on β-glu and LDH, moreover when MD was incorporated. Meanwhile, CAS, WPC–CAS and SM combined with MAN and GLY were capable of protecting ATPase effectively as compared to SPI; meanwhile MD showed a less protection effect on enzyme retaining (Fig. 9).

4. Discussion

Freezing is the most critical process which can cause damage to bacteria and protein denaturation (Bedu-Addo, 2004; Thammavongs, Corroler, Panoff, Auffray, & Boutibonnes, 1996), but its negative effect can be counteracted by applying proteins and sugars as encapsulant. pH changes can occur drastically during freezing (Pikal-Cleland, Rodríguez-Hornedo, Amidon, & Carpenter, 2000); the presence of calcium and phosphate in milk proteins (Ming et al., 2009) or proteins themselves as amphoteric molecules increase the survival rate during freezing due to their buffering capacity (Ugwu & Apte, 2004). Protective effect of microencapsulant on probiotic bacteria after freezing was in agreement with the result of Tsen, Huang, Lin, and King (2007). Although the authors used Ca-alginate and carrageenan as encapsulant materials instead of proteins, it was proven that macromolecules were capable of protecting probiotic bacteria during freezing as compared to free bacteria. However, matrix based on proteins or polysaccharides could be devastated during freezing due to the formation of larger ice crystals induced by freezing at higher temperatures (Bedu-Addo, 2004; Tsen et al., 2007); it might be the reason why decrease in viability still occurred after freezing at −18 °C (Fig. 1).

Destabilization of cell proteins and fusion of membrane liposomes can occur during freeze drying (Bedu-Addo, 2004). Water removal might increase van der Waal's interaction of non-polar site of phospholipid bilayers of cell membrane resulting in lateral phase transition (Crowe, Crowe, Carpenter, & Wistrom, 1987). Microencapsulating materials protected the cells from such devastations. Survival of *B. longum* 1941 after freeze drying depended on the type of proteins and their combination with sugars. The effectiveness of caseins as a probiotic bacterial protectant could be due to their high number of proline residues (hydrophobic) which cause caseins tend to be insoluble in water; whereas phosphate group esterified to serine residues promote a polar site of caseins (Livney, 2010). GLY and MAN as sugar alcohols appeared more effective than MD as cryoprotectant. It was due to their hydroxyl group allowing them to interact with polar sites of phospholipid bilayers of bacterial cell envelopes (Dianawati, Mishra, & Shah, 2012; Santivarangkna, Naumann, Kulozik, & Foerst, 2010). Compounds with three or six carbon atoms such as glycerol and mannitol appeared more effective in protecting bacteria than compounds with longer carbons such as polysaccharides (Mugnier & Jung, 1985). Maltodextrin might not be able to fully interact with the polar site of cell envelopes due to its high molecular weight; it was in agreement with Oldenhof, Wolkers, Fonseca, Passot, and Marin (2005). Similarly, incorporation of resistant starch to caseins perturbed the consistency of protein-matrix causing decrease in bacterial survival during freeze drying (Heidebach et al., 2010).

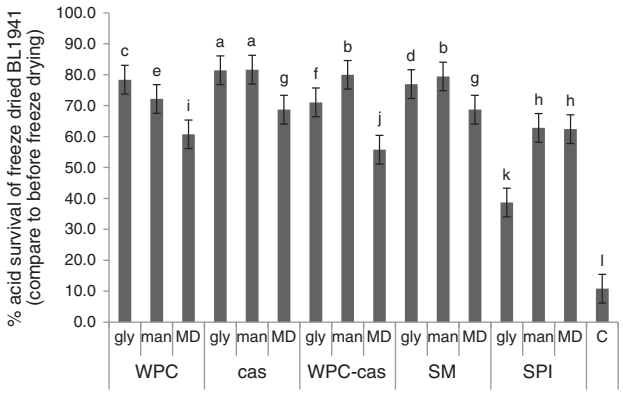

Fig. 4. Retention of freeze-dried *B. longum* 1941 in acid environment (pH 2.0; 2 h).

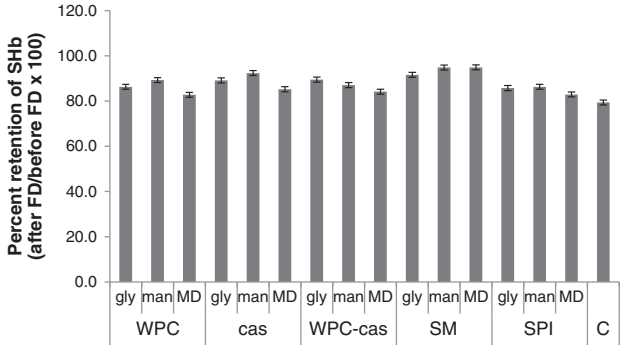

Fig. 6. Retention of surface hydrophobicity of freeze dried *B. longum* 1941.

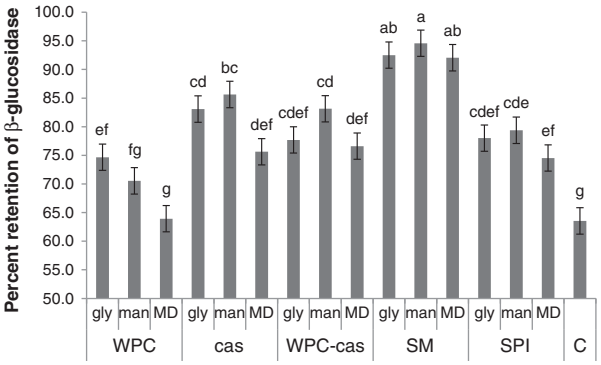

Fig. 7. Retention of β-glucosidase of freeze dried *B. longum* 1941.

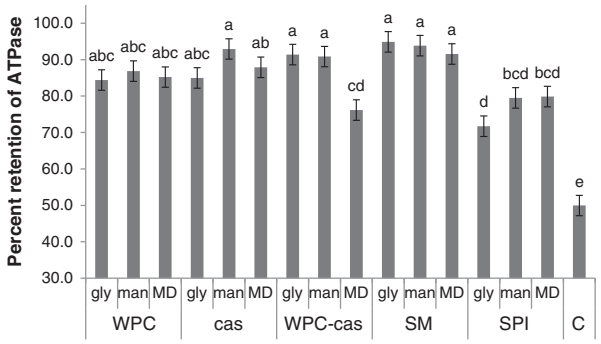

Fig. 9. Retention of adenosine triphosphatase of freeze dried *B. longum* 1941.

Our result demonstrated that a_ws of powder were higher than that of freeze dried *B. lactis* Bb12 protected with SPI–MD (Chavez and Ledeboer 2007); this difference could be due to bacterial strain, different types of proteins and of sugars, proportion of mixture of concentrated cells and the formulation, initial moisture content and set-up of freeze drier. However, storage of samples in powder form in −80 °C less than 7 days for analysis-purpose would not affect survival of microencapsulated bacteria. Tsen et al. (2007) demonstrated that storage of *L. reuteri* encapsulated with Ca-alginate gel in −80 °C during 2 weeks had no effect on bacterial viability; it remained stable at 10.57 log CFU/mL (=100% survival). In addition, Pearson correlation showed that there was no correlation between a_w and survival (=0.403 at P-value of 0.14>0.05); thus survival of freeze dried bacteria protected with different types of proteins and sugars was reliable to compare statistically regardless a_w variability. Microcapsules produced by WPC showed higher value of a_w than others (Fig. 3) regardless the type of incorporated sugars. It could be due to chemical structures of WPC. Whey proteins are made up of β-lactoglobulin (the level of which is approximately 50%), which has dominant amino acids such as aspartic acid, glutamic acid and lysine, which are hydrophilic (Gordon, Basch, & Kolan, 1961). On the other hand, the majority of amino acids in casein are proline, which is hydrophobic (Anonymous, 2012). Approximately 2.5 L of gastric juice having a pH of 1.8–2.0 is reported to be secreted in human stomach per day (Charteris, Kelly, Morelli, & Collins, 1998) and probiotic bacteria are expected to survive in such condition. Acid tolerance of freeze-dried *B. longum* 1941 in CAS–MAN and CAS–GLY was higher than that of Saarela et al. (2005). O'Riordan et al. (2001) found that the number of *Bifidobacterium* PL1 coated with modified starch decreased significantly to below 3.0 log CFU/g during 3 h exposure at pH 2.8 demonstrating ineffectiveness of polysaccharides. Protective effect of sugar alcohols on bacterial viability during exposure to low pH could be due to their chemical stability. Mannitol did not experience hydrolysis at low pH environment (Telang, Yu, & Suryanarayanan, 2003), although it was able to be protonated by strong acid (Boikess, Breslauer, & Edelson, 1986). On the other hand, MD can be hydrolyzed into glucose molecules (van der Veen, van der Goot, & Boom, 2005). In general, milk proteins provided good shielding for *Lactobacillus* and *Bifidobacterium* during their transit in acid environment of stomach due to their buffering capacity (Livney, 2010; Picot & Lacroix, 2004; Reid et al., 2005).

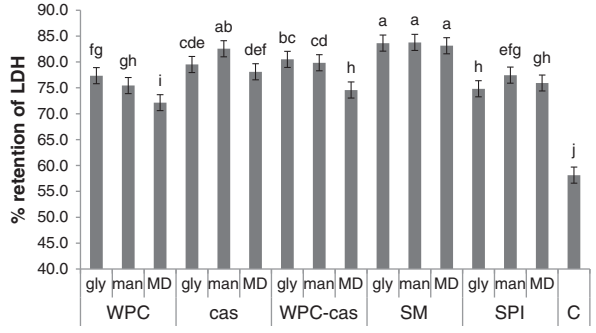

Fig. 8. Retention of lactate dehydrogenase of freeze dried *B. longum* 1941.

Following exposure to acid environment in gastrointestinal tract, bacteria are exposed to the bile environment in the small intestinal tract. Bile in small intestinal tract of the hosts has an important role as a surfactant and helps emulsify fats (Carey, 1992). The actual physiological concentration of human bile in duodenum is in the range of 0.3 to 0.5% (Vinderola & Reinheimer, 2003) and bile salts will be released once fatty meal is present. This natural phenomenon could be harmful for probiotic bacteria as their membrane composition comprises lipids and fatty acids (Liong & Shah, 2005b). However, all bifidobacteria survived well in the medium containing up to 0.5% conjugated bile salts (Noriega, Cuevas, Margolles, De-Los, & Clara, 2006). The presence of bile salts might aid release of bacteria from microcapsules due to its characteristic as a surfactant (Crittenden, Weerakkody, Sanguansri, & Augustin, 2006; Ding & Shah, 2009a). The hydrophobic characteristic of outmost surface probiotic bacteria has an important role in the attachment of bacteria to the surface of the colon of the host (Ali et al., 2009; Vinderola & Reinheimer, 2003). Determination of microbial adhesion using hydrocarbon such as hexadecane or xylene has been suggested by Kiely and Olson (2000) and Rahman, Kim, Kumura, and Shimazaki (2008). Our SHb result was higher than that of Pan, Li, and Liu (2006) using xylene as a hydrocarbon; but almost similar to the result of Vinderola and Reinheimer (2003) using hexadecane as hydrocarbon. SHb of *B. longum* showed a wide range between 51.5 and 97.3% depending upon the strains (Rahman et al., 2008).

Retention of β-glu was studied due to its important role in hydrolyzing the β-1,4-glycosidic linkage in various disaccharides, oligosaccharides, alkyl- and aryl-β-D-glucosides and isoflavone glycosides into their aglycones (Otieno et al., 2007; Yang, Wang, Yan, Jiang, & Li, 2009). LDH is the final enzyme in the glycolytic pathway in which conversion of pyruvate into lactate and oxidation of NADH (or vice versa) occurs in bifidobacteria (Ballongue, 1998). ATPase hydrolyses ATP into ADP and P along with pumping protons out of the intracellular cells (Axelsson, 1998; Matsumoto, Ohishi, & Benno, 2004). This mechanism improves the acid tolerance of gram positive bacteria (Corcoran, Stanton, Fitzgerald, & Ross, 2005).

The protection effect of proteins combined with sugars on retention of β-glu, LDH and ATPase of freeze-dried *B. longum* 1945 varied. In general, SM and CAS contributed higher retention of enzyme activities than SPI, and GLY or MAN was more effective than MD. Our current study demonstrated that microencapsulation using skim milk, regardless of the type of sugars, was more effective in protecting β-glu, LDH and ATPase of *Bifidobacterium* after freeze drying than that using alginate–mannitol (Dianawati & Shah, 2011a). Pearson correlation between survival and β-glu, LDH and ATPase was 0.327 (P=0.234), 0.592

(P = 0.02) and 0.723 (P = 0.002), respectively. It showed no correlation between β-glu activity and viability of microencapsulated bacteria. In contrast, a correlation between survival and either LDH or ATPase was found (both $P < P_{value}$ 0.05). It could be due to roles of LDH and ATPase in major fermentative pathways leading to viability of microencapsulated bacteria.

5. Conclusions

Proteins, sugars and an interaction between proteins and sugars had significant effects on acid tolerance, bile tolerance, and retention of β-glu, LDH and ATPase activities, while SHb was not influenced. SM, CAS and WPC–CAS were more effective in retaining functional properties of freeze dried *B. longum* 1941 than WPC, but overall milk proteins was more effective than SPI. GLY and MAN as sugar alcohols were more effective than MD in protecting those functional properties of freeze-dried bacteria. These observations provided new perspective for probiotic manufacturers to consider the use of various milk proteins and soy proteins combined with relatively cheap sugars (GLY, MAN and MD) as cryoprotectants. The method of preparation of the emulsion was simpler than conventional method of creating gel beads, hence easier for scaling up.

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Chapter 9 : Stability of microencapsulated *Lactobacillus acidophilus* and *Lactococcus lactis* ssp. *cremoris* during storage at room temperature at low a_w

Introduction

The purpose of this study was to study the effect of spray- or freeze-drying and 10 weeks of storage at 25^{o}C on microencapsulated *L. acidophilus* and *L. lactis* ssp. *cremoris* kept in an aluminium foil pouch containing adsorbent and to predict the maximum storage period in order to achieve minimum requirement of viable bacteria (10^7 CFU/g).

The following paper entitled "Stability of microencapsulated *Lactobacillus acidophilus* and *Lactococcus lactis* ssp. *cremoris* during storage at room temperature at low a_w" by D. Dianawati, V. Mishra and N. P. Shah has been published in *Food Research International*, 50, 259–265, 2013 (http://dx.doi.org/10.1016/j.foodres.2012.10.023).

