A low membrane lipid phase transition temperature is associated with a high cryotolerance of *Lactobacillus delbrueckii* subspecies *bulgaricus* CFL1

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ABSTRACT

The mechanisms of cellular damage that lactic acid bacteria incur during freeze-thaw processes have not been elucidated to date. Fourier transform infrared spectroscopy was used to investigate in situ the lipid phase transition behavior of the membrane of *Lactobacillus delbrueckii* ssp. *bulgaricus* CFL1 cells during the freeze-thaw process. Our objective was to relate the lipid membrane behavior to membrane integrity losses during freezing and to cell-freezing resistance. Cells were produced by using 2 different culture media: de Man, Rogosa, and Sharpe (MRS) broth (complex medium) or mild whey-based medium (minimal medium commonly used in the dairy industry), to obtain different membrane lipid compositions corresponding to different recovery rates of cell viability and functionality after freezing. The lipid membrane behavior studied by Fourier transform infrared spectroscopy was found to be different according to the cell lipid composition and cryotolerance. Freeze-resistant cells, exhibiting a higher content of unsaturated and cyclic fatty acids, presented a lower lipid phase transition temperature (T_s) during freezing ($T_s = -8^\circ\text{C}$), occurring within the same temperature range as the ice nucleation, than freeze-sensitive cells ($T_s = +22^\circ\text{C}$). A subzero value of lipid phase transition allowed the maintenance of the cell membrane in a relatively fluid state during freezing, thus facilitating water flux from the cell and the concomitant volume reduction following ice formation in the extracellular medium. In addition, the lipid phase transition of freeze-resistant cells occurred within a short temperature range, which could be ascribed to a reduced number of fatty acids, representing more than 80% of the total. This short lipid phase transition could be associated with a limited phenomenon of lateral phase separation and membrane permeabilization. This work highlights that membrane phase transitions occurring during freeze-thawing play a fundamental role

in the cryotolerance of *Lb. delbrueckii* ssp. *bulgaricus* CFL1 cells.

Key words: lactic acid bacteria, cryoresistance, fatty acid, membrane lipid phase transition

INTRODUCTION

Concentrates of lactic acid bacteria (LAB) are widely used in the food industry as starters for manufacturing cheeses, fermented milks, meats, vegetables, as well as for health-benefit products (probiotics). Freezing and storage at low temperatures (-50°C) are commonly applied to preserve the viability of concentrates while maintaining their technological properties (acidifying activity, production of aroma compounds, and contribution to product texture). *Lactobacillus delbrueckii* ssp. *bulgaricus* CFL1, a gram-positive LAB commonly used in fermented milk products is particularly sensitive to freezing, which generally leads to a low recovery rate of its technological properties upon thawing (Fonseca et al., 2000; Fonseca et al., 2003; Rault et al., 2010).

Direct cell injury following freezing has been explained by the 2-factor hypothesis of Mazur et al. (1972). This hypothesis suggests that, at low cooling rates, ice crystals are formed outside the cell, resulting in an increase in the solute concentration in the extracellular medium. This cryo-concentration of solutes induces water transport from the intracellular medium of the cell to the extracellular medium and, thus, leads to cell dehydration and cell injury. At high cooling rates, the formation of intracellular ice would be primarily responsible for the death of different kinds of eukaryote cells (Mazur, 1977; Karlsson et al., 1993). However, no direct evidence of intracellular ice formation in cryopreserved bacteria has been presented. In contrast, it was clearly established that at very high cooling rates, no intracellular ice was formed in LAB, but injuries were caused by cell plasmolysis occurring during thawing (Fonseca et al., 2006). Moreover, at low cooling rates, freeze-fracture electron microscopy highlights dense cell packing in the cryo-concentrated matrix. Therefore, direct contact between cells and ice is extremely reduced, thus allowing the neglect of mechanical damage caused by extracel-

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lular ice crystal constraints and favoring dehydration as the main stress incurred by cells at low cooling rates. Lactic acid bacteria survival during freezing is, thus, highly dependent on the cellular biophysical event of cell dehydration and particularly on the membrane permeability to water. The membrane permeability is influenced by its fluidity property, which is, in turn, governed by the composition and the structure of the lipid bilayer (Stubbs, 1983).

