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 (Tg) 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 Tg ≤25°C was detected. This study demonstrated that the changes in FA and secondary structures of the microencapsulated bacteria still occurred during storage at Tg above room temperature, indicating that the glassy state did not completely prevent chemical activities.

Key words: desiccant, glass transition temperature (Tg), 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)
stated that certain levels of water activity (a_w) at room

temperature contributed to conformational disorder of
diplytanoylphosphatidylcholine. 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 below a certain
level of water activity (a_w) might cause the changes in cell envelopes and sec-
dary structure of proteins of bacteria.

Apart from the molecular interaction between cell enve-
lopes and microencapsulants, the physical state of the
microcapsule matrix is also of great importance 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 (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 for-
mation 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 pel-
el 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), fructose-saccharides 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 super-
mart. 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 Dehumidi-
fier B296 (humidity 86%; temperature −3°C; Buchi).
The outlet temperature was 50°C, hence the inlet tem-
perature 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 tem-
perature 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 es-

tablished, 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 be-
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 stor-
age 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 CH₃ (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⁺(CH₃)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 Tg 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⁺(CH₃)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.
drogen bond) interaction (Cacela and Hincha, 2006). Frequencies at 1075 and 1073 cm\(^{-1}\) indicated the vibration of P=O symmetric of fresh \textit{Lb. acidophilus} and \textit{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 \textit{Lb. acidophilus} were shown in Table 1. A peak alteration of C–H asymmetric of \textit{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 \textit{Lb. acidophilus} at 2967 for spray-dried \textit{Lb. acidophilus}, along with peak broadening. Conversely, no obvious alteration was detected of C–H frequency of microencapsulated \textit{Lc. cremoris} during storage at low \(a_w\) of microencapsulant on phospholipid bilayers of \textit{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 Santivaramkna 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 \textit{Lb. acidophilus} and \textit{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 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 (Gradelnik and Hadzi, 1998) via sugar hydroxyl–lipid headgroups (Ricker et al., 2003).

The \(N^+\text{(CH}_3\text{)}_3\) asymmetric stretching vibration of the choline terminal of spray- and freeze-dried \textit{Lb. acidophilus} after storage, as well as that of spray-dried and freeze-dried \textit{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 \textit{Lb. acidophilus} and 947 for \textit{Lc. cremoris}). The \(N^+\text{(CH}_3\text{)}_3\) of spray-dried \textit{Lb. acidophilus} demonstrated a higher frequencies than that of freeze-dried \textit{Lb. acidophilus}. The \(N^+\text{(CH}_3\text{)}_3\) of freeze-dried \textit{Lc. cremoris} was commonly higher than that of spray-dried \textit{Lc. cremoris}, respectively.
### Table 1. Second derivative of spectra of cell envelopes of microencapsulated *Lactobacillus acidophilus* (La; means ± SD)\(^1\)

<table>
<thead>
<tr>
<th>Functional group(^2)</th>
<th>Freeze-dried (FD)</th>
<th>Spray-dried (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After FD</td>
<td>NaOH</td>
</tr>
<tr>
<td>CH(_3) asym</td>
<td>2.972 ± 0.3</td>
<td>2.971 ± 0.3</td>
</tr>
<tr>
<td>CH(_3) sym</td>
<td>2.884 ± 0.5</td>
<td>2.886 ± 0.5</td>
</tr>
<tr>
<td>N(^+)(CH(_3))(_3) asym</td>
<td>968 ± 0.3</td>
<td>967 ± 0.5</td>
</tr>
<tr>
<td>P=O sym</td>
<td>1.047 ± 0.2</td>
<td>1.014 ± 0.5</td>
</tr>
<tr>
<td>C=O</td>
<td>1.747 ± 0.5</td>
<td>1.747 ± 0.3</td>
</tr>
</tbody>
</table>

\(^1\)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 without 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 microencapsulated *Lactococcus lactis* ssp. *cremoris* (Lc; means ± SD)\(^1\)

<table>
<thead>
<tr>
<th>Functional group(^2)</th>
<th>Freeze-dried (FD)</th>
<th>Spray-dried (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After FD</td>
<td>NaOH</td>
</tr>
<tr>
<td>CH(_3) asym</td>
<td>2.965 ± 0.2</td>
<td>2.964 ± 0.3</td>
</tr>
<tr>
<td>CH(_3) sym</td>
<td>2.881 ± 0.2</td>
<td>2.880 ± 0.2</td>
</tr>
<tr>
<td>N(^+)(CH(_3))(_3) asym</td>
<td>981 ± 0.2</td>
<td>992 ± 0.3</td>
</tr>
<tr>
<td>P=O sym</td>
<td>1.055 ± 0.1</td>
<td>1.055 ± 0.1</td>
</tr>
<tr>
<td>C=O</td>
<td>1.720 ± 0.5</td>
<td>1.720 ± 0.5</td>
</tr>
</tbody>
</table>

\(^1\)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 without 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 *L. cremoris* in silica gel or without any desicant 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+(CH₃)₃ stretching vibration of freeze- or spray-dried *Lb. acidophilus* and *L. 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 (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+(CH₃)₃ stretching vibration as a part of polar/apolar interfacial of bacterial phospholipids varies between 1716 and 1750 cm⁻¹ (Erulkhovitch 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.
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 aw 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 aw 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 N₂ 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).