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PART B: DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by (candidate name): DIANAWATI DIANAWATI **Signature:** [redacted] **Date:** 11/3/2013

Paper Title

Stability of microencapsulated *Lactobacillus acidophilus* and *Lactococcus lactis* ssp. *cremoris* during storage at room temperature at low a_w

In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Dianawati Dianawati	70	Designed the experiment Performed the sample analyses Performed statistical analysis using MINITAB 16 Prepared the major part of the manuscript
Nagendra P. Shah	20	Contribution to writing of paper and journal submission
Vijay Mishra	10	Contribution to writing of paper

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
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Stability of microencapsulated *Lactobacillus acidophilus* and *Lactococcus lactis* ssp. *cremoris* during storage at room temperature at low a_w

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ABSTRACT

The effect of freeze drying or spray drying, the use of desiccants to maintain the low a_w and the period of storage (at 25 °C) of *Lactobacillus acidophilus* and *Lactococcus lactis* ssp. *cremoris* on survival, acid tolerance, bile tolerance, retention of surface hydrophobicity and retention of β-galactosidase was studied; an estimation of the maximum storage period was also carried out. Sodium caseinate, vegetable oil, glucose, mannitol and fructooligosaccharides were used as protectant of *L. acidophilus* and *L. cremoris* during freeze drying or spray drying and during subsequent storage. NaOH, LiCl and silica gel were used as desiccants during 10 weeks of storage of microencapsulated *L. acidophilus* and *L. cremoris* kept in an aluminum foil pouch. The results showed that mainly freeze dried *L. acidophilus* and *L. cremoris* kept in foil pouch containing NaOH (a_w 0.07) or LiCl (a_w 0.1) showed higher survival (89–94%) than spray dried bacteria kept under the same conditions (86–90%) after 10 weeks of storage ($P = 0.0005$). Similar results were also showed by acid tolerance, bile tolerance and surface hydrophobicity of freeze-dried or spray-dried *L. acidophilus* and *L. cremoris*. Silica gel was less effective in protecting the functional properties of microencapsulated *L. acidophilus* or *L. cremoris* with percentage of survival between 81 and 87% at week 10 of the storage. However, retention of β-galactosidase was only influenced by a_w adjusted by desiccators ($P<0.05$). Based on forecasting using linear regression, the predicted storage period for freeze dried *L. acidophilus*, spray dried *L. acidophilus* and freeze dried *L. cremoris* kept in foil pouch containing NaOH would be 46, 42 and 42 weeks, respectively; while spray dried *L. cremoris* under LiCl desiccant would require 39 weeks to achieve minimum required bacterial population of 10^7 CFU/g.

1. Introduction

Microencapsulation of probiotic bacteria has been studied extensively, including the use of microencapsulating materials, application of drying techniques and effect of storage at room temperature (Rokka & Rantamäki, 2010). The most two widespread techniques to preserve bacteria are freeze drying and spray drying; but both methods could have any adverse effects on the cells. Freezing and freeze drying steps are critical points for bacterial viability (Coulibaly et al., 2010) while cell damage due to heat and osmotic stress during spray drying might occur (Boza, Barbin, & Scamparini, 2004).

An emulsion-based system, sugars or their combination has been proven effective in improving survival of bacteria during freeze drying (Ming, Rahim, Wan, & Ariff, 2009; Savini, Cecchini, Verdenelli, Silvi, & Orpianesi, 2010) or spray drying (Crittenden, Weerakkody, Sanguansri, & Augustin, 2006). Mannitol was proven effective in protecting bacteria during storage at room temperature (Ndoye, Weekers, Diawara, Guiro, & Thonart, 2007) due to its role as hydroxyl radical scavenger (Efiuvwevwere, Gorris, Smid, & Kets, 1999); its incorporation into emulsion system could improve bacterial stability during drying and during storage.

Storage at room temperature instead of refrigeration temperature has been developed to decrease storage and transportation cost. Storage of dried bacteria at low water activity (a_w) in jars using saturated salt solutions to adjust expected a_w has been proven effective in improving bacterial survival during long storage period (Kurtmann, Carlsen, Skibted, & Risbo, 2009; Ying et al., 2010). However, it was likely impracticable for commercial application. In this study, we developed the storage method to improve the probiotic bacterial survival during storage at room temperature. Aluminum foil pouch with zero permeability rate was used for packing spray- or freeze-dried probiotic bacteria after their a_w had been reduced by keeping them in a jar containing saturated salt solution. The packed solid salt was put in the aluminum foil pouch as an adsorbent to maintain the expected a_w of microcapsule powder. The purpose of this study was to compare some functional properties (such as viability, acid tolerance, bile tolerance, surface hydrophobicity (SHb) and β-glalactosidase activity) of spray dried or freeze dried two sensitive bacteria (*Lactobacillus acidophilus*

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and *Lactococcus lactis* ssp. *cremoris*) kept in an aluminum foil pouch containing adsorbent during long term storage at room temperature and to predict the maximum storage period at such conditions.

2. Materials and methods

2.1. L. lactis subsp. cremoris R-704 and L. acidophilus 2401 and their cultivation

Pure cultures of *L. lactis* subsp. *cremoris* R-704 (*L. cremoris*) and *L. acidophilus* 2401 (*L. acidophilus*) were obtained from Victoria University stock culture. The morphology of microorganisms was confirmed using gram staining (Ding & Shah, 2009). *L. acidophilus* and *L. cremoris* were grown as per method of Riveros, Ferrer, and Borquez (2009) and Kimoto, Nomura, Kobayashi, Mizumachi, and Okamoto (2003), respectively. The bacterial cells were concentrated as per method of Vinderola and Reinheimer (2003), and the cell pellet was washed twice with 0.85% of sterilized saline solution. The cell pellet was then resuspended in the same solution (10 mL cell pellet was added by 10 mL of saline solution). The initial population of concentrated bacteria was 1.1×10^{10} CFU/mL for *L. cremoris* and was 3.1×10^{10} CFU/mL for *L. acidophilus*.

2.2. Preparation of microcapsules

Microencapsulation was performed using two types of oil-in-water (O/W) emulsion system containing vegetable oil (10% w/v), sodium caseinate (6% w/v), fructooligosaccharides (FOS) (2% w/v), D-glucose (3% w/v) and mannitol (3% w/v). All of the materials were from Sigma Aldrich Corp except the vegetable oil which was obtained from the local market. Once the materials had been mixed using magnetic stirrer, the emulsion obtained was heated to 95 °C for 30 min to encourage the formation of Maillard substances. The mixture was then cooled to 10 °C and one fifth of concentrated bacteria (*L. acidophilus* or *L. cremoris*) were added directly to the emulsion system prior to spray drying or freeze drying. The emulsion was spray dried using a modified method of O'Riordan, Andrews, Buckle, and Conway (2001) using a Buchi Mini spray drier B290 Switzerland with Dehumidifier B296 (humidity 86%; temperature −3 °C). The purposed outlet temperature was 50 °C, hence the inlet temperature was set up to 99 °C with peristaltic pump of 27% (feeding rate = 7.1 mL/min) for emulsion system containing *L. acidophilus* and was set up to 80 °C with peristaltic pump of 20% (feeding rate = 3.0 mL/min) for emulsion system containing *L. cremoris*. The powder collected from the product collection vessel was then transferred thoroughly onto petri-disks before keeping them in desiccators. Freeze drying of formulation containing bacteria was carried out in petri disks. Frozen samples were loaded into a freeze drier (model FD-300, Airvac Engineering Pty. Ltd., Dandenong, Australia) which was set to achieve −100 Torr of internal pressure before freezing drying at temperature of −88 °C, 44 h of primary freeze drying, and 4 h of secondary freeze drying. Either freeze-dried or spray-dried products (*L. acidophilus* and *L. cremoris*) on the petri-disks were kept in desiccators (at 25 °C) containing saturated solution of NaOH ($a_w = 0.07$), saturated solution of LiCl ($a_w = 0.11$) or silica gel for 2 weeks to reach the equilibrium between vapor pressure of the samples and that of surrounding. Once equilibrium was achieved, the products were transferred in to aluminum foil pouch and NaOH, LiCl and silica gel (in crystal form) packed inside the semi-permeable membrane were put inside the foil pouch. All the products kept in foil pouch containing desiccants were put in the incubators (25 °C) and was considered as 0th week. Spray- or freeze dried samples kept in foil pouch without any desiccant were used as control. The viability, acid tolerance, bile tolerance, SHb and β-gal activity of microencapsulated *L. acidophilus* and *L. cremoris* was determined immediately after freeze drying or spray drying. The measurement of viability, acid tolerance and bile tolerance was measured every 2 weeks up to 10th week of storage, while SHb and β-gal activity was measured after 10th week of storage.

2.3. Estimation of storage period to achieve the population of 10^7 CFU/g

The estimation of the longest storage period to achieve the minimum requirement of bacterial population (10^7 CFU/g) was carried out after viability of bacteria during 10 weeks of storage had been collected. The forecasting was achieved by applying linear regression as model. R^2 was determined to fit the model with actual data supported by analysis of variance of regression. The linear regression formulation $y = ax + b$ was determined to calculate the estimated period of storage by including the minimum number of population (7 log CFU/g) to the formulation, thus x referring to predicted period of storage was calculated. RMSE was determined to measure the differences between values predicted by a model and the observed values.

2.4. Survival of microencapsulated bacteria

Survival of the microencapsulated bacteria during storage was carried out according to the modified method of Kimoto et al. (2003) and Crittenden et al. (2006). One gram of microencapsulated probiotic bacteria was suspended in sodium phosphate buffer 0.1 M before serial dilutions using peptone water solution (1.5 g peptone in 1 L water) were carried out. Plating was carried out using pour-plate method using M17 agar supplemented with 0.5% glucose for *L. cremoris* and using MRS agar for *L. acidophilus*. Plates were incubated at 30 °C and 37 °C for *L. cremoris* and *L. acidophilus*, respectively, for 48 h before colony counting.

The survival of microencapsulated bacteria after freeze drying or spray drying was expressed as the CFU/g. All survival experiments were repeated 3 times and all analyses were carried out in duplicate ($n = 6$) for statistical analysis (Otieno, Shah, & Ashton, 2007). The percentage survival of microencapsulated bacteria during 10 week storage was determined as the colony forming units (CFU) after 0, 2, 4, 6, 8 and 10 week storage (CFU), relative to the CFU after freeze drying or spray drying, multiplied by 100%.

2.5. Acid tolerance

Acid tolerance of microencapsulated bacteria during storage was carried out according to the method of Ding and Shah (2009). Simulated gastric environment was prepared by adjusting pH of MRS broth to pH 2.0 using 5 M HCl. One gram of encapsulated probiotic organisms was inoculated into 9 mL of acidified MRS broth, incubated at 37 °C for 2 h for both *L. acidophilus* and *L. cremoris* to imitate actual condition of the digestive tract (Kimoto et al., 2003). Subsequent serial dilution and plating was carried as described above. Replication of survival experiments and the percentage survival of microencapsulated bacteria in acid environment were carried out as mentioned in 2.4.

2.6. Bile tolerance

Bile tolerance of microencapsulated bacteria during storage was carried out according to the method of Ding and Shah (2009). MRS broth supplied with 0.3% w/v of taurocholic acid was adjusted to pH 5.8 using 5 M HCl. One gram of encapsulated probiotic was inoculated into 9 mL of the MRS broth, incubated at 37 °C for up to 8 h. Subsequent serial dilution and plating was carried as described above. Replication of survival experiments and the percentage survival of microencapsulated bacteria in bile environment were carried out as mentioned in 2.4.

2.7. Surface hydrophobicity

The surface hydrophobicity (SHb) measurement was carried out according to the modified method of Vinderola and Reinheimer

(2003); Riveros et al. (2009). Probiotic bacteria in microcapsules were suspended in 50 mM potassium phosphate buffer (pH 7.0). The suspension was centrifuged at 14,000 ×*g* for 5 min at 4 °C. Pellets were collected, washed twice and then resuspended with the same buffer. Absorbance at 560 was measured and considered as A_o. One milliliter of the suspension was mixed with 200 μL of n-hexadecane by vortexing for 120 s. Changes in the absorbance of probiotic bacterial suspension was recorded at 560 nm using a UV–vis spectrophotometer. Surface hydrophobicity (SHb%) was determined using the following formula:

$$SHb\% = [(A_o - A)/A_o] \times 100$$

where A_o and A are the absorbances before and after extraction with n-hexadecane, respectively.

The SHb retention was measured by comparing the SHb after 10 weeks of storage with SHb after freeze drying or spray drying; the result was expressed in percent. All SHb experiments were repeated 3 times for statistical analysis.

2.8. β-Galactosidase assay

One gram of microencapsulated *L. acidophilus* or *L. cremoris* was dissolved in 9 mL of 100 mmol/L of sodium phosphate buffer (pH 7.0) for the preparation of β-gal assay. The cells were then disrupted by a sonicator (Unisonics, Pty. Ltd., Sydney, Australia) for 2×2 min at 50% duty cycle, output at 5. Disrupted cells of sonicated suspensions were centrifuged as per method of Germain, Toukourou, and Donaduzzi (1986). The supernatant was then filtered through 0.45 μm millipore filter (Izutsu, Yoshioka, & Terao, 1993) and used as the enzymatic extract. All experiments were performed at 4 °C. The determination of β-gal and calculation of percent retention was carried out as per method of Dianawati and Shah (2011a).

2.9. Statistical analysis

A randomized full factorial design was applied. Drying method (freeze drying and spray drying), type of dessicant (NaOH, LiCl, silica gel and the control) and storage period (from week 0 to week 10) were independent variables; while survival, acid tolerance, bile tolerance, SHb and β-gal retention were dependent variables. This microencapsulation study worked with two species of bacteria namely *L. acidophilus* and *L. cremoris* ssp. *lactis*, but these bacteria were not compared. Results from at least three replications were used for ANOVA analysis. Analysis of variance (ANOVA) was used to ascertain which factor has a significant effect on dependent variables and to examine whether there was an interaction between factors. The analysis was performed using GLM procedure for each parameter. Multiple comparisons between the means of samples were determined using Tukey HSD test. All statistical analysis was carried out using Minitab 16 released 2010.