The cell membrane thus plays a fundamental role in the freezing resistance of LAB. Some authors have established a correlation between membrane lipid composition and freeze tolerance of different strains. The viability after cell freezing of *Lb. delbrueckii* ssp. *bulgaricus* increases with the cyclopropane FA concentration in membranes (Smittle et al., 1974) and an increase in the ratio between unsaturated FA (UFA) and SFA is related to an improvement in the cryotolerance of *Lactococcus lactis* ssp. *lactis* and *Lactobacillus* spp. (Goldberg and Eschar, 1977) and *Streptococcus thermophilus* (Beal et al., 2001). The FA composition of the cellular membrane depends upon several environmental factors, including growth temperature, pH, culture medium, and composition, among others. Several researchers have, thus, attempted to modulate the membrane FA composition to improve cryotolerance of LAB by modifying fermentation conditions (Fonseca et al., 2001; Wang et al., 2005; Streit et al., 2007). It is also well established that the cell membrane is one of the primary sites for freezing injuries (Beney and Gervais, 2001; Moussa et al., 2008). However, research is still needed to elucidate the biophysical mechanisms responsible for cell degradation following freezing, and especially to relate them to membrane properties. Previous studies have established that freezing alters the physical state of lipids, thus modifying lipid organization and membrane fluidity (Wolkers et al., 2007; Balasubramanian et al., 2009; Ragoonanan et al., 2010). Biological membranes often exhibit a liquid crystalline-to-gel phase transition during cooling and vice versa during heating (Crowe et al., 1989; Drobnis et al., 1993). The consequences of such lipid phase transition are thought to include increased membrane leakage and lateral phase separation of membrane components (Drobnis et al., 1993; Tablin et al., 2001).

Fourier transform infrared (FTIR) spectroscopy is a suitable technique for studying in situ LAB cells (Oldenhof et al., 2005). Moreover, it has already been used to study freezing- and thawing-induced changes in the membrane lipid bilayer structure of mammalian cells (Oldenhof et al., 2011, 2013). The symmetric CH₂ stretching vibrations of lipids are commonly used to detect phase transitions in isolated biological membranes

and in whole cells (Crowe et al., 1989; Hoekstra and Golovina, 1999).

The aim of the present work was to relate membrane physical behavior to the freezing resistance of *Lb. delbrueckii* ssp. *bulgaricus* CFL1 cells. Two cell populations presenting different lipid membrane compositions and different resistances to freezing were generated by using 2 different culture media. Biological responses of frozen-thawed bacterial cells (cultivability, acidifying activity, and membrane integrity) were compared with those of fresh cells. Fourier transform infrared spectroscopy was used to investigate in situ changes in membrane lipid phase behavior of cells during freeze-thawing. The results presented here expand our understanding of the physical events occurring in LAB cell membranes during freeze-thaw cycles and their connection to cell cryoinjury.

MATERIALS AND METHODS

Materials, Reagents, and Chemicals

de Man, Rogosa, and Sharpe (MRS) broth and agar, mild whey powder, yeast extract, and skim milk powder were purchased from Biokar Diagnostics (Beauvais, France), Euroserum (Port-sur-Saône, France), Organotechnie SAS (La Courneuve, France), and EPI Ingredients (Ancenis, France), respectively. Propidium iodide (PI) was obtained from Sigma-Aldrich Chimie S.a.r.l. (Lyon, France).

Diatomaceous earth and cellulose filters were furnished by Dionex (Voisins-le-Bretonneux, France). Sand of 50- to 70-mesh particle size and trimethylsulfonium hydroxide (0.25 M in methanol) were provided by Sigma-Aldrich Chimie S.a.r.l. Potassium chloride, methanol, and chloroform stabilized with 0.6% ethanol were supplied by VWR International SAS (Fontenay-sous-Bois, France). Solvents were Normapur/analytical grade. Fatty acid standards were purchased either from Sigma-Aldrich Chimie S.a.r.l. or from Interchim (Montluçon, France) with a purity $\geq 99\%$.

Bacterial Strain and Growth Conditions

The lactic acid bacterium *Lb. delbrueckii* ssp. *bulgaricus* CFL1 was chosen due to its sensitivity to freezing (Fonseca et al., 2000; Rault et al., 2007). It was provided by the Laboratoire de Génie et Microbiologie des Procédés Alimentaires (INRA, Thiverval-Grignon, France). Inocula were stored at -80°C and were thawed for 5 min before inoculation at 40°C . Two different culture media [MRS broth (a rich medium) or mild whey-based medium (a less rich medium than MRS)]

were used to obtain bacterial populations exhibiting different membrane properties. The mild whey medium was composed of 60 g of whey powder/L and was heated at 110°C for 20 min. The supernatant obtained after centrifugation ($17,700 \times g$ for 30 min at 4°C) and filtration (330 nm) was supplemented with 5 g of yeast extract/L. The mild whey medium was then sterilized at 110°C for 20 min. The MRS broth was prepared by following the explanations written on the commercial box and sterilized at 118°C for 20 min. Preliminary experiments were carried out to determine the kinetics of bacterial growth in both culture media to ensure that the cells were harvested at the end of the exponential phase. Absorbance measurements at 600 nm were used to follow bacterial growth. These experiments made it possible to define the inoculation rates and the duration of the preculture and culture steps. So, after a preculture of 10 h in MRS broth at 40°C without shaking and with an inoculation at around 10^8 cfu/mL, 10 mL and 150 μ L of preculture were introduced into 300 mL of MRS broth and mild whey medium, respectively. *Lactobacillus delbrueckii* ssp. *bulgaricus* CFL1 was grown without shaking at 40°C until the end of the exponential phase was reached {10 h for mild whey medium [specific growth rate (μ) = 1.3 h^{-1}] and 13 h for MRS broth (μ = 0.4 h^{-1})}. The cell suspensions were then cooled down at 4°C in an ice-water bath and harvested by centrifugation ($17,700 \times g$ for 10 min at 4°C). The cellular pellets obtained either from the MRS or the mild whey cultures were resuspended at 4°C in the same weight of a sterile protective medium (i.e., 50% wt/wt). The protective medium was composed of 20% (wt/wt) sucrose in saline solution (8.9 g of NaCl/L). These protected bacterial suspensions were used for biological activity assessment and freezing experiments.