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

<table>
<thead>
<tr>
<th>Species and treatment¹</th>
<th>Wavenumber (cm⁻¹)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lb. acidophilus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After FD</td>
<td>1,650.3 ± 0.3</td>
<td>α-Helix</td>
</tr>
<tr>
<td>FD: NaOH</td>
<td>1,650.2 ± 0.3</td>
<td>α-Helix</td>
</tr>
<tr>
<td>FD: LiCl</td>
<td>1,624.2 ± 0.2</td>
<td>β-Sheet</td>
</tr>
<tr>
<td>FD: silica gel</td>
<td>1,629.3 ± 0.3</td>
<td>β-Sheet</td>
</tr>
<tr>
<td>FD: control</td>
<td>1,629.9 ± 0.4</td>
<td>β-Sheet</td>
</tr>
<tr>
<td></td>
<td>1,668.0 ± 0.2</td>
<td>β-Turn</td>
</tr>
<tr>
<td>After SD</td>
<td>1,649.2 ± 0.2</td>
<td>α-Helix</td>
</tr>
<tr>
<td>SD: NaOH</td>
<td>1,655.1 ± 0.1</td>
<td>α-Helix</td>
</tr>
<tr>
<td>SD: LiCl</td>
<td>1,656.3 ± 0.3</td>
<td>α-Helix</td>
</tr>
<tr>
<td>SD: silica gel</td>
<td>1,638.0 ± 0.3</td>
<td>β-Sheet</td>
</tr>
<tr>
<td>SD: control</td>
<td>1,633.0 ± 0.2</td>
<td>β-Sheet</td>
</tr>
<tr>
<td></td>
<td>1,670.0 ± 0.5</td>
<td>β-Turn</td>
</tr>
<tr>
<td>Fresh</td>
<td>1,654.2 ± 0.1</td>
<td>α-Helix</td>
</tr>
<tr>
<td><strong>Lc. cremoris</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After FD</td>
<td>1,649.8 ± 0.5</td>
<td>No order</td>
</tr>
<tr>
<td></td>
<td>1,092.0 ± 0.2</td>
<td>β-Sheet</td>
</tr>
<tr>
<td>FD: NaOH</td>
<td>1,649.9 ± 0.2</td>
<td>No order</td>
</tr>
<tr>
<td>FD: LiCl</td>
<td>1,689.0 ± 0.2</td>
<td>β-Sheet</td>
</tr>
<tr>
<td>FD: silica gel</td>
<td>1,650.1 ± 0.2</td>
<td>α-Helix</td>
</tr>
<tr>
<td>FD: control</td>
<td>1,642.0 ± 0.2</td>
<td>No order</td>
</tr>
<tr>
<td></td>
<td>1,689.5 ± 0.5</td>
<td>β-Turn</td>
</tr>
<tr>
<td>After SD</td>
<td>1,656.1 ± 0.2</td>
<td>α-Helix</td>
</tr>
<tr>
<td>SD: NaOH</td>
<td>1,646.0 ± 0.2</td>
<td>No order</td>
</tr>
<tr>
<td>SD: LiCl</td>
<td>1,684.4 ± 0.4</td>
<td>β-Turn</td>
</tr>
<tr>
<td>SD: silica gel</td>
<td>1,642.2 ± 0.2</td>
<td>No order</td>
</tr>
<tr>
<td>SD: control</td>
<td>1,648.2 ± 0.2</td>
<td>No order</td>
</tr>
<tr>
<td></td>
<td>1,688.3 ± 0.3</td>
<td>β-Turn</td>
</tr>
<tr>
<td>Fresh</td>
<td>1,651.1 ± 0.1</td>
<td>α-Helix</td>
</tr>
</tbody>
</table>

¹After 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.
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 different 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 clusters (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 aw 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 desiccants) are shown in Table 5. The Tg 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 aw was essential. Similarly, RM of spray- or freeze-dried microcapsules increased significantly (P = 0.0006), along with significant decrease in Tg (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 Tg of microcapsules than Tg 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 Tg >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 (≥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 Tg), 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 Tg is considered to be useful in maintaining products in their amorphous state.

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

<table>
<thead>
<tr>
<th>Item</th>
<th>Lb. acidophilus</th>
<th>Lc. cremoris</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tg (°C)</td>
<td>RM (%)</td>
</tr>
<tr>
<td>After FD</td>
<td>50.0^a</td>
<td>3.0^a</td>
</tr>
<tr>
<td>After SD</td>
<td>41.2^b</td>
<td>4.0^b</td>
</tr>
<tr>
<td>SEM</td>
<td>2.56</td>
<td>0.34</td>
</tr>
</tbody>
</table>

Means followed by the same letters indicate no statistical difference (P ≥ 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

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**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.