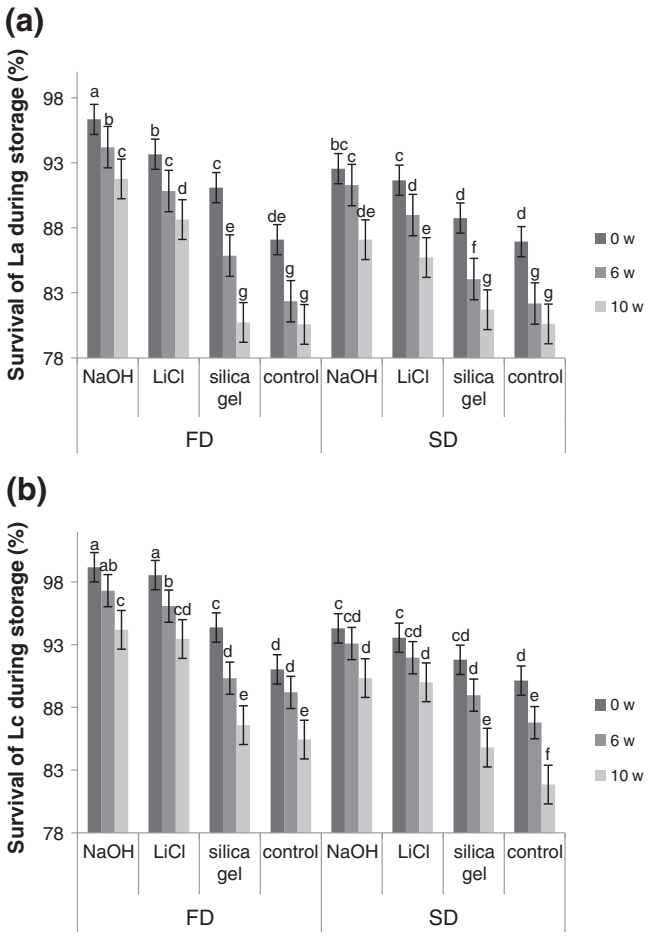

Fig. 1. Survival percentage of microencapsulated *L. acidophilus* (a) and *L. lactis* ssp. *cremoris* (b) during 10 weeks of storage at room temperature. FD = Freeze dried cells; SD = Spray dried cells.

3. Results

Selected functional properties of *L. acidophilus* and *L. cremoris* after spray- or freeze-drying are shown in Table 1. *L. cremoris* appeared more sensitive to drying process than *L. acidophilus*; however, there was no different effect between spray- and freeze-drying on *L. cremoris*. Both dehydrated bacteria are bile tolerant, but *L. cremoris* is acid intolerance. Drying method had no significant effect on β-gal of both dehydrated bacteria, but it influenced SHb of *L. cremoris* (Table 1).

Survival of freeze dried or spray dried *L. acidophilus* kept in foil pouch using various desiccants during 10 week storage at room temperature was shown in Fig. 1a. Analysis of variance showed that freeze dried *L. acidophilus* provided higher survival than spray dried *L. acidophilus* ($P = 0.0005$); the use of NaOH and LiCl as desiccants was better than silica gel or without any desiccants ($P < 0.0001$); while

Table 1
Selected functional properties of *L. acidophilus* and *L. cremoris* after drying.

	Viability (log CFU/g)	Acid tolerance (log CFU/g)	Bile tolerance (log CFU/g)	β-Gal (U/mL)	SHb (%)
FD *L. acidophilus*	$9.39 \pm 0.49^{b*}$	8.55 ± 0.52^{a}	9.12 ± 0.56^{a}	1.590 ± 0.03^{a}	44.7 ± 1.12^{a}
SD *L. acidophilus*	9.71 ± 0.56^{a}	7.95 ± 0.72^{b}	8.66 ± 0.52^{b}	1.586 ± 0.04^{a}	45.3 ± 1.21^{a}
FD *L. cremoris*	8.94 ± 0.37^{A}	4.69 ± 0.51^{A}	8.81 ± 0.44^{A}	1.168 ± 0.12^{A}	22.1 ± 0.03^{A}
SD *L. cremoris*	8.76 ± 0.40^{A}	4.67 ± 0.49^{A}	8.17 ± 0.28^{B}	1.163 ± 0.11^{A}	21.6 ± 0.47^{B}

Means with different superscripts are significantly different ($P<0.05$). Upper case is used for comparing *L. cremoris*; lower case is for comparing *L. acidophilus*. FD = Freeze dried cells; SD = Spray dried cells.

* Showed standard deviation of each treatment.

storage period had a significant influence on dehydrated *L. acidophilus* (P = 0.0003). There was a significant difference between freeze-dried and spray-dried *L. acidophilus* kept either using NaOH or LiCl desiccant at week 10 of storage as demonstrated by different letters. Storage of freeze dried or spray dried *L. acidophilus* in room temperature using NaOH and LiCl as desiccants provided higher survival than that using silica gel.

Survival trend of microencapsulated *L. cremoris* during 10 weeks of storage kept at various desiccants was demonstrated in Fig. 1b. In general, freeze drying showed a superior effect on *L. cremoris* survival than spray drying (P<0.0001); the use of NaOH and LiCl as desiccants was better than silica gel (P = 0.0004); however, decrease in survival of microencapsulated *L. cremoris* was inevitable (P = 0.0006). ANOVA also demonstrated that the type of desiccator, drying method and period of storage influenced the survival of *L. acidophilus* and *L. cremoris* significantly (all P<0.001). There was no significant different at the beginning of the storage periods (up to 4 weeks) between freeze dried *L. cremoris* kept in foil pouch using NaOH or LiCl as desiccants, but decrease was taken place from 6th week to 10th week of storage in such conditions. Comparable trend was also detected in spray dried *L. cremoris* using NaOH and LiCl as desiccants during 10 week period of storage.

A linear regression model was used to estimate the period of storage at such circumstances in which dehydrated *L. acidophilus* and *L. cremoris* would reach the population number of 10^7 CFU/g. The coefficient of determination R^2 of freeze dried *L. acidophilus*, spray dried *L. acidophilus*, freeze dried *L. cremoris*, spray dried *L. cremoris* kept at different desiccants was between 86.2% and 99.1% (Fig. 2a–d); these were also confirmed by RMSE. All of ANOVA of regression showed P values<0.05 indicating a good fit to the data. It could be predicted that the longest storage of freeze dried *L. acidophilus*, spray dried *L. acidophilus* and freeze dried *L. cremoris* kept in foil pouch containing NaOH would be 46, 42 and 42 weeks, respectively (Fig. 2a–c); while spray dried *L. cremoris* kept in foil pouch containing LiCl would require storage period of 39 weeks (Fig. 2d) to reach 10^7 CFU/g. The linear trend of decrease in freeze dried and spray dried microencapsulated bacteria during storage at 25 °C was in agreement with Riveros et al. (2009) and Tsen, Chen, and King (2002).

Acid tolerance of freeze dried or spray dried *L. acidophilus* and *L. lactis* ssp. *cremoris* during 10 weeks of storage at various desiccants at 25 °C was demonstrated in Fig. 3a and b, respectively. ANOVA demonstrated that desiccators, drying method and period of storage had a significant effect on acid tolerance of microencapsulated *L. acidophilus* stored at 25 °C (P<0.0001; P = 0.0007; P<0.0001) and there was an interaction between those 3 factors (P = 0.009). Similar ANOVA result was also found in microencapsulated *L. cremoris*; an interaction between those 3 factors was also obvious (P<0.0009). Acid tolerance of freeze dried *L. acidophilus* kept in foil pouch using NaOH as desiccant showed significant decrease between 0th week to week 10 of storage (Fig. 3a). Comparable trend during storage was also shown by acid tolerance of spray dried *L. acidophilus* kept in foil pouch containing NaOH or LiCl. Acid tolerance of the freeze dried *L. cremoris* kept in foil pouch containing LiCl or NaOH was significantly higher than that of the spray dried *L. cremoris* under the same desiccants after 10th week of storage (Fig. 3b).

Bile tolerance of microencapsulated *L. acidophilus* and *L. cremoris* during 8 h exposure to MRS broth containing 0.3% taurocholic acid (pH 5.8) was demonstrated in Fig. 4a and b, respectively. ANOVA showed that dessicators, drying method and storage period had a significant effect on bile tolerance of microencapsulated *L. acidophilus* (P = 0.0003; P<0.0001 and P = 0.0005, respectively), while a significant interaction was noticed between drying method and storage period (P = 0.004). On the other hand, microencapsulated *L. cremoris* appeared more sensitive in bile environment due to an interaction effect of desiccators, drying method and storage period (P = 0.0008). Bile tolerance of freeze dried *L. acidophilus* kept in foil pouch containing either NaOH or LiCl was relatively stable from 0th to 10th week (Fig. 4a); while freeze dried *L. cremoris* kept under such conditions appeared more sensitive (Fig. 4b). In contrast, significant

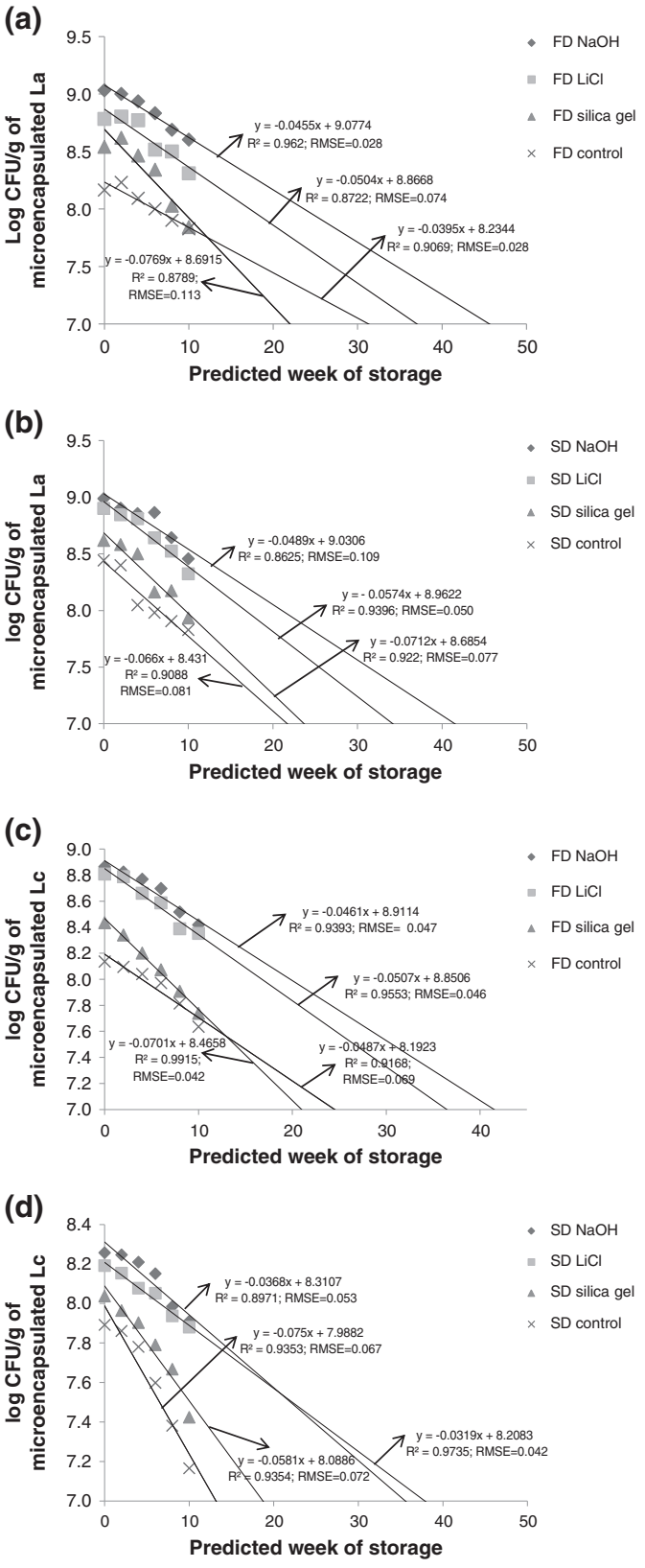

Fig. 2. Estimation of storage period for freeze dried *L. acidophilus* (a); spray dried *L. acidophilus* (b); freeze dried *L. cremoris* (c); and spray dried *L. cremoris* (d) to achieve minimum requirement (7.0 log CFU/g) of bacterial population.

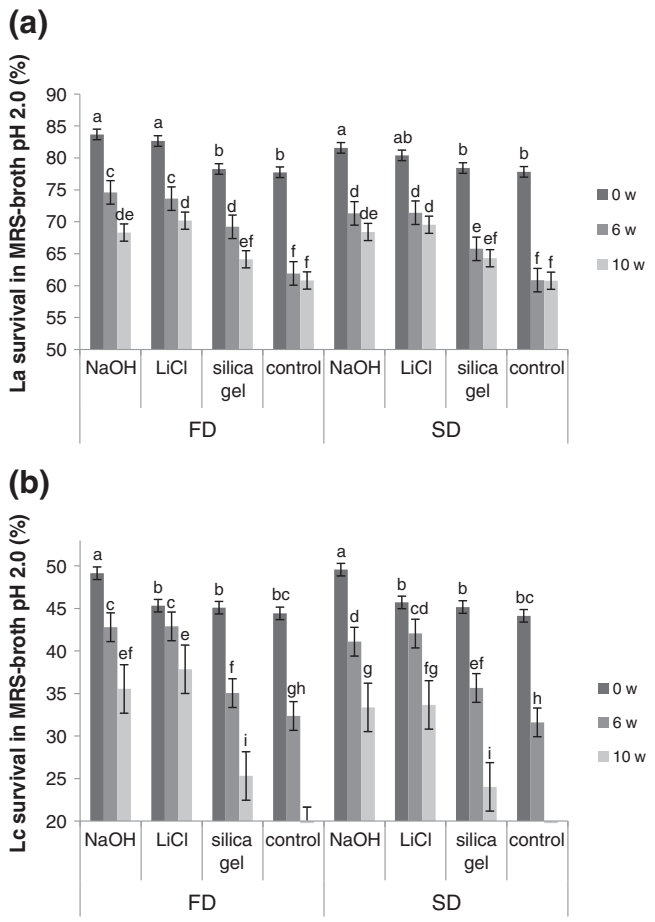

Fig. 3. Survival of *L. acidophilus* (a) and *L. lactis* ssp. *cremoris* (b) in MRS-broth pH 2.0 for 2 h during 10 weeks of storage at room temperature. FD = Freeze dried cells; SD = Spray dried cells.