Lipid Extraction

Cellular lipids were extracted by means of accelerated solvent extraction (ASE 350; Dionex). The procedure was adapted from previously described work (Cescut et al., 2011). For each fermentation medium (MRS broth or mild whey medium), 3 cultures were subjected to extraction and for each culture, 3 extractions were performed. At the end of the fermentation, bacterial cells were collected by centrifugation ($17,700 \times g$ for 10 min at 4°C) and washed twice with saline solution at 4°C. About 0.1 to 0.2 g of washed pellet was precisely weighed and mixed with 2.0 g of diatomaceous earth as dispersant and desiccant to improve extraction performance. The mix was introduced into a stainless steel cell (5 mL). Three extraction cycles using 3 different chloroform/methanol solvent mixtures: 2:1; 1:1, and 1:2 (vol/vol) were performed. For each solvent mixture, 2

static cycles of 15 min were applied. The extraction solvent was pumped through the extraction cell fitted with a cellulose filter and a stainless steel frit at the outlet. Each stainless steel cell was maintained at a pressure of 10^7 Pa and a temperature of 100°C throughout the extraction. All resulting extracts of a sample were automatically combined in the same vial. At the end of the extraction, extracts were first evaporated to dryness in a vacuum centrifugal evaporator (RC 10-22) combined to a refrigerated trap (RCT 90; Jouan, Saint-Herblain, France), at 1,200 rpm and approximately 55°C. Dry samples were resuspended in 3 mL of chloroform and 3 mL of a potassium chloride solution [0.88% (wt/vol) KCl] was added. After vigorous manual shaking, the extracts were centrifuged (vacuum centrifugal evaporator; $480 \times g$ for 5 min at 25°C) to separate aqueous and organic phases. The organic phase containing membrane lipids was dried (vacuum centrifugal evaporator; RC 10-22; Jouan, Saint-Herblain, France; 1,200 rpm, heating at approximately 55°C). Dry lipids were then resuspended in 300 μ L of chloroform before GC-MS analysis. Fifty microliters of this solution was mixed with 25 μ L of internal standard C9:0 and 50 μ L of methylation reagent trimethylsulfonium hydroxide in vials for GC-MS analysis.

FA Analysis by GC-MS

Gas chromatography-mass spectrometry analysis was carried out on a Hewlett-Packard 6890 gas chromatograph equipped with a capillary column packed with 70% cyanopropyl polysilphenylene-siloxane BPX70 (60 m $\times$ 0.25 mm $\times$ 0.25 μ m; SGE Analytical Science Pty Ltd., Victoria, Australia), coupled to a mass selective detector (5973; Agilent Technologies, Avondale, PA). The carrier gas was helium (1.2 mL/min) and the column pressure was 1.3×10^5 Pa. Injection of 1 μ L of the vial was done splitless at an injector temperature of 250°C. The oven temperature was held for 1 min at 35°C and then increased from 35 to 100°C at 40°C/min, held for 1 min at 100°C and then increased from 100 to 130°C at 5°C/min, followed by an increase from 130 to 180°C at 1.5°C/min and finally from 180 to 240°C at 5°C/min. The detection temperature was 230°C. The FA methyl esters were first identified by comparison of their retention times with those of known standards. Identification of FA methyl esters was confirmed by using the mass selective detector at a scan rate of 3.14 scans/s, with data collected in the range of 33 to 500 amu. The mass spectra of the FA methyl esters were compared with the data bank Wiley 275.L (Hewlett-Packard, Gaithersburg, MD). The quantification of FA was calculated on target ions using external calibration performed with serial dilutions of commercial standards,

with C9:0 as internal standard. As Δ C19:0 (cyclic FA) could not be found as pure standard, its concentration was approximated using the response coefficient of the C18:1 FA (for which the response coefficient was equal to 0.25). All quantification results were converted into the mass of FA per cell mass unit on a dry basis.

DM of Cell Pellets

Freshly harvested cell pellets were washed twice with saline solution at 4°C. About 0.5 to 1 g of washed pellet was weighed precisely (± 0.1 mg) and placed at 104°C for 24 h. The dried pellet was weighed again and the DM could be calculated. This was done in triplicate for the 2 cell culture media (MRS broth and mild whey medium).

Freezing Experiments

Two milliliters of the protected bacterial suspensions was dispersed into 5-mL sterile tubes. Samples were then placed into a freezer at -80°C . The temperature inside the samples was monitored with thermocouples (type T; 0.2-mm diameter) to quantify the cooling rate, which was determined as $4^{\circ}\text{C}/\text{min}$. The samples were then stored at -80°C for at least 24 h before biological analysis. The samples were thawed for 2 min in a 42°C temperature-controlled water bath just before performing analysis.