<table>
<thead>
<tr>
<th>Desiccator</th>
<th>Lb. acidophilus</th>
<th>Lc. cremoris</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( T_g ) (°C)</td>
<td>RM (%)</td>
</tr>
<tr>
<td>NaOH</td>
<td>SD 47.6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>53.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>FD 53.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.2&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>LiCl</td>
<td>SD 42.8&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>42.7&lt;sup&gt;bd&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>FD 42.7&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>3.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Silica gel</td>
<td>SD 41.1&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>35.2&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>FD 41.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>SD 39.8&lt;sup&gt;de&lt;/sup&gt;</td>
<td>36.5&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>FD 39.8&lt;sup&gt;de&lt;/sup&gt;</td>
<td>5.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>SEM</td>
<td>1.46</td>
<td>0.27</td>
</tr>
</tbody>
</table>

<sup>a–e</sup>Means followed by the same letters indicate no statistical difference (\( P \geq 0.05 \)).
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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REFERENCES


Cluster analysis of protein Fourier transform infrared spectra.
Biopolymers 27:1831–1839.

Lu, J., X.-J. Wang, Y.-X. Liu, and C.-B. Ching. 2007. Thermal and
FTIR investigation of freeze-dried protein-excipient mixtures. J.


Mauer, A. 2006. Secondary structural changes of spray dried pro-
teins with Fourier transform infrared spectroscopy: Doctoral Diss.
Department of Pharmaceutics, Friedrich-Alexander University Er-
uni-erlangen.de/publications/12_Mauerer_06.pdf.

Spray-drying of proteins: effects of sorbitol and trehalose on ag-
gregation and FT-IR amide I spectrum of an immunoglobulin G.

Mobili, P., A. Londero, T. M. R. Maria, M. E. S. Eusebio, G. L. D.
Antoni, R. Fausto, and A. Gomez-Zavaglia. 2009. Characterization of S-layer proteins of Lactobacillus by FTIR spectroscopy and dif-

Oldenhof, H., W. F. Wolkers, F. Fonseca, S. Passot, and M. Marin.
2005. Effect of sucrose and maltodextrin on the physical proper-
ties and survival of air-dried Lactobacillus bulgaricus: An in situ

2008. State transition ad physicochemical aspects of cryoprotec-
tion and stabilization in freeze-drying of L. rhamnosus GG (LGG).

Papova, A. V., and D. Hincha. 2007. Effects of cholesterol on dry bi-
layers: interactions between phosphatidylcholine unsaturation and
glycolipid or free sugar. Biophys. J. 93:1204–1214.

Popova, A. V., and D. Hincha. 2003. Intermolecular interactions in dry and rehydrated pure and mixed bilayers of phosphatidylchlo-
line and digalactosyldiacylglycerol: A Fourier transform infrared

Rahman, M. S. 2010. Food stability determination by macro-micro
region concept in the state diagram and by defining a critical tem-

Ricker, J. V., N. M. Tsvetkova, W. F. Wolkers, C. Leidy, F. Tablin, M.
Longo, and J. H. Crowe. 2003. Trehalose maintains phase separa-
tion in an air-dried binary lipid mixture. Biophys. J. 84:3045–
3051.

Riveros, B., J. Ferrer, and R. Borquez. 2009. Spray-drying of a vagi-
nal probiotic strain of Lactobacillus acidophilus. Drying Technol.

Roos, Y. H. 2002. Importance of glass transition and water activity
to spray-drying and stability of dairy powders. Lait 82:475–484.

Santivarangkna, C., M. Aschenbrener, U. Kulozik, and P. Foerst.
2011. Role of glassy state on stabilities of freeze-dried probiotics.
J. Food Sci. 76:R152–R156.

Protective effects of sorbitol during the vacuum-drying of Lacto-

Damage of cell envelope of Lactobacillus helveticus during vacuum-

Savic, D., N. Jokovic, and L. Topisirovic. 2008. Multivariate statisti-
cal methods for discrimination of lactobacilli based on their FTIR

Schille, S., W. Friess, K. Bechhold-Peters, and P. Garidel. 2007. Con-
formational analysis of protein secondary structure during spray-
drying of antibody/mannitol formulations. Eur. J. Pharm. Bio-
pharm. 65:1–9.

on protein-mannitol interactions: The physical state of mannitol
and protein structure in the dried state. AAPS PharmSciTech
5:58–69.

interactions in lyophilized amorphous mixtures. J. Pharm. Sci.
87:1615–1621.

Telang, C., L. Yu, and R. Suryanarayanan. 2003. Effective inhibition of
mannitol crystallization in frozen solutions by sodium chloride.

Tzannis, S. T., and J. J. Prestrelski. 1999. Activity-stability consider-
ations of trypsinogen during spray-drying: Effects of sucrose. J.

van den Bogaart, G., N. Hermans, V. Krasnikov, A. H. d. Vries, and
B. Poolman. 2007. On the decrease in lateral mobility of phospho-

Vinderola, C. G., and J. A. Reinheimer. 2003. Lactic acid starter and
probioitic bacteria, a comparative “in vitro” study of probiotic
characteristics and biological barrier resistance. Food Res. Int.
36:895–904.

glass properties of D-mannitol using sorbitol as an impurity.

ure content on