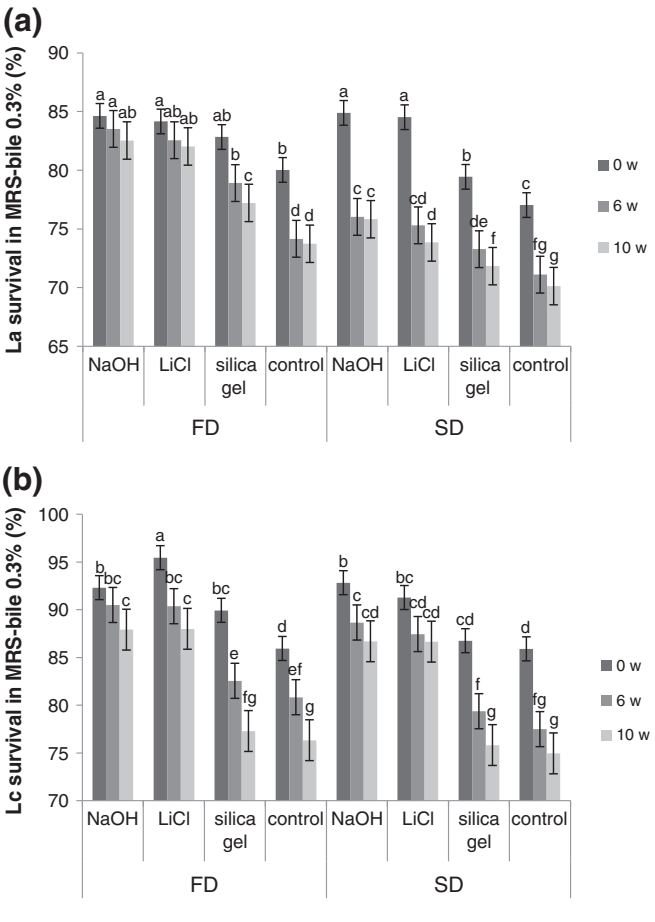

Fig. 4. Survival of *L. acidophilus* (a) and *L. lactis* ssp. *cremoris* (b) in MRS-bile (0.3%, pH 5.8; 8 h) during 10 weeks of storage at room temperature. FD = Freeze dried cells; SD = Spray dried cells.

decrease in spray dried *L. acidophilus* under the same conditions during 10 weeks of storage was unavoidable. After 10th week of storage, freeze dried *L. cremoris* with a_w controlled by NaOH or LiCl showed higher bile tolerance than spray dried *L. cremoris* with a_w controlled by the same desiccants.

SHb retention of microencapsulated *L. acidophilus* and *L. cremoris* after 10 weeks of storage can be seen in Fig. 5. ANOVA showed that drying method and desiccants had a significant effect on microencapsulated *L. acidophilus* ($P = 0.013$ and $P = 0.0002$, respectively), but there was no interaction between drying method and desiccant. Similar result of ANOVA of SHb retention was obtained for microencapsulated *L. cremoris*. Freeze drying *L. acidophilus* contributed a higher retention of SHb than spray dried *L. acidophilus* after 10 weeks of storage; but the difference was not significant. NaOH and LiCl desiccant were superior to silica gel in maintaining the SHb of microencapsulated *L. acidophilus* and *L. cremoris*.

β-Gal retention of microencapsulated *L. acidophilus* and *L. cremoris* after 10 weeks of storage was shown in Fig. 6. ANOVA of microencapsulated *L. acidophilus* and *L. cremoris* demonstrated the similar result. There was no significant effect of drying method on β-gal retention of microencapsulated *L. acidophilus* and *L. cremoris* and there was no interaction between drying method and desiccant; but desiccant had a significant effect on β-gal retention of *L. acidophilus* ($P = 0.003$) and that of *L. cremoris* ($P = 0.005$). These results showed that β-gal activity of *L. acidophilus* and *L. cremoris* was not sensitive to drying method and water activity, as long as adsorbent was still present to stabilize the low moisture content of microcapsule.

4. Discussion

Many efforts have been carried out to improve stability of probiotic bacteria during storage as well as to minimize transportation and storage costs. Dehydration such as freeze drying or spray drying is a promising method to increase bacterial survival during storage at room temperature. However, drying procedure might cause injure of cell envelopes resulting in releasing of some intracellular materials (Riveros et al., 2009). Ananta, Volkert, and Knorr (2005) demonstrated that survival of *Lactobacillus rhamnosus* GG was 60% (using outlet

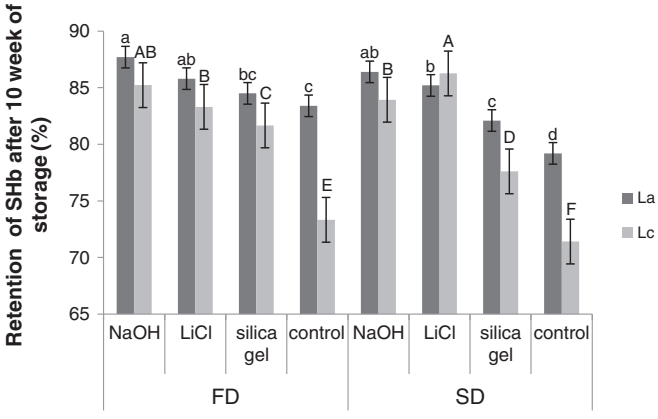

Fig. 5. Retention of SHb of microencapsulated *L. acidophilus* (La) and *L. cremoris* (Lc) after 10 weeks of storage. FD = Freeze dried cells; SD = Spray dried cells.

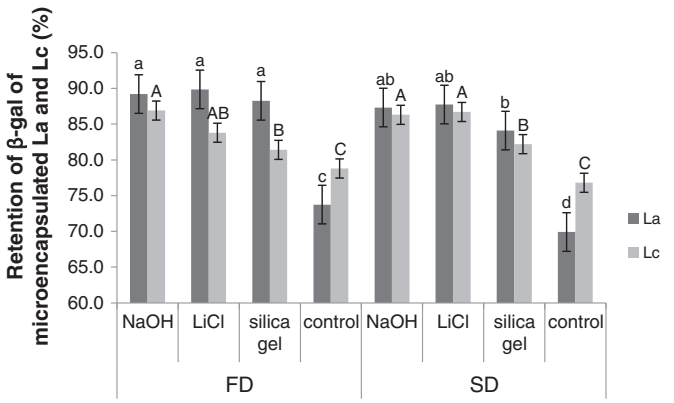

Fig. 6. Retention of β-galactosidase of microencapsulated *L. acidophilus* (La) and *L. cremoris* (Lc) after 10 weeks of storage. FD = Freeze dried cells; SD = Spray dried cells.

temperature of 80 °C) after spray drying using reconstituted skim milk (RSM) combined with prebiotic; however, stability of lactobacillus due to spray drying and subsequent storage (25 °C, without a_w adjustment) was strain-dependent (Corcoran, Ross, Fitzgerald, & Stanton, 2004) On the other hand, freeze drying also demonstrated significant contribution on cell membrane damage and on protein denaturation (Leslie, Israeli, Lighthart, Crowe, & Crowe, 1995; Zayed & Roos, 2004). Our study demonstrated that survival of freeze dried *L. acidophilus* and *L. cremoris* was higher than spray dried ones. This result was in agreement with Leja, Dembczyński, Białas, and Jankowski (2009), Wong et al. (2010) but on the contrary with that of Zamora, Carretero, and Parés (2006).

Storage of freeze dried and spray dried *L. acidophilus* in an aluminum foil pouch at room temperature preserved bacterial stability as long as a_w was maintained at 0.07–0.1. This is in agreement with the result of Ying et al. (2010). Viability of freeze-dried *Lactobacillus paracasei* ssp. *paracasei* and *L. acidophilus* using lactose or sucrose as lyoprotectants was relatively stable during long term of storage at 20 °C at a_w of 0.07 or 0.1 (Higl et al., 2007). In addition, decrease in viability of spray dried *L. rhamnosus* GG using RSM combined with prebiotics as microencapsulant was only about 0.5 (log N/No) during 6 weeks of storage at 25 °C at a_w of 0.1 (Ananta et al., 2005).

Comprehensive studies related storage of freeze dried and spray dried *L. lactis* ssp. *cremoris* at room temperature at low a_w have never been reported so far. Berner and Viernstein (2006) demonstrated that a high proportion of mannitol (10% w/v) had an adverse effect on bacterial survival possibly due to the formation of crystalline mannitol, as suggested by Constantino et al. (1998). Alternatively, the use of skim milk, lactose, sucrose, trehalose combined with soy milk improved the viability of *L. lactis* (Nanasombat & Sriwong, 2007); this result was higher than that of Carcoba and Rodriguez (2000) using RSM as medium.

Stability of *L. acidophilus* in simulated gastrointestinal tract has been studied by Mirlohi, Soleimanian-Zad, Dokhani, Sheikh-Zeinodin, and Abghary (2009), Pan, Chen, Wu, Tang, and Zhao (2009), Pozza, Miglioranza, Garcia, Garcia, and Pozza (2011). The results were various depending upon strains, for example Mirlohi et al. (2009) found that *L. acidophilus* strains survival in pH 2.5 for 2 h was between 6.0 and 7.8 log CFU/mL; while no growth of *L. acidophilus* NIT was found after 2 h exposure to pH 2.0 (Pan et al., 2009). On the other hand, *L. lactis* ssp. *cremoris* showed relatively high sensitiveness in harsh acid environment (Kim, Ren, & Dunn, 1999; Bakari, Tatsadjieu, Mbawala, & Mbofung, 2011). However, the growth ability indicated that the microcapsule was still able to protect the very sensitive lactic acid bacteria as long as storage at low a_w was carried out. In fact, *L. lactis* ssp. *cremoris* and *L. acidophilus* were detected after 12 h in mouse intestinal tract (about 6 log CFU/g feces) indicating that they were able to survive during passage through gastric juice (Kimoto et al., 2003). *L. acidophilus* showed a relatively high survival during exposure to bile environment (Pozza et al., 2011). In contrast, *L. cremoris* appeared more sensitive to this simulated small intestinal tract (Kim et al., 1999; Sabir, Beyatli, Cokmus, & Onal-Darilmaz, 2010). Interesting result was found by Bakari et al. (2011) who demonstrated that survival of *L. cremoris* NPL was 45% after 4 h exposure to 0.4% bile salts; but an increase to 70% was taken place after 24 h exposure in the same environment indicating that they might be able to grow in intestinal tract.

It has been recognized that probiotic bacteria should be able to adhere to intestinal wall after reaching the intestine. Surface hydrophobicity has been proven as an effective in vitro method for bacterial adhesion assay (Pan, Li, & Liu, 2006; Rahman, Kim, Kumura, & Shimazaki, 2008). Kos et al. (2003) stated that *L. acidophilus* is a hydrophobic bacterium with its surface hydrophobicity of 71%; similar result was reported by Colloca, Ahumada, Lopez, and Nader-Macıas (2000). SHb retention of our spray dried *L. acidophilus* was higher than that of Riveros et al. (2009). On the other hand, SHb of *L. lactis* ssp. *cremoris* strains were highly various (Sijtsma, Jansen, Hazeleger, Wouters, & Hellingwerf, 1990; Giaouris, Chapot-Chartier, & Briandet, 2009) indicating that SHb of *L. cremoris* was highly strain-dependent. Neither SHb of freeze dried probiotic bacteria nor that of spray or freeze dried *L. lactis* has been reported.

The ability of probiotic and LAB to hydrolyze lactose through their β-gal activity is essential for conquering the lactose intolerance problem. Our study was concord with that of Vasiljevic and Jelen (2003). The use of skim milk or casein as protectant appeared successful in retaining β-gal activity during freeze drying or spray drying (with low T_{outlet}) and during subsequent storage. In terms of the role of a_w during storage, freeze dried β-gal protected with reduced lactose whey + maltodextrin (50:50) showed high stability during 60 days of storage at 70 °C at $a_w = 0.00$; while its stability decreased to 80% only for 10 days of storage when storage at a_w of 0.22 was carried out at 70 °C (Burin, Buera, Hough, & Chirife, 2002). No study related to freeze- or spray-dried β-gal kept at low a_w at room temperature has been developed.

In general, our current study demonstrated that the use of casein-based formulation fortified by glucose, mannitol and prebiotic improved bacterial stability, particularly during exposure to acid and bile environment as compared to the use of Ca-alginate-based microencapsulation (Dianawati & Shah, 2011b). However, this work might not be able to compare straightforwardly since we used different species of probiotic bacteria besides the difference in microencapsulation technique.

5. Conclusion

The use of emulsion-based formulation (sodium caseinate) fortified with glucose and mannitol as microcapsule materials was effective in protecting *L. acidophilus* and *L. cremoris* during freeze drying or spray drying process (with $T_{outlet} = 50$ °C) as well as during 10 weeks of storage at 25 °C at low a_w (0.07 and 0.1). In general, freeze drying contributed higher stability of microencapsulated *L. acidophilus* and *L. cremoris* than spray drying even though the survival of spray dried *L. acidophilus* and *L. cremoris* was still relatively high as long as they were kept in an aluminum foil pouch using NaOH or LiCl as desiccant. The acid and bile tolerance was influenced by the drying method, desiccant and period of storage. Spray- or freeze-dried bacteria kept in foil pouch using NaOH or LiCl desiccants increased the acid and bile tolerance. Drying method and desiccant also influenced the SHb of *L. acidophilus* and *L. cremoris* after 10 weeks of storage. Stability of β-gal after 10 weeks of storage was influenced by a_w adjusted by desiccant; but spray drying or freeze drying showed no difference effect on it. The linear regression model demonstrated that the maximum storage period for freeze dried or spray dried *L. acidophilus* or *L. cremoris* kept in foil pouch

containing NaOH or LiCl as desiccant would be about 39–46 weeks to reach minimum bacterial population of 10^7 CFU/g.

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Chapter 10 : Effect of drying methods on microencapsulated *Lactobacillus acidophilus* and *Lactococcus lactis* ssp. *cremoris* on secondary protein structure and glass transition temperature as studied by Fourier transform infrared and differential scanning calorimetry

Introduction

This study observed interactions between polar sites of phospholipid bilayers of cell envelopes and mannitol as part of microencapsulating material, ascertains the changes in the structure of secondary proteins of intact cells of *L. acidophilus* and *L. cremoris* ssp. *lLactis.* Glass transition temperatures of microcaspules after spray- or freeze-drying and after 10 week of storage in aluminium foil pouch containing desiccants at 25^{o}C were measured.

The following paper entitled "Effect of drying methods on microencapsulated *Lactobacillus acidophilus* and *Lactococcus lactis* ssp. *cremoris* on secondary protein structure and glass transition temperature as studied by Fourier transform infrared and differential scanning calorimetry" by D. Dianawati, V. Mishra and N. P. Shah has been published by *Journal of Dairy Science*, 96, 1419–1430 (http://dx.doi.org/10.3168/jds.2012-6058).