Analysis of the Biological Activity

To determine the biological activity of the protected bacterial suspensions before and after freezing, 2 properties were measured: (1) the specific acidifying activity (t_{spe}) and (2) the membrane integrity. The measurements were carried out before and after freezing and thawing on the protected bacterial suspensions.

Specific Acidifying Activity Measurements. The Cinac system (AMS, Frépillon, France) was used to measure the acidifying activity of the protected suspensions of LAB before and after freezing (Spinnler et al., 1989; Picque et al., 1992; Beal et al., 2001). Acidifying activity measurements were performed in triplicate, using reconstituted dry skim milk at 42°C . The pH of inoculated milk samples was continuously measured, which allowed the calculation of its first-time derivative, representing the acidifying rate. For each sample, the time necessary to reach the maximum acidifying rate in milk (t_{m} ; in min) was used to characterize the acidifying activity of bacterial suspensions. The higher the t_{m} , the longer the latency phase and the lower the acidifying activity was.

Cell concentration (cfu/mL) was evaluated by plate counts. After serial dilutions in saline water, cells were plated onto solid MRS agar and incubated under anaerobic conditions (GENbox 96124; bioMérieux, Marcy l'Étoile, France) at 42°C for 48 h before cell counting. Results corresponded to geometrical means of at least 3 counts.

The t_{spe} [in min/log (cfu/mL)] was defined as the ratio of t_{m} to the corresponding log of the cell concentration (Streit et al., 2007). Consequently, the t_{spe} give an accurate and meaningful measurement of the biological activity of LAB, including the physiological state and viability. The t_{spe} was calculated before ($t_{\text{spe}c}$; in min) and after freezing ($t_{\text{spe}f}$; in min). As a result, dt_{spe} ($t_{\text{spe}f} - t_{\text{spe}c}$) characterized the loss in t_{spe} during freezing. An increase in the dt_{spe} corresponds to an increase of loss in t_{spe} during freezing.

Membrane Integrity Measurements. Propidium iodide uptake was successfully used to measure membrane permeabilization by flow cytometry in relation with inactivation kinetics of different microbial strains after various treatments (Ulmer et al., 2000; Wouters et al., 2001; Rault et al., 2007). This probe cannot penetrate within cells having intact membranes but can enter when cell membranes have been damaged. Inside the cells, it binds to the DNA and produces a red fluorescence after excitation at 488 nm. Before staining, cell suspensions of *Lb. delbrueckii* ssp. *bulgaricus* CFL1 were diluted in McIlvaine buffer (pH 7.3) to reach approximately 10^6 cells/mL. The McIlvaine buffer is composed of 0.1 M $\text{C}_6\text{H}_8\text{O}_7$ (citric acid) and 0.2 M Na_2HPO_4 (sodium phosphate). One milliliter of the diluted suspension was supplemented with 10 μL of PI solution (1.5 mM in distilled water) and then incubated for 10 min at 40°C . Fluorescence was measured by flow cytometry using a BactiFlow cytometer (AES Chemunex, Bruz, France). Data were collected and analyzed with FlowMax software (Partec S.a.r.l., Sainte-Geneviève-des-Bois, France). This software allowed the calculation of the percentage of stained cells. The loss in membrane integrity was then evaluated by calculating the difference between the percentage of stained cells after and before freezing (Rault et al., 2007). The lower the difference, the lower the loss in membrane integrity during freezing was. All fluorescence measurements were performed at least in triplicate from independent cultures.

FTIR Spectroscopy Studies

Infrared absorption measurements were carried out with a Nicolet Magna 750 FTIR spectrometer (Thermo Fisher Scientific, Madison, WI) equipped with a nar-

row-band mercury/cadmium/telluride (MCT) infrared detector and a Specac variable temperature cell cooled by liquid nitrogen (Specac Ltd., Orpington, Kent, UK). The temperature of the sample was recorded separately using an extra thermocouple that was located close to the sample. The optical bench was continuously purged with dry air (Balston, Haverhill, MA). The acquisition parameters were 4 cm^{-1} resolution, 32 co-added interferograms, and a 4,000 to 900 cm^{-1} wavenumber range. Fresh bacterial pellets were washed twice in saline water. The washed bacterial pellet was then sandwiched between 2 CaF_2 windows. Samples were mounted into the Specac cell. The temperature dependence of the FTIR spectra was studied by cooling the sample from 50°C down to -50°C at a rate of 2°C/min and then by heating the sample from -50 to 80°C at 2°C/min. Experiments were performed 8 times. Spectral analysis and display were carried out using OMNIC software (Thermo Fisher Scientific, Madison, WI).

