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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

Dianawati Dianawati,* Vijay Mishra,† and Nagendra P. Shah‡[1]
*Tribhuwana Tunggadewi University, Jalan Telaga Warna, Malang 65145, East Java, Indonesia
†School of Biomedical and Health Sciences, Victoria University, Werribee campus, PO Box 14428, Melbourne, Victoria 8001, Australia
‡Food and Nutrition Science, School of Biological Science, The University of Hong Kong, Pokfulam Road, Hong Kong

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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[1]Corresponding author: npshah@hku.hk

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).

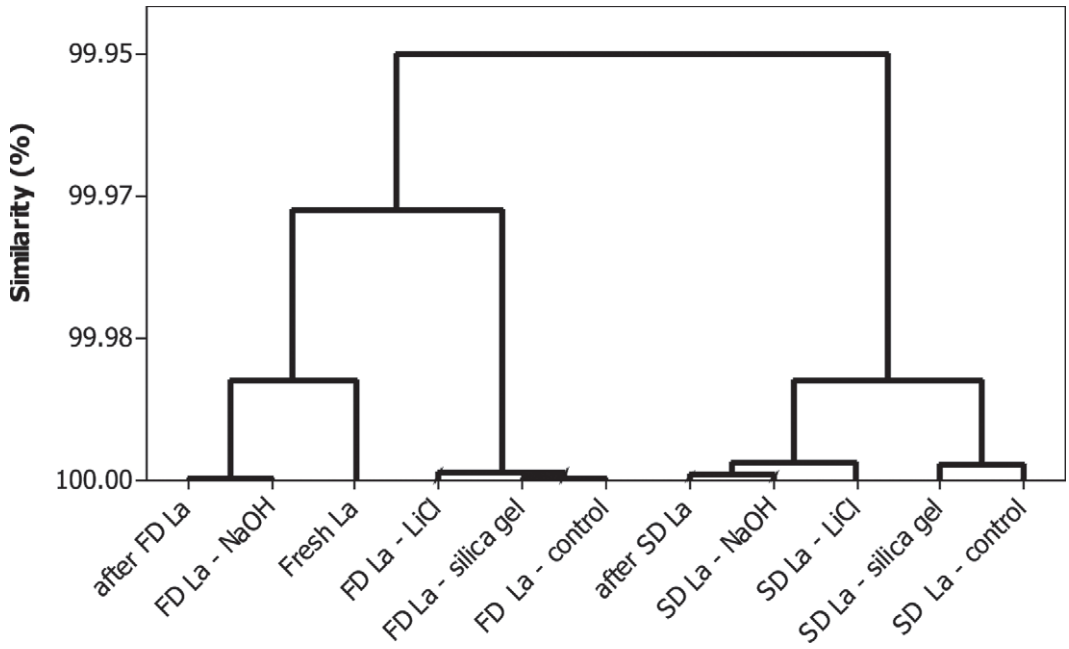

Figure 1. Classification of microencapsulated *Lactobacillus acidophilus* (La) after drying and after storage at room temperature. After FD/after SD La = microencapsulated La after freeze drying/spray drying; FD/SD La - NaOH = microencapsulated La (under FD/SD) after storage in foil pouch containing 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.

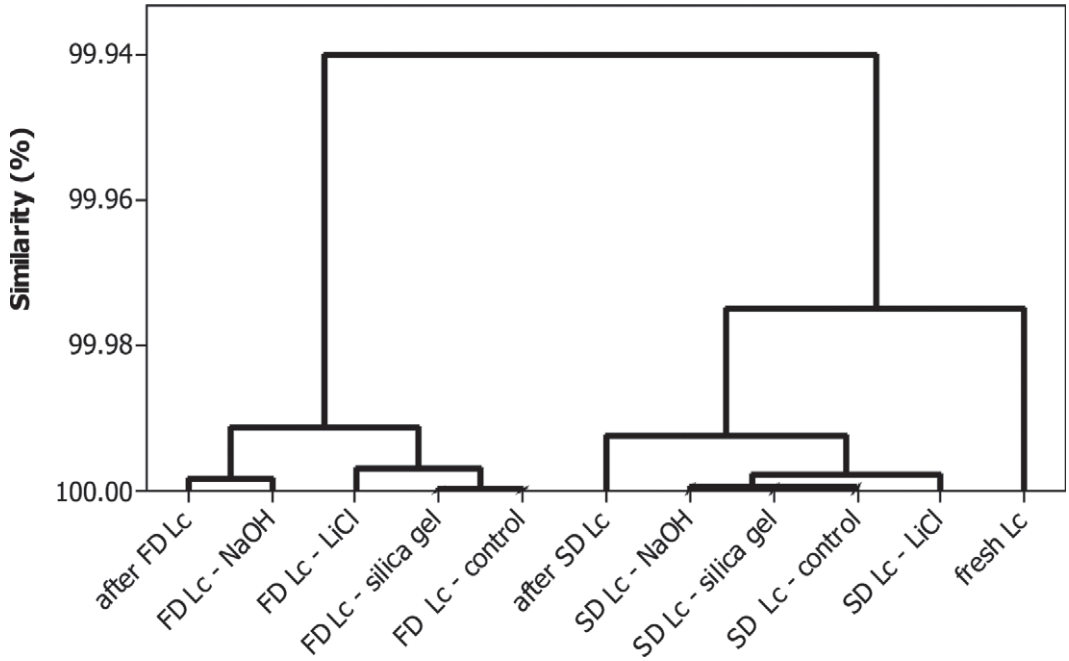

Figure 2. Classification of microencapsulated *Lactococcus lactis* ssp. *cremoris* (Lc) after drying and after storage at room temperature. After FD/after SD Lc = microencapsulated Lc after freeze drying/spray drying; FD/SD Lc - NaOH = microencapsulated Lc (under FD/SD) after storage in foil pouch containing 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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