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PART B: DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by (candidate name): DIANAWATI DIANAWATI **Signature:** [redacted] **Date:** 11/3/2013

Paper Title

Effect of drying methods on microencapsulated *Lactobacillus acidophilus* and *Lactococcus lactis* ssp. *cremoris* on secondary protein structure and glass transition temperature as studied by Fourier transform infrared and differential scanning calorimetry

In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Dianawati Dianawati	70	Designed the experiment Performed the sample analyses Performed statistical analysis using MINITAB 16 and data presentation Prepared major part of the manuscript
Nagendra P. Shah	20	Contribution to writing of paper and journal submission
Vijay Mishra	10	Contribution to writing of paper

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to **a)** granting bodies, **b)** the editor or publisher of journals or other publications, and **c)** the head of the responsible academic unit; and
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Effect of drying methods of microencapsulated *Lactobacillus acidophilus* and *Lactococcus lactis* ssp. *cremoris* on secondary protein structure and glass transition temperature as studied by Fourier transform infrared and differential scanning calorimetry

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ABSTRACT

Protective mechanisms of casein-based microcapsules containing mannitol on *Lactobacillus acidophilus* and *Lactococcus lactis* ssp. *cremoris*, changes in their secondary protein structures, and glass transition of the microcapsules were studied after spray- or freeze-drying and after 10 wk of storage in aluminum foil pouches containing different desiccants (NaOH, LiCl, or silica gel) at 25°C. An in situ Fourier transform infrared analysis was carried out to recognize any changes in fatty acids (FA) of bacterial cell envelopes, interaction between polar site of cell envelopes and microcapsules, and alteration of their secondary protein structures. Differential scanning calorimetry was used to determine glass transition of microcapsules based on glass transition temperature (T_g) values. Hierarchical cluster analysis based on functional groups of cell envelopes and secondary protein structures was also carried out to classify the microencapsulated bacteria due to the effects of spray- or freeze-drying and storage for 10 wk. The results showed that drying process did not affect FA and secondary protein structures of bacteria; however, those structures were affected during storage depending upon the type of desiccant used. Interaction between exterior of bacterial cell envelopes and microencapsulant occurred after spray- or freeze-drying; however, these structures were maintained after storage in foil pouch containing sodium hydroxide. Method of drying and type of desiccants influenced the level of similarities of microencapsulated bacteria. Desiccants and method of drying affected glass transition, yet no $T_g \leq 25$°C was detected. This study demonstrated that the changes in FA and secondary structures of the microencapsulated bacteria still occurred during storage at T_g above room temperature, indicating that the glassy state did not completely prevent chemical activities.

Key words: desiccant, glass transition temperature (T_g), cell envelope, secondary protein

INTRODUCTION

The use of particular drying methods to preserve probiotic bacteria provides some advantages besides its ease of handling, including low cost of transportation and storage at room temperature. Freeze-drying and spray-drying are 2 common drying methods for preservation of bacteria; however, these have many adverse effects on cell envelopes and secondary protein structures (Leslie et al., 1995; Mauerer, 2006). Microencapsulation technology has been developed to overcome these problems. The application of sodium caseinate-glucose to form a glassy Maillard substance, combined with mannitol, is effective in protecting spray-dried probiotic bacteria (Crittenden et al., 2006). Mannitol is excellent in protecting probiotic bacteria during storage and exposure to a simulated gastric environment due to its radical scavenging ability and structural stability in low pH (Efiuvwevwere et al., 1999; Telang et al., 2003), in spite of its tendency to crystallize (Izutsu and Kojima, 2002).

Mechanisms of dehydrated bacterial protection by sugars can be explained by water replacement theory (Crowe et al., 1988) or the formation of amorphous state (Santivarangkna et al., 2011). The Fourier transform infrared (**FTIR**) technique has been used to investigate the role of sugars in retarding conformational changes of bacterial cell envelopes and proteins (Leslie et al., 1995; Oldenhof et al., 2005; Santivarangkna et al., 2010). The wave number alteration indicated that the protective mechanism of cell envelopes of bacteria occurs through sugar interaction with phospholipid headgroups via hydrogen bond (Crowe et al., 1988; Grdadolnik and Hadzi, 1998). Gauger et al. (2002)

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stated that certain levels of water activity ($\mathbf{a_w}$) at room temperature contributed to conformational disorder of diphytanoylphosphatidylcholine. Protein conformation was also affected by freeze- and spray-drying (Garzon-Rodriguez et al., 2004; Schüle et al., 2007); drying process and storage at room temperature at low a_w might cause the changes in cell envelopes and secondary structure of proteins of bacteria.

Apart from molecular interaction between cell envelopes and microencapsulants, the physical state of microcapsule matrix is also crucial for bacterial stability. The extremely high viscosity of dehydrated products in the amorphous state is capable of decreasing molecular mobility reducing adverse chemical reactions; however, this solid state is metastable and strongly depends on the glass transition temperature ($\mathbf{T_g}$). Storage at room temperature above T_g might increase the chance of glass transition (Santivarangkna et al., 2011), in which molecular mobility would increase along with the formation of crystalline state. Glass transition temperature is also influenced by a_w of storage: an increase in a_w results in a decrease in T_g (Higl et al., 2007; Kurtmann et al., 2009). The mechanism of bacterial protection by sugars during dehydration has been established, but the effect of long-term storage at room temperature on the changes in phospholipid bilayers and secondary protein structures of bacterial cells has not. The aims of this study were to ascertain the interaction between cell envelopes of bacteria and encapsulant, as well as to determine the changes in the structure of secondary proteins and to establish T_g and moisture content of microcapsules after spray- or freeze-drying and after 10 wk of storage in aluminum foil pouches containing different desiccants at 25°C. One probiotic bacteria (*Lactobacillus acidophilus*) and one sensitive lactic acid bacteria (*Lactococcus cremoris* ssp. *lactis*) were used as models in this study.

MATERIALS AND METHODS

Lb. acidophilus 2401 and Lc. lactis ssp. cremoris R-704 and Their Cultivation

Pure cultures of *Lactobacillus acidophilus* 2401 (*Lb. acidophilus*) and *Lactococcus lactis* ssp. *cremoris* R-704 (*Lc. cremoris*) were obtained from Victoria University stock culture and were confirmed using Gram staining (Ding and Shah, 2009). *Lactobacillus acidophilus* was grown in de Man, Rogosa, and Sharpe broth at 37°C for 18 h (Riveros et al., 2009), whereas *Lc. cremoris* was grown in M17 supplemented with 0.5% glucose at 30°C for 18 h (Kimoto et al., 2003); both organisms were subcultured 3 times. The cells were concentrated by centrifuging the broth at 14,000 × *g* for 15 min at 4°C (Vinderola and Reinheimer, 2003). The resultant cell pellet was washed twice with 0.85% of sterilized saline solution and then resuspended in the same solution (10 mL of cell pellet was added by 10 mL of saline solution). The initial population of concentrated bacteria was 3.1×10^{10} cfu/mL for *Lb. acidophilus* and 1.1×10^{10} cfu/mL for *Lc. cremoris.*

Preparation of Microcapsules

Microencapsulation was performed using an oil-in-water emulsion system comprising vegetable oil (10% wt/vol), sodium caseinate (6% wt/vol), fructooligosaccharides from chicory (2% wt/vol), D-glucose (3% wt/vol), and mannitol (3% wt/vol). All of the materials were from Sigma Aldrich Corp. (St. Louis, MO) except vegetable oil, which was obtained from a local supermarket. The materials were mixed and homogenized using a magnetic stirrer, and were heated at 95°C for 30 min to initiate the Maillard reaction. One-fifth of the concentrated bacteria were incorporated to the cold emulsion system (10°C) before spray- or freeze-drying. The emulsion was spray-dried using a Buchi Mini spray drier (model B290, Bern, Switzerland) with Dehumidifier B296 (humidity 86%; temperature −3°C; Buchi). The outlet temperature was 50°C, hence the inlet temperature was set to 99°C with pump 27% (feeding rate = 7.14 mL/min) for the emulsion system containing *Lb. acidophilus*, and was set to 80°C with pump 20% (feeding rate = 3.03 mL/min) for the emulsion system containing *Lc. cremoris*. The powder gathered from the collection vessel was then stored in desiccators. For freeze-drying, frozen microcapsules were loaded into a freeze-drier (model FD-300, Airvac Engineering Pty. Ltd., Dandenong, Australia) set to achieve −13,332.2 Pa of internal pressure before freeze-drying at a temperature of −88°C, with 44 h of primary freeze-drying, and 4 h of secondary freeze-drying. Each of the freeze-dried and spray-dried products (*Lb. acidophilus* and *Lc. cremoris*) were placed on Petri-disks and kept in desiccators containing a saturated solution of sodium hydroxide (NaOH; $a_w = 0.07$), a saturated solution of lithium chloride (LiCl; $a_w = 0.11$), or silica gel for 2 wk to reach the equilibrium. Once equilibrium was established, the products were transferred to aluminum foil pouches, and NaOH, LiCl, or silica gel was packed inside a semi-permeable membrane and placed inside the pouch. Controls were stored without desiccant, fresh samples were freshly harvested bacteria after being grown in media for 18 h, and prestorage samples were after freeze drying/after spray drying. Storage at 25°C was carried out for 10 wk; after the end of storage period, samples were kept at −80°C until further analysis.

Sample Preparation for FTIR Spectroscopy

Solid sample preparation was carried out according to Izutsu and Kojima (2002) and Sharma and Kalonia (2004). The powdered sample (10 mg) of dehydrated, microencapsulated bacteria was mixed with 100 mg of dried KBr powder. A transparent pellet of the sample KBr mixture was obtained by pressing the mixture under vacuum at 10 tons of hydraulic pressure. The spectra of microcapsules without bacteria were subtracted from those of samples with bacteria (Mauerer, 2006; Han et al., 2007). Measurement of spectra of functional groups was carried out at room temperature (~25°C) using an FTIR combined with infrared solution software (Type 8400S, Shimadzu, Kyoto, Japan). All FTIR spectra were recorded using a resolution of 4 cm^{-1} and 20 scans. Air spectra were recorded before each experiment to correct background effects for all spectra recorded. Spectra were collected from 3 different batches of samples. Smoothing and normalization of the second derivatives of deconvoluted spectra were carried out to develop clearer separation of complex bands (Santivarangkna et al., 2007). Spectra of freshly harvested *Lb. acidophilus* and *Lc. cremoris* were used as controls to recognize whether cell envelope and secondary structure of proteins of microencapsulated bacteria experienced a change in frequency. Ten microliters of washed bacterial cell suspension was spread onto the surface of a calcium difluoride window and the spectra of cells were determined after being dehydrated in desiccators containing phosphorus pentoxide (Oldenhof et al., 2005) to reduce interfering spectra of water. All FTIR measurements were repeated 3 times.

Determination of State of Cell Envelopes and Secondary Proteins of Microencapsulated Lb. acidophilus and Lc. cremoris Using FTIR Spectroscopy

Wavenumbers (cm^{-1}) of molecular vibrations were detected based on the functional groups of cell envelopes and secondary protein structures of the 2 bacteria. Hydrophobic sites consisted of CH_3 (asymmetric and symmetric vibration) of FA in the range of 2950 to 2990 and 2860 to 2890 cm^{-1}, respectively (Davis and Mauer, 2010). Hydrophilic sites consisted of choline group, $N^+(CH_3)_3$ asymmetric stretching vibration at ~970 cm^{-1} (Popova and Hincha, 2003), and P=O symmetric stretching of phosphodiesters in phospholipids at ~1080 cm^{-1} (Davis and Mauer, 2010). Polar/apolar site of C=O stretching of lipid ester was detected at 1715 to 1740 cm^{-1} (Santivarangkna et al., 2010). Secondary proteins were detected in the wide range of 1620 to 1700 cm^{-1} (amide I) reflecting β-sheet, α-helix, β-turn, or unordered structures (Kong and Yu, 2007).

Cluster Analysis

Hierarchical cluster analysis was carried out according to the modified method of Dziuba et al. (2007). The Ward's algorithm method (Lipkus et al., 1988) was used to analyze the similarities between bacterial spectra (for each *Lb. acidophilus* and *Lc. cremoris*) after drying and after 10 wk of storage at room temperature using various desiccants. The fresh bacteria were used as a control.

Differential Scanning Calorimetry and Residual Moisture Content

Differential scanning calorimetry (**DSC**) was performed using a PerkinElmer DSC 7 (PerkinElmer, San Jose, CA) to determine T_g of the samples; samples (8–12 mg) were pressed in standard sealed aluminum DSC pans. Pressed samples were scanned from 5 to 170°C at a heating rate of 5°C/min (Zimeri and Kokini, 2002); measurements were carried out in duplicate. Glass transition temperature was obtained from the temperature of the midpoint of the change in heat capacity scanned at 10°C/min as suggested by Kalichevsky and Blanshard (1992). Residual moisture content of spray- or freeze-dried products was determined gravimetrically at 105°C (Mauer et al., 2000; Lu et al., 2007).

RESULTS AND DISCUSSION

Cell Envelopes and Secondary Protein Structures of Microencapsulated Bacteria

The second derivative of spectra of cell envelopes of fresh and microencapsulated *Lb. acidophilus* and *Lc. lactis* after spray- or freeze-drying and subsequent storage are shown in Tables 1 and 2, respectively. The $N^+(CH_3)_3$ asymmetric stretching of choline of fresh *Lb. acidophilus* and *Lc. lactis* were indicated at 957 and 947 cm^{-1}, respectively. Frequencies of C–H asymmetric and symmetric stretching vibration of FA of cell envelopes of fresh *Lb. acidophilus* were at ~2963 and ~2882 cm^{-1}, respectively; whereas those of fresh *Lc. lactis* were at ~2964 and ~2883 cm^{-1}, respectively. A band of 1768 to 1776 cm^{-1} indicated C=O located in interface between the polar site of headgroups and the apolar site of tailgroups of phospholipid bilayers (Davis and Mauer, 2010). This functional group is dependent on a hydrogen bond; a decrease in frequencies indicated a stronger C=O···H–O bonding (dotted bond = hy-

drogen bond) interaction (Cacela and Hincha, 2006). Frequencies at 1075 and 1073 cm^{-1} indicated the vibration of P=O symmetric of fresh *Lb. acidophilus* and *Lc. lactis*, respectively.