Following Crowe et al. (1989), membrane phase behavior was monitored by observing the position of the symmetric CH_2 stretching band at approximately 2,850 cm^{-1} . To determine the peak location, the second-derivative spectrum analysis was performed with a Savitzky-Golay derivative function of third degree following 9-point smoothing (Savitzky and Golay, 1964) and was then multiplied by -1. Wavenumber of the symmetric CH_2 stretching peak (νCH_2 symmetric) versus temperature plots were constructed for freezing and thawing. The plots were fitted with a curve based on an asymmetric sigmoid transition function and the first derivative was calculated. The maximum of the first derivative of the fitted curves was taken as the lipid phase transition temperature (**Ts**: lipid solidification following freezing; **Tm**: lipid melting following thawing).

The ice nucleation and melting temperatures were determined by following the peak position of the water libration and bending combination band in the region 2,250 to 2,000 cm^{-1} . The phase change of water into ice leads to a shift to higher wavenumbers due to the changes in hydrogen-bonding interactions that occur as water solidifies. Wavenumber of the position of the H_2O -libration and -bending band at approximately 2,200 cm^{-1} ($\nu\text{H}_2\text{O}$) versus temperature plots were constructed to determine the ice nucleation and melting temperatures as the onset-temperature at which the H_2O band shifted to higher values or lower values, respectively.

Statistical Analysis

Significance of results was assessed at a 95% confidence level. Statistical tests were carried out using

XLSTAT software (Addinsoft, Paris, France). Exact 2-sample permutation tests were conducted to contrast data obtained from cells cultivated in MRS broth versus data obtained from cells cultivated in mild whey medium.

RESULTS

Membrane FA Composition of *Lb. delbrueckii* ssp. *bulgaricus* CFL1 Cells

To quantify the physiological changes that are induced by different compositions of culture media (MRS broth or mild whey medium), the membrane FA composition of *Lb. delbrueckii* ssp. *bulgaricus* CFL1 cells was analyzed just after harvesting at the end of the exponential phase. Results are summarized in Table 1. The type and total content of FA were comparable for both culture conditions (13 FA in common of 14 FA found; about 6 mg of FA/g of bacteria, dry basis); C16:0 and C18:1 were the 2 major FA for *Lb. delbrueckii* ssp. *bulgaricus* CFL1 cell populations, whatever the culture medium. However, marked differences were observed in the membrane FA composition of cells according to these culture conditions. For cells grown in MRS broth, only 3 FA represented more than 80% of the total FA: C18:1 (48%), C16:0 (18%), and $\Delta\text{C19:0}$ (cyclic FA, 14%). For cells grown in mild whey-based medium, 5 FA accounted for more than 80% of the total FA: C16:0 (38%), C18:1 (22%), C14:0 (9%), C16:1 (7%), and C18:0 (7%). Cells grown in MRS broth exhibited a higher ratio of UFA:SFA than cells grown in mild whey (1.6 and 0.5, respectively). In addition, $\Delta\text{C19:0}$ represents 14% of the cell FA content for cells grown in MRS broth but only 2% of the total FA of cells grown in whey medium. Growth in MRS broth permitted cells to enrich significantly their membrane in unsaturated and cyclic FA compared with growth in mild whey.

Freezing Resistance of *Lb. delbrueckii* ssp. *bulgaricus* CFL1 Cells

The t_{spe} [in min/log (cfu/mL)] was determined before and after freezing for both culture conditions. Freezing resistance was assessed by calculating the loss in t_{spe} [dt_{spe} , in min/log (cfu/mL)] following freezing as the difference between t_{spe} values before and after freezing (Table 2). Cells grown in MRS broth exhibited a significantly lower loss in t_{spe} during freezing [dt_{spe} = 2 min/log (cfu/mL)] than cells grown in mild whey medium [dt_{spe} = 6 min/log (cfu/mL); $P < 0.05$]. Cells grown in MRS broth appeared, thus, more resistant to freezing than cells grown in mild whey.

Furthermore, when considering the measurement of membrane integrity following freezing, an increase

Table 1. Fatty acid composition ($\mu\text{g/g}$ of dry pellet and relative percentages; means $\pm$ SD¹) in *Lactobacillus delbrueckii* ssp. *bulgaricus* CFL1 cells grown in de Man, Rogosa, and Sharpe (MRS) broth or in mild whey medium and harvested at the end of the exponential growth phase