Frequency increase in C–H frequencies and asymmetric and symmetric stretching of CH_3 of FA of cell envelopes of spray- or freeze-dried *Lb. acidophilus* were shown in Table 1. A peak alteration of C–H asymmetric of *Lb. acidophilus* was detected after freeze- and spray-drying from 2963 (fresh cells) to 2972 and 2968, respectively. Storage in a foil pouch containing silica gel appeared less effective than storage in pouches containing NaOH or LiCl, as reflected by the shift to higher wavenumbers, such as 2987 for freeze-dried *Lb. acidophilus* and 2967 for spray-dried *Lb. acidophilus*, along with peak broadening. Conversely, no obvious alteration was detected of C–H frequency of microencapsulated *Lc. cremoris* during storage at low a_w (NaOH and LiCl desiccants) compared with that of freshly harvested *Lc. cremoris* (Table 2). However, spray-dried *Lc. cremoris* kept in foil pouch without using desiccant (control), as well as freeze-dried *Lc. cremoris* kept in foil pouch containing silica gel or the control (freeze-dried *Lc. cremoris* without desiccant), demonstrated an alteration to the higher frequencies. Similar behavior was also observed of peak alteration of C–H symmetric of *Lb. acidophilus* and *Lc. cremoris* due to drying and storage at different a_w (Gauger et al., 2002).

The IR frequency of ~2955 and of ~2880 indicates C–H stretching of $–CH_3$ in FA of cell envelopes of bacteria (Davis and Mauer, 2010); however, it depends on the bacterial species and strains. An upshift in C–H frequencies indicated FA conformational changes from lyotropic gel into liquid crystalline phase (Goodrich et al., 1991; Grdadolnik and Hadzi, 1998; Popova and Hincha, 2003, 2007). This occurrence is known as chain melting, which could be induced by the change in water content (Gauger et al., 2001). The intermediate level of hydration, such as relative humidity of 20% (a_w = 0.22), causes a steep increase in frequencies reflecting conformational disorder of the acyl chains (Gauger et al., 2002). In our study, the rehydration phenomena could be due to ineffectiveness of silica gel as an adsorbent (a_w microcapsules = 0.28), hence, the moisture adsorption from environment by the microcapsules can take place; the presence of water causes adverse chemical activities (Labuza, 1984).

The P=O frequencies of microencapsulated *Lb. acidophilus* after freeze- and spray-drying were 1047 and 1048, respectively; whereas those after 10 wk of storage were in the range of 1044 to 1059, depending upon the desiccant type placed in the foil pouch (Table 1a). A slight increase in frequencies of P=O was detected when the freeze- or spray-dried *Lb. acidophilus* were kept in foil pouch containing silica gel. It showed similar frequency with that kept in foil pouch without desiccant as control. However, all of the P=O symmetric frequencies were lower than that of freshly harvested *Lb. acidophilus* (1075). Similarly, an interaction of phospholipid bilayers of *Lc. cremoris* with the polar group of microencapsule materials during storage appears depending on the presence of moisture (Table 1b). The frequencies of P=O symmetric of *Lc. cremoris* after freeze- and spray-drying were 1045 and 1056, respectively, whereas those of *Lc. cremoris* after subsequent storage were between 1055 and 1057.

Decrease in P=O wavenumbers in our findings was in agreement with that of Leslie et al. (1995), Oldenhof et al. (2005), and Santivarangkna et al. (2010). In the dehydrated form, an interaction takes place between molecules of sugars and the polar site of lipids, which decreases the chance of lateral lipid movement (van den Bogaart et al., 2007). The role of sugars to replace water during dehydration is important in cell envelope protection mechanism (Goodrich et al., 1991). This theory might explain the relationship between the stability of FA of tailgroups of cell envelopes of *Lb. acidophilus* and *Lc. cremoris* kept at low a_w (using NaOH, LiCl, or silica gel) and the interaction of microcapsule substances with lipid headgroups. In terms of the protection effect of microencapsulant on phospholipid bilayers, one possible explanation might come from the fortification of mannitol in the formulation. Glucose interacted with proteins (caseins) through formation of Maillard complex substances during microcapsule preparation (Crittenden et al., 2006) via interaction between glucose carbonyl groups and primary amino groups of proteins (Blei and Odian, 2000); thus, the chance of glucose to interact directly with P=O of lipid headgroups might be lower than mannitol. Mannitol, which is not able to take a part in Maillard formation, might have more chance to interact with polar surface of phospholipid bilayers. The interaction could be through mannitol's role as a proton donor; hence, a strong hydrogen bond was formed (Grdadolnik and Hadzi, 1998) via sugar hydroxyl–lipid headgroups (Ricker et al., 2003).

The $N^+(CH_3)_3$ asymmetric stretching vibration of the choline terminal of spray- and freeze-dried *Lb. acidophilus* after storage, as well as that of spray-dried and freeze-dried *Lc. cremoris,* is also demonstrated in Tables 1 and 2, respectively. All of the frequencies were higher than that of freshly harvested bacteria (957 for *Lb. acidophilus* and 947 for *Lc. cremoris*). The $N^+(CH_3)_3$ of spray-dried *Lb. acidophilus* demonstrated a higher frequencies than that of freeze-dried *Lb. acidophilus*. The $N^+(CH_3)_3$ of freeze-dried *Lc. cremoris* was commonly higher than that of spray-dried *Lc. cremoris*,

Table 1. Second derivative of spectra of cell envelopes of microencaspulated *Lactobacillus acidophilus* (La; means ± SD)[1]

Functional group[2]	Freeze-dried (FD)					Spray-dried (SD)					
	After FD	NaOH	LiCl	Silica gel	Control	After SD	NaOH	LiCl	Silica gel	Control	Fresh
CH_3 asym	2,972.2 ± 0.3	2,971.7 ± 0.3	2,976.5 ± 0.5	2,986.7 ± 0.3	2,988.5 ± 0.5	2,967.5 ± 0.5	2,966.3 ± 0.3	2,966.5 ± 0.5	2,967.2 ± 0.3	2,978.0 ± 0.5	2,963.2 ± 0.3
CH_3 sym	2,884.0 ± 0.5	2,886.0 ± 0.5	2,887.0 ± 0.5	2,888.8 ± 0.3	2,890.5 ± 0.5	2,888.5 ± 0.5	2,889.1 ± 0.2	2,898.5 ± 0.5	2,900.5 ± 0.5	2,901.8 ± 0.8	2,882.2 ± 0.3
$N^+(CH_3)_3$ asym	958.2 ± 0.3	957.7 ± 0.8	966.5 ± 0.5	968.5 ± 0.5	970.6 ± 0.4	968.7 ± 0.3	967.5 ± 0.5	967.4 ± 0.4	972.5 ± 0.5	974.5 ± 0.5	957.3 ± 0.2
P=O sym	1,047.1 ± 0.2	1,044.5 ± 0.5	1,055.5 ± 0.5	1,058.1 ± 0.2	1,058.5 ± 0.5	1,048.2 ± 0.3	1,047.0 ± 0.4	1,057.4 ± 0.4	1,057.5 ± 0.5	1,058.7 ± 0.3	1,075.2 ± 0.3
C=O	1,747.5 ± 0.5	1,747.3 ± 0.3	1,748.0 ± 0.5	1,749.2 ± 0.3	1,750.5 ± 0.5	1,716.8 ± 0.8	1,717.5 ± 0.5	1,721.5 ± 0.5	1,722.7 ± 0.6	1,735.5 ± 0.5	1,767.8 ± 0.3

[1]CH_3 asym = CH_3 asymmetric stretching vibration of FA; CH_3 sym = CH_3 symmetric stretching vibration of FA; $N^+(CH_3)_3$ asym = $N^+(CH_3)_3$ asymmetric stretching vibration of choline group; P=O sym = P=O symmetric stretching vibration of phosphate group of phospholipids; C=O = C=O stretching vibration of carboxylic ester.

[2]After FD/after SD = microencapsulated La after freeze drying/spray drying; NaOH = microencapsulated La (under FD/SD) after storage in foil pouch containing NaOH as desiccant; LiCl = microencapsulated La (under FD/SD) after storage in foil pouch containing LiCl as desiccant; silica gel = microencapsulated La (under FD/SD) after storage in foil pouch containing silica gel as desiccant; control = microencapsulated La (under FD/SD) after storage in foil pouch without desiccant; fresh = freshly harvested La after being grown in the medium for 18 h.

Table 2. Second derivative of spectra of cell envelopes of microencaspulated *Lactococcus lactis* ssp. *cremoris* (Lc; means ± SD)[1]

Functional group[2]	Freeze-dried (FD)					Spray-dried (SD)					
	After FD	NaOH	LiCl	Silica gel	Control	After SD	NaOH	LiCl	Silica gel	Control	Fresh
CH_3 asym	2,965.3 ± 0.2	2,964.2 ± 0.3	2,965.5 ± 0.4	2,985.5 ± 0.5	2,988.8 ± 0.2	2,963.0 ± 0.2	2,965.2 ± 0.2	2,964.5 ± 0.5	2,965.2 ± 0.3	2,973.2 ± 0.2	2,964.1 ± 0.2
CH_3 sym	2,883.2 ± 0.2	2,881.0 ± 0.2	2,887.0 ± 0.5	2,895.9 ± 0.2	2,904.0 ± 0.6	2,883.0 ± 0.3	2,888.0 ± 0.1	2,900.7 ± 0.4	2,900.4 ± 0.4	2,901.3 ± 0.3	2,883.2 ± 0.3
$N^+(CH_3)_3$ asym	984.2 ± 0.2	982.2 ± 0.3	989.0 ± 0.3	995.9 ± 0.3	998.0 ± 0.3	950.3 ± 0.3	950.2 ± 0.2	952.3 ± 0.3	952.4 ± 0.4	952.7 ± 0.6	947.1 ± 0.1
P=O sym	1,045.1 ± 0.1	1,055.0 ± 0.4	1,055.1 ± 0.3	1,055.9 ± 0.2	1,057.2 ± 0.2	1,056.0 ± 0.3	1,055.2 ± 0.3	1,056.0 ± 0.2	1,056.9 ± 0.2	1,057.3 ± 0.3	1,073.1 ± 0.1
C=O	1,723.2 ± 0.2	1,720.5 ± 0.5	1,744.0 ± 0.3	1,744.0 ± 0.2	1,746.2 ± 0.2	1,729.2 ± 0.3	1,741.5 ± 0.5	1,743.3 ± 0.3	1,743.9 ± 0.3	1,744.0 ± 0.5	1,776.2 ± 0.3

[1]CH_3 asym = CH_3 asymmetric stretching vibration of FA; CH_3 sym = CH_3 symmetric stretching vibration of FA; $N^+(CH_3)_3$ asym = $N^+(CH_3)_3$ asymmetric stretching vibration of choline group; P=O sym = P=O symmetric stretching vibration of phosphate group of phospholipids; C=O = C=O stretching vibration of carboxylic ester.

[2]After FD/after SD = microencapsulated Lc after freeze drying/spray drying; NaOH = microencapsulated Lc (under FD/SD) after storage in foil pouch containing NaOH as desiccant; LiCl = microencapsulated Lc (under FD/SD) after storage in foil pouch containing LiCl as desiccant; silica gel = microencapsulated Lc (under FD/SD) after storage in foil pouch containing silica gel as desiccant; control = microencapsulated Lc (under FD/SD) after storage in foil pouch without desiccant; fresh = freshly harvested Lc after being grown in the medium for 18 h.

which might reflect a stronger interaction with sugars. In addition, storage of spray- or freeze-dried *Lc. cremoris* in silica gel or without any desiccant showed higher frequencies, which could be due to moisture adsorption from surroundings. The use of silica gel as a desiccant increased wavenumbers indicating an interference of moisture from the environment.

Our study showed that frequencies of asymmetric $N^+(CH_3)_3$ stretching vibration of freeze- or spray-dried *Lb. acidophilus* and *Lc. cremoris* were higher than that of freshly harvested ones. This might be due to dipolar interaction between choline functional groups and sugars (Popova and Hincha, 2003). However, storage in silica gel showed higher wavenumbers, which might be related to the ineffectiveness of the desiccant; therefore, the surrounding moisture could be adsorbed and increase the wavenumbers slightly. Both sugars and moisture interaction result in almost similar peak alteration (Cacela and Hincha, 2006). Our results were in agreement with that of Grdadolnik and Hadzi (1998) and Popova and Hincha (2003), who demonstrated a shift to higher wavenumbers due to an interaction between sugars, such as glycerol or glucose, and polar site of phosphatidylcholine. A possible mechanism of frequency alteration of $N^+(CH_3)_3$ stretching vibration could be explained by the torsional angles theory as proposed by Grdadolnik and Hadzi (1998). The authors suggested that the presence of sugars such as sorbitol or moisture alters rotamer population, resulting in an *ap* torsion angle increase along with an *sc* torsion angle decrease, thus indicating an increase in H-bond. This might explain the difference of choline frequencies in our study due to the storage at low a_w using various desiccants.

The C=O double bond of microencapsulated *Lb. acidophilus* and *Lc. cremoris* was also demonstrated in Tables 1 and 2, respectively. The frequencies of C=O were lower after freeze- or spray-drying, and after storage in a foil pouch containing NaOH or LiCl, compared to the C=O of the fresh bacteria. Trend of peak alteration of C=O was almost similar to that of P=O, as mentioned above. The wavenumber of C=O stretching vibration of ester carbonyl group as a part of polar/apolar interfacial of bacterial pospholipids varies between 1716 and 1750 cm^{-1} (Erukhimovitch et al., 2005; Santivarangkna et al., 2010). Nonhydrogen-bonded or weak and strong hydrogen-bonded C=O were indicated by higher and lower frequencies, respectively (Lewis and McElhaney, 1998). Decrease in C=O frequencies of freeze- or spray-dried bacteria could be due to water removal along with replacement by sugars (Santivarangkna et al., 2010).

Amide I band is mainly related to C=O stretching vibration (70–85%) and C–N group (10–20%). It showed the secondary structure of peptide components such as α-helix, β-sheet and β-turn (Gallagher, 2011); determination of secondary protein structures was based on Chirgadze and Nevskaya (1976), Kong and Yu (2007), and Mobili et al. (2009). Elements of amide I reflecting secondary protein structures of spray-dried *Lc. cremoris*, freeze-dried *Lc. cremoris*, spray-dried *Lb. acidophilus*, and freeze-dried *Lb. acidophilus* are shown in Table 3. Wave numbers of *Lb. acidophilus* after freeze- or spray-drying, as well as freeze-dried *Lb. acidophilus* kept in foil pouch containing NaOH and spray-dried *Lb. acidophilus* kept in a pouch containing either NaOH or LiCl, indicated the presence of α-helix (from 1649 to 1657 cm^{-1}); whereas storage using silica gel caused the conformational changes from α-helix to β-sheet or β-turn. On the contrary, *Lc. cremoris* appeared more sensitive to drying processes as indicated by the formation of no-order and β-sheet after freeze-drying, whereas the α-helix structure of *Lc. cremoris* was maintained after spray-drying. However, storage at room temperature for a long period affected the secondary protein structures of microencapsulated *Lc. cremoris*, as indicated by frequency changes along with the presence of a new peak (Table 3). For instance, spray-dried *Lc. cremoris* kept in a foil pouch containing NaOH showed 2 peaks at 1646 and 1684, whereas freeze-dried *Lc. cremoris* under the same conditions showed peaks at 1650 and 1689 (frequency of fresh *Lc. cremoris* = 1651).

Table 3 demonstrated that the structure of secondary proteins of microencapsulated bacteria was retained after dehydration. This result was in agreement with that of Oldenhof et al. (2005) and Garzon-Rodriguez et al. (2004). These authors stated that the use of sugars, such as sucrose, maltodextrin, or disaccharides, combined with starch maintains the native-like secondary protein components after freeze-drying. In addition, Schüle et al. (2007) found that mannitol at relatively low concentration protected antibodies during spray-drying with inlet and outlet temperatures of 90°C and 50°C, which was similar to our spray-drying procedure. Similar results were demonstrated by Tzannis and Prestrelski (1999) and Liao et al. (2002) using different sugars as the protein protectant. The protective mechanism during freeze- or spray-drying of protein models in those studies is taken place through water replacement via H-bond (Maury et al., 2005); hence preservation of protein folding occurs (Garzon-Rodriguez et al., 2004). The protective mechanism might be different with ours, as bacterial proteins could be embedded on the surface or within the cell. In our study, an encapsulant containing mannitol and glucose interacted with the polar site of phospholipid bilayers, thus protection effect on proteins from dehydration should be indirect.

Table 3. Assignment of components of secondary protein structures of microencapsulated *Lactobacillus acidophilus* and *Lactococcus lactis* ssp. *cremoris* (means ± SD)

Species and treatment[1]	Wavenumber (cm^{-1})	Assignment
Lb. acidophilus		
After FD	1,650.3 ± 0.3	α-Helix
FD: NaOH	1,650.2 ± 0.3	α-Helix
FD: LiCl	1,624.2 ± 0.2	β-Sheet
FD: silica gel	1,629.3 ± 0.3	β-Sheet
	1,667.2 ± 0.3	β-Turn
FD: control	1,629.9 ± 0.4	β-Sheet
	1,668.0 ± 0.2	β-Turn
After SD	1,649.2 ± 0.2	α-Helix
SD: NaOH	1,655.1 ± 0.1	α-Helix
SD: LiCl	1,656.3 ± 0.3	α-Helix
SD: silica gel	1,638.0 ± 0.3	β-Sheet
	1,672.9 ± 0.1	β-Sheet
SD: control	1,633.0 ± 0.2	β-Sheet
	1,670.0 ± 0.5	β-Turn
Fresh	1,654.2 ± 0.1	α-Helix
Lc. cremoris		
After FD	1,649.8 ± 0.5	No order
	1,692.0 ± 0.2	β-Sheet
FD: NaOH	1,649.9 ± 0.2	No order
	1,689.0 ± 0.2	β-Sheet
FD: LiCl	1,650.1 ± 0.2	α-Helix
	1,690.0 ± 0.2	β-Sheet
FD: silica gel	1,642.0 ± 0.2	No order
	1,689.5 ± 0.5	β-Turn
FD: control	1,640.1 ± 0.2	β-Sheet
	1,688.4 ± 0.5	β-Turn
After SD	1,656.1 ± 0.2	α-Helix
SD: NaOH	1,646.0 ± 0.2	No order
	1,684.4 ± 0.4	β-Turn
SD: LiCl	1,644.2 ± 0.2	No order
	1,671.2 ± 0.2	β-Turn
SD: silica gel	1,648.2 ± 0.2	No order
	1,688.3 ± 0.3	β-Turn
SD: control	1,647.1 ± 0.1	No order
	1,683.2 ± 0.2	β-Turn
Fresh	1,651.1 ± 0.1	α-Helix

[1]After FD/after SD = microencapsulated bacteria after freeze drying/spray drying; FD/SD: NaOH = microencapsulated bacteria (under FD/SD) after storage in foil pouch containing NaOH as desiccant; FD/SD: LiCl = microencapsulated bacteria (under FD/SD) after storage in foil pouch containing LiCl as desiccant; FD/SD: silica gel = microencapsulated bacteria (under FD/SD) after storage in foil pouch containing silica gel as desiccant; FD/SD: control = microencapsulated bacteria (under FD/SD) after storage in foil pouch without desiccant; fresh = freshly harvested bacteria after being grown in the medium for 18 h.

In terms of storage, Garzon-Rodriguez et al. (2004) found that perturbation of freeze-dried proteins takes place during storage at a temperature of 40°C for 6 mo with no a_w adjusted. The change of protein structures was indicated by disappearing of band of α-helix along with the extension of the β-sheet. Their findings might be in agreement with our study regarding the role of low a_w in increasing protein stability, however, the difference in storage temperature should be considered. In addition, Maury et al. (2005) revealed that amide I spectra of spray-dried immunoglobulin G protected by sorbitol and trehalose sealed under dry N_2 was not altered after 12 mo at 25°C.

Hierarchical cluster analysis was used determine the similarities of bacterial spectra and to categorize them into a cluster (Dziuba et al., 2007). Second-derivative spectra are commonly used for bacterial classification. A second-derivative spectrum helps in separation and resolution of bacterial spectra; thus classification can be done more easily (Davis and Mauer, 2010). Ward's algorithm is a frequent method for cluster analysis algorithms to develop a dendrogram (Lipkus et al., 1988).

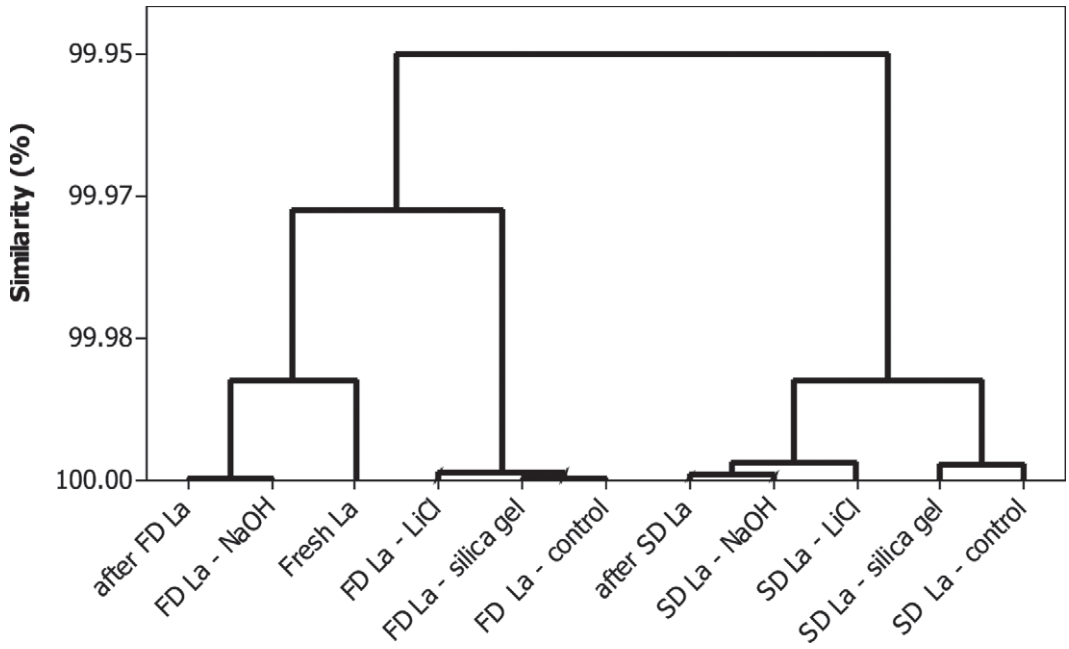

Figure 1. Classification of microencapsulated *Lactobacillus acidophilus* (La) after drying and after storage at room temperature. After FD/after SD La = microencapsulated La after freeze drying/spray drying; FD/SD La - NaOH = microencapsulated La (under FD/SD) after storage in foil pouch containing NaOH as desiccant; FD/SD La - LiCl = microencapsulated La (under FD/SD) after storage in foil pouch containing LiCl as desiccant; FD/SD La - silica gel = microencapsulated La (under FD/SD) after storage in foil pouch containing silica gel as desiccant; FD/SD La - control = microencapsulated La (under FD/SD) after storage in foil pouch without desiccant; fresh La = freshly harvested La after being grown in the medium for 18 h.

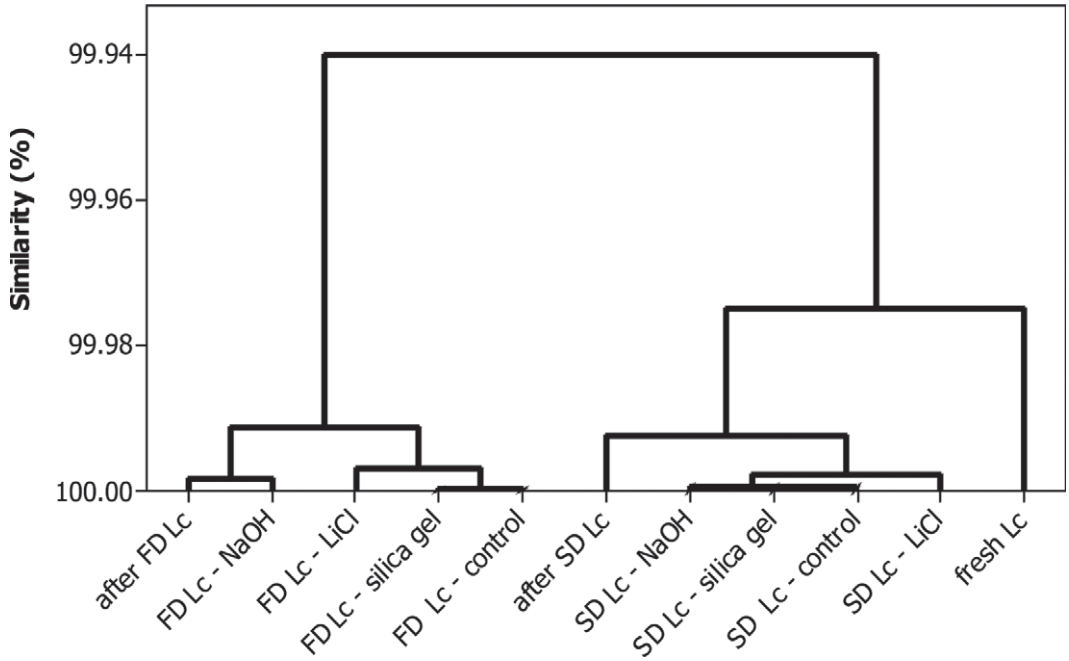

Figure 2. Classification of microencapsulated *Lactococcus lactis* ssp. *cremoris* (Lc) after drying and after storage at room temperature. After FD/after SD Lc = microencapsulated Lc after freeze drying/spray drying; FD/SD Lc - NaOH = microencapsulated Lc (under FD/SD) after storage in foil pouch containing NaOH as desiccant; FD/SD Lc - LiCl = microencapsulated Lc (under FD/SD) after storage in foil pouch containing LiCl as desiccant; FD/SD Lc - silica gel = microencapsulated Lc (under FD/SD) after storage in foil pouch containing silica gel as desiccant; FD/SD Lc - control = microencapsulated Lc (under FD/SD) after storage in foil pouch without desiccant; fresh Lc = freshly harvested Lc after being grown in the medium for 18 h.

A dendrogram, or tree diagram, is commonly used to depict the clusters calculated by clustering algorithm (Davis and Mauer, 2010). Fourier transform infrared bands were used to classify 41 strains of 6 lactobacilli isolated from cheese using the hierarchical cluster analysis method (Savic et al., 2008). More specifically, the use of cluster analysis has been developed to categorize characteristics of the lactobacilli S-layer (Mobili et al., 2009). In this study, we classified microencapsulated *Lb. acidophilus* (Figure 1) and *Lc. cremoris* (Figure 2) after spray- or freeze-drying and after 10 wk of storage based on the similarities of the cell envelopes and secondary structure of proteins.

Microencapsulated *Lb. acidophilus* after freeze-drying and long-term storage formed different clusters than *Lb. acidophilus* after spray-drying and storage (Figure 1). *Lb. acidophilus* after freeze-drying had a high similarity with freeze-dried *Lb. acidophilus* after storage in foil pouch containing NaOH (which was in one cluster with fresh *Lb. acidophilus*); whereas *Lb. acidophilus* after storage in a foil pouch containing either LiCl or silica gel had similarities with the control. Conversely, microencapsulated *Lb. acidophilus* after spray-drying showed similar characteristics of cell envelopes and secondary protein structures with spray-dried *Lb. acidophilus* kept in a foil pouch containing either NaOH or LiCl. Freeze-dried *Lc. cremoris* also indicated different characteristics with spray-dried *Lc. cremoris*, as demonstrated by the formation of different cluster (Figure 2). *Lactococcus cremoris* after freeze-drying and freeze-dried *Lc. cremoris* after storage for 10 wk in a foil pouch containing NaOH showed high similarities. Freeze-dried *Lc. cremoris* after storage in a foil pouch containing silica gel had similar characteristics with that of the control, and was in one cluster with *Lc. cremoris* after storage in a foil pouch containing LiCl. Interestingly, microencapsulated *Lc. cremoris* after spray-drying showed different characteristics than spray-dried *Lc. cremoris* after storage regardless of a_w adjustment, as demonstrated by the formation of different sub clusters. In addition, fresh *Lc. cremoris* was the most isolated, indicating its difference in characteristics as compared to microencapsulated *Lc. cremoris* after drying and subsequent storage. Even though classification of bacteria based on their cell envelopes and secondary protein structures has been established by Helm et al. (1991) and Dziuba et al. (2007), specific studies related to the similarities of microencapsulated bacteria after dehydration and after subsequent storage have never been carried out.

Glass Transition Temperature and Residual Moisture Content of Microcapsules

Glass transition temperature and residual moisture content (**RM**) of microcapsules (containing *Lb. acidophilus* and *Lc. cremoris*) after spray- or freeze-drying are shown in Table 4, whereas those of freeze- or spray-dried microcapsules after storage (10 wk, 25°C, in foil pouches containing different desiccators) are shown in Table 5. The T_g of the microcapsules after spray-drying was lower than that of microcapsules after freeze-drying, whereas the opposite trend occurred for RM. Higher RM of microcapsules after spray-drying than that of microcapsules after freeze-drying was due to relatively low outlet temperature of spray drying (50°C); therefore, reducing the residual water by storage at low a_w was essential. Similarly, RM of spray- or freeze-dried microcapsules increased significantly ($P = 0.0006$), along with significant decrease in T_g ($P = 0.0008$) due to storage in a foil pouch using different desiccators. Storage in a foil pouch using NaOH or LiCl resulted in relatively higher T_g of microcapsules than T_g using silica gel, with the exception of storage of freeze-dried microcapsules containing *Lc. cremoris* kept under LiCl. However, all of the different desiccants showed microcapsule T_g >25°C.