FA	MRS broth		Mild whey	
	$\mu\text{g/g}$	%	$\mu\text{g/g}$	%
C11:0	<DL ²		<DL	
C12:0	<DL		184 $\pm$ 19	3 $\pm$ 0.2
C13:0	118 $\pm$ 39	2 $\pm$ 0.1	48 $\pm$ 10	1 $\pm$ 0.2
C14:0	353 $\pm$ 110	5 $\pm$ 0.9	484 $\pm$ 51	9 $\pm$ 0.8
C15:0	111 $\pm$ 17	2 $\pm$ 0.1	97 $\pm$ 14	2 $\pm$ 0.3
C16:0	1,294 $\pm$ 287	18 $\pm$ 1.5	2,146 $\pm$ 378	38 $\pm$ 0.5
C16:1	159 $\pm$ 27	2 $\pm$ 0.5	378 $\pm$ 135	7 $\pm$ 1.8
C17:0	18 $\pm$ 2	0 $\pm$ 0	36 $\pm$ 5	0 $\pm$ 0
C18:0	191 $\pm$ 21	3 $\pm$ 0.1	385 $\pm$ 65	7 $\pm$ 0.3
C18:1	3,459 $\pm$ 394	48 $\pm$ 2.3	1,242 $\pm$ 266	22 $\pm$ 3.7
C18:2	235 $\pm$ 92	3 $\pm$ 0.6	223 $\pm$ 3	4 $\pm$ 0.7
C19:0	203 $\pm$ 54	3 $\pm$ 0.1	79 $\pm$ 18	1 $\pm$ 0.3
Δ C19:0	1,023 $\pm$ 20	14 $\pm$ 1.8	119 $\pm$ 43	2 $\pm$ 1.1
C20:0	10 $\pm$ 4	0 $\pm$ 0.1	29 $\pm$ 5	0 $\pm$ 0
C22:0	40 $\pm$ 23	0 $\pm$ 0.1	208 $\pm$ 50	4 $\pm$ 0.5
C18:2 conjugated	<DL		<DL	
Total SFA	2,341 $\pm$ 514		3,698 $\pm$ 584	
Total UFA ³	3,853 $\pm$ 459		1,843 $\pm$ 367	
UFA:SFA	1.60 $\pm$ 0.17		0.50 $\pm$ 0.02	

¹Data are the averages of 3 separate experiments.

²DL = detection limit.

³UFA = unsaturated FA (cyclic FA Δ C19:0 not included in the UFA).

in the percentage of PI-stained cells was observed for both culture media (Table 2), indicating membrane permeabilization. These results are in agreement with other work reporting membrane permeabilization when the freezing process was applied to yeast and bacteria (Moussa et al., 2008; Cao-Hoang et al., 2010) or sperm cells (Holt et al., 1992; Klimowicz-Bodys et al., 2012). The loss in membrane integrity following freezing was lower for cells grown in MRS broth (2.8%) compared with cells grown in whey (6%; $P < 0.05$). Membrane damage following freezing appeared, thus, significantly less important for cells grown in MRS broth than for cells grown in mild whey medium ($P < 0.05$).

Furthermore, the initial acidifying activity (t_{spe}) and membrane integrity (% of PI-stained cells) were different according to the culture conditions. The best cryo-

tolerance was observed for cells presenting the highest t_{spe} and membrane integrity (MRS broth; Table 2).

FTIR Spectra of *Lb. delbrueckii* ssp. *bulgaricus* CFL1 Cells Before and After Freezing

Figure 1 depicts the infrared absorbance spectra of *Lb. delbrueckii* ssp. *bulgaricus* CFL1 pellets obtained at ambient temperature and in the frozen state at -50°C for the 2 culture conditions. The infrared spectra of the cells were dominated by the signal of absorption bands arising from water (either in liquid or in solid state). Water exhibited strong vibrational bands at $3,400\text{ cm}^{-1}$, $2,200\text{ cm}^{-1}$, and $1,650\text{ cm}^{-1}$, arising from the combination of stretching, libration and bending, and scissoring vibrational modes, respectively. The

Table 2. Specific acidifying activity [t_{spe} ; in min/log (cfu/mL)], percentage of propidium iodide (PI)-stained cells determined before and after freezing and losses in specific acidifying activity [dt_{spe} ; in min/log (cfu/mL)] and in membrane integrity during freezing of *Lactobacillus delbrueckii* ssp. *bulgaricus* CFL1 grown in de Man, Rogosa, and Sharpe (MRS) broth or in mild whey medium and harvested at the end of the exponential growth phase (data are the means of 3 separate experiments)

Item	MRS broth		Mild whey	
	Before freezing	After freezing	Before freezing	After freezing
t_{spe}	14 ^a	16 ^a	29 ^b	35 ^c
dt_{spe}		2 ^a		6 ^b
% of PI-stained cells	2.2 ^a	5 ^b	5.4 ^b	11 ^c
Loss in membrane integrity (%)		2.8 ^a		6 ^b

^{a-c}Means within a row with different superscript letters differ ($P < 0.05$).

Figure 1. In situ infrared absorption spectra of *Lactobacillus delbrueckii* ssp. *bulgaricus* CFL1 cells. Spectra are shown for unfrozen (20°C, solid line) and frozen (−50°C, dashed line) cells. Characteristic molecular group vibrations are indicated. MRS = de Man, Rogosa, and Sharpe broth.

band at $3,400\text{ cm}^{-1}$ appears saturated. At $2,200\text{ cm}^{-1}$, the frozen sample exhibited clear differences in spectral shape compared with the sample at ambient temperature. This is mostly due to shape changes of the water absorption bands upon transition into ice. In the $3,000$ to $2,800\text{ cm}^{-1}$ region, the symmetric and asymmetric CH_2 stretching vibrations of lipid acyl chains were present. Characteristic protein bands were observed at $1,655\text{ cm}^{-1}$ (amide I), at $1,550\text{ cm}^{-1}$ (amide II), and at $1,250\text{ cm}^{-1}$ (amide III).