It has been widely reported that in a glassy or amorphous state, dehydrated products have liquid characteristics, with random molecule position but high viscosity ($\geq 10^{12}$ Pa·s); thus, molecular mobility is limited (Roos, 2002). This state is unstable and is temperature-dependent. At a certain temperature (known as T_g), the transformation from a solid-like to a liquid-like state initiates along with an increase in molecular mobility; this phenomenon is recognized as a glass transition (Santivarangkna et al., 2011). Therefore, storage at temperature below T_g is considered to be useful in maintaining products in their amorphous

Table 4. Glass transition temperature (T_g) and residual moisture content (RM) of *Lactobacillus acidophilus* and *Lactococcus lactis* ssp. *cremoris* after freeze-drying (FD) and spray-drying (SD)

	Lb. acidophilus		*Lc. cremoris*	
Item	T_g (°C)	RM (%)	T_g (°C)	RM (%)
After FD	50.0[a]	3.0[b]	42.0[a]	2.9[b]
After SD	41.2[b]	4.0[a]	40.3[a]	3.2[a]
SEM	2.56	0.34	1.58	0.08

[a,b]Means followed by the same letters indicate no statistical difference ($P \geq 0.05$).

Table 5. Glass transition temperature (T_g) and residual moisture content (RM) of freeze-dried (FD) or spray-dried (SD) *Lactobacillus acidophilus* and *Lactococcus lactis* ssp. *cremoris* after 10 wk of storage (25°C) in foil pouches containing different desiccators

	Lb. acidophilus				*Lc. cremoris*			
	T_g (°C)		RM (%)		T_g (°C)		RM (%)	
Desiccator	SD	FD	SD	FD	SD	FD	SD	FD
NaOH	47.6^{ab}	53.5^{a}	2.6^{de}	2.2^{e}	50.3^{a}	47.3^{ab}	1.7^{d}	2.2^{d}
LiCl	42.8^{bc}	42.7^{bcd}	3.3^{cd}	3.4^{cd}	47.4^{ab}	30.0^{d}	2.7^{cd}	4.5^{ab}
Silica gel	41.1^{cde}	35.2^{e}	4.2^{bc}	4.5^{ab}	44.6^{b}	28.1^{d}	3.6^{bc}	5.0^{ab}
Control	39.8^{cde}	36.5^{de}	5.2^{a}	5.0^{ab}	40.8^{c}	27.7^{d}	4.5^{ab}	5.2^{a}
SEM	1.46		0.27		2.29		0.33	

[a–e]Means followed by the same letters indicate no statistical difference ($P \geq 0.05$).

state. In addition to T_g, storage at low a_w, particularly at its monolayer state, was effective in extending the shelf life of products (Rahman, 2010). Water activity and T_g of freeze-dried matrix containing lactobacilli has been proven to influence the survival of lactobacilli (Kurtmann et al., 2009). An increase in a_w and moisture content results in decrease in T_g (Kurtmann et al., 2009, Pehkonen et al., 2008, Roos, 1995), and vice versa. The second order transition, from a glassy to a rubbery state, likely occurs due to moisture adsorption during storage at higher a_w. Therefore, we hypothesized that freeze- or spray-dried bacteria kept at low a_w (using desiccators) would have relatively higher T_g of mixture than storage temperature, hence glass transition would not have taken place during room temperature storage. Our results showed that all T_g were higher than room temperature, which might reflect that no glass transition occurred at 25°C.

Glass transition temperature determination is critical for this study, as we fortified mannitol into the formulation, whereas mannitol has a low T_g (i.e., 12.6°C; Yu et al., 1998). However, due to the presence of casein as a main component (T_g of 120°C at a_w 0.11 at storage at 22.5°C; Mauer et al., 2000), we expected the T_g of the mixture would be higher than the room temperature we used in this study. The combination of mannitol with sodium caseinate appeared useful to increase T_g of the mixture due to the high T_g of sodium caseinate. A similar study showed that incorporation of skim milk into disaccharides increased T_g of freeze-dried *Geotrichum candidum* (Hamoudi et al., 2007). In our study, no T_g of pure crystalline mannitol (at 10°C) was detected, indicating that mannitol strongly interacted with other substances (Kalichevsky and Blanshard, 1992; Taylor and Zografi, 1998). However, storage above room temperature does not ensure the stability of encapsulated products, as amorphous matrix of microencapsulants is one of several factors influencing the stability of the bacteria (Ananta et al., 2005; Higl et al., 2007). It is still controversial whether glass transition is more important than molecular interaction in preserving dehydrated biomaterials, or vice versa; the relationship between those factors has been proposed by Taylor and Zografi (1998). The authors stated that lower glass transition of matrix could be due to less hydrogen bonding involvement in glassy state, and, thus, it affected T_g; our results are in agreement with those of Taylor and Zografi (1998), Garzon-Rodriguez et al., (2004), and Maury et al. (2005). For instance, storage at low a_w using NaOH as a desiccant provided a relatively high T_g (Table 5) as well as lower frequencies of P=O symmetrical (Table 1 and 2), indicating stronger hydrogen bonding interaction between P=O of cell envelopes and sugars (Santivarangkna et al., 2010). However, Breen et al. (2001) stated that T_g is more important than chemical interaction to protect cells; this is in disagreement with our results. In fact, alteration of wavenumbers of FA (Table 1, 2) and secondary proteins (Table 3) still occurred after 10 wk of storage in a foil pouch using different desiccants, even though all T_g values were higher than room temperature of storage. Water activity appeared to have an important role on these phenomena; in this regard our results are similar to that of Garzon-Rodriguez et al. (2004). In addition, Maury et al. (2005) demonstrated that protein stabilization by sorbitol and trehalose occurred through water replacement mechanism instead of amorphous state. Yet, storage in a foil pouch using NaOH is likely preferable to preserve the glassy state of freeze- or spray-dried microcapsules owing to a wide range of actual room temperatures (20–35°C).

CONCLUSIONS

Our FTIR study showed that all microcapsules interacted with P=O of phospholipid bilayers of the cell envelopes of *Lb. acidophilus* and *Lc. cremoris* after spray- or freeze-drying. After 10 wk of storage, the type of

desiccant used (indicating the difference in a_w) seemed to affect the FA and secondary protein structures of microencapsulated bacteria. Study on glass transition using DSC demonstrated that T_g of encapsulated *Lb. acidophilus* and *Lc. cremoris* after freeze-drying was higher than that after spray-drying. The type of desiccant used during 10 wk of storage had significant effect on T_g of dehydrated *Lb. acidophilus* and *Lc. cremoris*. This study demonstrated that even though no glass transition was detected at storage at 25°C, changes in cell envelopes and secondary protein structures could still occur.

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Chapter 11: General conclusions and future directions

This study was carried out to investigate the influence of microencapsulating materials and drying methods on survival, acid and bile tolerance, surface hydrophobicity and retention of some enzymes of probiotic bacteria during storage for 10 weeks at room temperature (25 ^{o}C) at low a_ws. Protective mechanism and microstructures of microencapsulating materials containing mannitol were also studied. *B. animalis* ssp. *lactis* Bb12, *B. longum* 1941, *L. acidophilus* 2401 and *L. lactis* subsp. *cremoris* R-704 were used in this study.

Alginate-based formulation was not effective as coating materials in improving survival, acid and bile tolerance and SHb of *B. animalis* ssp. *lactis* Bb12 after freeze drying; its viability in alginate-mannitol after freeze drying was only 6.6 log CFU/g. Freeze drying also decreased activity of β-glu, β-gal, LDH, PK, HK, ATPase; percent enzyme activity retention was enzyme-dependent. Incorporation of mannitol into alginate system improved stability of freeze dried bacteria during storage at low a_ws as survival was higher after storage at a_w of 0.07, 0.1 and 0.2 than that in aluminium foil (control). Alginate alone and alginate-mannitol microcapsules and a_w affected the retention of *β*-glu, *β*-gal, HK, and ATPase, but not of LDH and PK. On the other hand, GM and G formulations maintained viability of *B. animalis* ssp *lactis* Bb12 after spray drying (8.9 and 8.7 log CFU/g, respectively); but G was less effective than GM in preserving the bacteria during 10 weeks of storage (25^{o}C). Storage of spray dried *B. animalis* Bb12 in GM formulation at a_w of 0.07 increased bacterial survival, acid and bile tolerance as well as maintained the surface hydrophobicity and activities of β-glu, β-gal, LDH, PK, HK, ATPase during 10 week storage.

To ascertain the effectiveness of casein and mannitol as coating materials, the effect of protein-based (SM, CAS, WPC-CAS, WPC) or SPI combined with sugar-based (GLY, MAN or

MD) formulations on survival of *B. longum* 1941 was observed. Milk proteins along with sugar alcohols appeared more effective in improving the bacterial survival, acid and bile tolerance as well as in maintaining SHb, β-glu, LDH and ATPase activities during freeze drying as compared to SPI and MD. This explains why sodium caseinate – based formulation combined with mannitol were the most effective in improving stability of *B. animalis* ssp *lactis* Bb12.

The effectiveness of GM in increasing survival of bifidobacteria led us to apply this formulation to *L. acidophilus* (probiotic bacteria) and *L. lactis* ssp. *cremoris* (very sensitive LAB to acid and bile environment). Storage of freeze- or spray-dried bacteria coated with GM kept in aluminium foil pouch containing different desiccants was carried out. Survival, acid and bile tolerance, SHb and β-gal of freeze dried and spray dried *L. acidophilus* and *L. lactis* ssp. *cremoris* were relatively high when packed in foil pouch containing NaOH or LiCl. Freeze drying was superior to spray drying in preserving microencapsulated bacteria. The longest storage period at 25^{o}C to achieve a minimum requirement of bacterial population of 10^{7} CFU/g is predicted to be between 39-46 weeks depending upon the type of bacteria and a_w used for storage.

The protective mechanism of microencapsulation as studied by FTIR showed that mannitol in alginate-based system was able to protect cell envelopes of *B. animalis* ssp. *lactis* Bb12 during freeze drying through interaction of polar sites of phospholipid bilayers of bacterial cell envelopes *via* H-bond, but conformational changes of secondary protein structures still occurred. Even though alginate-mannitol showed less alteration of wavenumbers of functional groups of C-H and amide II as compared to alginate only, changes in fatty acids and secondary protein structures after 10 week of storage at low a_w (0.07-0.2) were noticed. This suggests that the decrease in survival occurred after the storage regardless of the type of microcapsules and a_w.

Interactions between P=O of phospholipid bilayers of cell envelopes of *B. animalis* ssp. *lactis* Bb12 and mannitol of GM formulation were also detected. Fatty acids and secondary proteins of spray dried *B. animalis* ssp. *lactis* Bb12 in GM formulation were preserved after drying and after 10-week storage at a_w of 0.07; this might contribute to high survival of the bacteria after the storage. Storage at a_w of 0.2 and without controlled a_w resulted in conformational changes of cell envelope and secondary proteins of spray dried bacteria in GM or G formulation. Similarly, interactions between GM microcapsules and P=O of cell envelopes of *L. acidophilus* and *L. cremoris* were observed after spray- or freeze-drying. Storage of the microcapsules within foil pouch containing NaOH or LiCl as desiccants maintained the stability of fatty acids of cell envelopes, while changes in fatty acids occurred in foil pouch containing silica gel. Secondary protein structures of *L. cremoris* were more sensitive to drying method and storage at various desiccants than that of *L. acidophilus* as indicated by alteration of wavenumbers of amide I. Hierarchical cluster analysis demonstrated that the functional groups of the bacteria after freeze-drying or spray-drying and those of *L. acidophilus* or *L. cremoris* after storage at a_w of 0.07 and 0.1 had high similarities. Glass transition analysis showed that T_g of GM microcapsules after freeze drying and spray drying were different. The type of desiccant influenced T_g and moisture content of *L. acidophilus* after 10 weeks of storage; while drying method affected T_g of *L. cremoris* after storage for the same period. This study demonstrated that conformational changes in fatty acids and secondary protein structures of *L. acidophilus* and *L. cremoris* took place at T_g above 25 ^oC.

Microstructure of alginate-based and casein-based microcapsules of *B. animalis* ssp *lactis* Bb12 afer freeze- or spray drying, respectively, and after storage at a_w of 0.07 was also studied. Surfaces of alginate-based microcapsules appeared dense and relatively rough after freeze-drying

with few wrinkles. After 10 weeks of storage, the wrinkles were more obvious due to residual moisture removal during storage at a_w of 0.07. Incorporation of mannitol into alginate gel improved the smoothness of bead surface. The absence of *B. animalis* ssp *lactis* Bb12 on the microcapsule surface indicated that they were trapped within the alginate matrices. On the other hand, spray drying resulted in globular structures of microcaspules coating *B. animalis* ssp *lactis* Bb12 within; GM resulted in relatively larger size of microcapsule than G formulation. Storage at a_w of 0.07 of spray dried microcapsules produced compact structure of microcapsules; the compactness decreased and the size was relatively larger when spray dried GM were kept in an aluminium foil without a_w adjustment.

Microencapsulation using casein-based system combined with mannitol was the most effective in protecting *B. animalis* ssp *lactis* Bb12, *B. longum* 1941, *L. acidophilus* and *L. lactis* ssp. *cremoris* after spray- or freeze drying followed by storage at room temperature at low a_w and during exposure to acid and bile environments. This study focused on *in vitro* environments which were set up at extreme conditions prevalent in gastrointestinal tract. Future studies should (i) consider stability of microencapsulated probiotic bacteria *in vivo* (stomach, duodenum and colon) using animals as model, (ii) assess aspects of packaging for microencapsulated bacteria to minimize contact with atmospheric moisture and O_2; the presence of salts within semipermeable membrane as desiccant may not be necessary, (iii) expand the scope of the study by including storage temperatures other than 25^oC and identify maximum temperature of storage corresponding to the desired viability. To ascertain the *in vivo* stability, the microencapsulated probiotic bacteria with known concentration should be delivered to the stomach of fasting animals through force-feeding method. As a comparison, a certain proportion of microencapsulated probiotic incorporated into meal could also be observed to study the effect of

foods on viability of the observed bacteria in the stomach. The total contents of stomach, duodenum and colon should be directly collected after slaughtering for isolation and determination of the probiotic bacteria.