Because the water band slightly overlaps with the lipid band, water spectra at 20°C and −50°C were subtracted from raw spectra at 20 and −50°C, respectively (Figures 2A and 2B). The subtracted spectra plotted in the $3,000$ to $2,800\text{ cm}^{-1}$ region displayed 4 peaks corresponding to the CH stretching vibrations of lipids (symmetric and asymmetric CH_3 stretching vibrations at $2,958$ and $2,872\text{ cm}^{-1}$, respectively, and symmetric and asymmetric CH_2 stretching vibrations at $2,925$ and $2,852\text{ cm}^{-1}$, respectively). The symmetric CH_2 stretching vibration is currently used to follow lipid phase transition (Crowe et al., 1989; Leslie et al., 1995; Tablin et al., 2001). For accurately determining the peak positions of lipids, the second derivative was calculated (Figures 2C and 2D). The shift of the symmetric CH_2 stretching peak toward lower wavenumbers can be clearly observed between the unfrozen and frozen samples.

FTIR Assessment of the Lipid Membrane Phase Behavior During Freezing and Thawing

Fourier transform infrared spectroscopy was used to characterize changes in the membrane phase behavior

during freezing and thawing. Spectra were acquired during cooling of cell pellets from 50 to −50°C, with a cooling rate of $2^\circ\text{C}/\text{min}$ (Figures 3A and 3B) and during heating from −50 to 80°C , with a heating rate of $2^\circ\text{C}/\text{min}$ (Figures 3C and 3D). Changes in the position of the H_2O libration and bending combination band and in the lipid symmetric CH_2 stretching band were determined and plotted as a function of the sample temperature to assess water phase transition and membrane phase behavior of bacterial cells, respectively.

Ice formation was visible during cooling as an abrupt increase in the water band position ($\nu\text{H}_2\text{O}$) from around $2,140$ to $2,200\text{ cm}^{-1}$, which occurred spontaneously at around -11°C for both culture conditions (Figures 3A and 3B). During heating, an abrupt decrease in the water band position was conversely observed associated with ice melting at around -2°C (Figures 3C and 3D). A significant degree of supercooling, which corresponds to the difference between the ice melting temperature and the ice nucleation temperature, was obtained during the freezing experiments (9°C).

The membrane phase behavior was monitored by tracking the shift in the wavenumber position of the symmetric CH_2 stretching band at approximately $2,850\text{ cm}^{-1}$ (νCH_2 symmetric) with cooling and subsequent heating. Considering both culture media, a shift in νCH_2 symmetric from $2,854\text{ cm}^{-1}$ to lower wavenumbers ($2,851\text{ cm}^{-1}$ for MRS broth and $2,850\text{ cm}^{-1}$ for mild whey medium) was observed with decreasing temperature (Figures 3A and 3B). This decrease in wavenumbers denotes membrane phase transition from a relatively disordered fluid lipid phase, called the liquid crystalline phase, to an ordered rigid lipid phase,

Figure 2. (A and B) Fourier transform infrared (FTIR) absorption spectra after subtraction of the water spectrum of unfrozen (20°C, solid line) and frozen (−50°C, dashed line) *Lactobacillus delbrueckii* ssp. *bulgaricus* CFL1 cells in the lipid region (3,000–2,800 cm^{-1}). Arrows indicate the different lipid stretching vibrations occurring in this region: CH_3 symmetric (CH_3 sym) and asymmetric (CH_3 asym) and CH_2 symmetric (CH_2 sym) and asymmetric (CH_2 asym). (C and D) The second-derivative profile of previous spectra in the 2,900 to 2,800 cm^{-1} region was included to highlight the decrease in wavenumber of the symmetric CH_2 stretching band during freezing. MRS = de Man, Rogosa, and Sharpe broth.

called the gel phase. Changes in frequency are known to reflect isomerization events in the hydrocarbon chains that occur as the lipids are transformed from straight chains, predominantly gauche conformation in liquid crystalline fluid phase to *trans* conformation in the gel phase (Crowe et al., 1989). The first derivative of the νCH_2 symmetric versus temperature curve was plotted for the determination of the lipid phase transition temperature during freezing (T_s) and is also represented in Figures 3A and 3B. Cells grown in MRS broth exhibited a lower T_s value ($-8^\circ\text{C} \pm 4^\circ\text{C}$) than cells grown

in mild whey medium ($22^\circ\text{C} \pm 5^\circ\text{C}$) during freezing. Furthermore, when considering the MRS culture conditions, it clearly appears in Figure 2A that membrane lipid phase transition occurred simultaneously with ice nucleation in a quite narrow temperature range (21°C) during cell freezing. Conversely, for cells grown in mild whey medium (Figure 3B), the membrane had already reached the gel phase, a rigid ordered phase when ice nucleation occurred, and the membrane lipid phase transition took place over a broad temperature range (more than 50°C). In addition, cells grown in MRS

Figure 3. Membrane lipid phase behavior (■ CH_2 symmetric) and ice nucleation/melting (◇ H_2O) versus temperature plots of *Lactobacillus delbrueckii* ssp. *bulgaricus* CFL1 cells after culture in de Man, Rogosa, and Sharpe (MRS) broth (A and C) or in whey (B and D). Cells were frozen from 50°C to -50°C (A and B) and they were heated from -50°C to 80°C (C and D) at $2^\circ\text{C}/\text{min}$. The maximum of the first derivative of the wavenumber of the symmetric CH_2 stretching peak (νCH_2) versus temperature plot (solid line) allows the determination of the lipid phase transition temperature (T_s : lipid solidification following freezing; T_m : lipid melting following heating). Vertical arrows indicate the ice nucleation temperatures and horizontal arrows the ice melting temperatures. All represented spectra are the averages of 8 separate experiments. Error bars represent the SD of the 8 experiments. $\nu\text{H}_2\text{O}$ corresponds to the position of the H_2O -libration and -bending combination band.

broth showed a higher wavenumber of the symmetric CH_2 stretching vibration in the frozen state than cells grown in mild whey medium ($2,850.5\text{ cm}^{-1}$ and $2,849.5\text{ cm}^{-1}$, respectively). This suggests that even in the gel phase region, a less rigid membrane (less conformational order) was obtained in the frozen state in the case of cells grown in MRS broth than in the case of cells grown in mild whey medium.

Figures 3C and 3D represent the membrane phase behavior during thawing, as usually reported in the literature (Crowe et al., 1989; Leslie et al., 1995; Oldenhof et al., 2005). When comparing the νCH_2 symmetric curves for cells cultivated in MRS broth during cooling (Figure 3A) and heating (Figure 3C) no significant difference was observed in membrane behavior ($T_m = -2 \pm 5^\circ\text{C}$). However, a shift of the lipid phase transition temperature to a higher value was observed during thawing of cells grown in mild whey ($T_m = 33 \pm 5^\circ\text{C}$).

DISCUSSION

The cell membrane is a critical site of damage during cryopreservation. Freezing induces membrane permeabilization, which can result in cell death. Many studies have already related the lipid membrane composition to the stress adaptation and the improvement of the cell's freezing resistance, in particular for LAB (Smittle et al., 1974; Beal et al., 2001; Streit et al., 2007). However, to our knowledge, no study has investigated the complex biophysical behavior of membranes of LAB during freezing and thawing relative to both the membrane FA composition and integrity and the resulting freezing resistance.

Cells grown in MRS broth, a complex medium leading to freeze-resistant cells, exhibited a higher UFA:SFA ratio (1.6) and content of $\Delta\text{C19:0}$ cyclic FA (14%) than cells grown in mild whey medium (0.5 and 2%, respectively). These results are in agreement with previous

studies reporting a positive relationship between high levels of unsaturated and cyclic FA and the cryoresistance of LAB (Gómez Zavaglia et al., 2000; Beal et al., 2001; Wang et al., 2005). By adding UFA, such as oleic acid derivatives, to the culture medium, it is possible to promote the synthesis of high contents of unsaturated and cyclic FA (Johnson and Etzel, 1995; Muller et al., 2011). The addition of Tween 80 (oleic acid derivative) has proved to enhance the resistance of *Lb. delbrueckii* ssp. *bulgaricus* (Smittle et al., 1974; Fonseca et al., 2001) and *Strep. thermophilus* (Beal et al., 2001) to freezing. Furthermore, the UFA:SFA ratio is commonly used for assessing membrane fluidity (Beal et al., 2001; Denich et al., 2003; Tymczyszyn et al., 2005). A high UFA:SFA ratio is usually associated with high membrane fluidity. Membranes of cells grown in MRS broth would, thus, be more fluid than those of cells grown in whey. In addition, the $\Delta C19:0$ cyclic FA is often associated with increasing membrane fluidity and freezing resistance of LAB (Smittle et al., 1974; Gómez Zavaglia et al., 2000; Wang et al., 2005). Even if its role is still poorly understood, its chemical structure suggests that it can act in a comparable way to *cis* C18:1 FA. These results confirmed that modulating the membrane lipid composition can induce LAB cryotolerance.

The phase behavior in cellular membranes of both bacterial populations exhibiting different lipid compositions and concomitant different freezing resistances was investigated by FTIR spectroscopy. The position of the lipid symmetric CH_2 stretching vibration band around $2,850\text{ cm}^{-1}$ is a measure of membrane lipid state generally related to membrane fluidity. Whatever the culture medium used (MRS broth or mild whey medium), membranes exhibited a liquid crystalline-to-gel phase transition during cooling and vice versa during warming but significant differences in membrane biophysical behaviors were observed. Freeze-tolerant cells grown in MRS medium exhibited a low value of T_m (-2°C during thawing). Similarly, *Lactobacillus plantarum*, which is reported to be a freeze-resistant bacterium (Dumont et al., 2004), also presented a low T_m value (4°C) when cultivated in enriched MRS broth (Linders et al., 1997). It has been demonstrated that T_m values depend on the saturation level of the cytoplasmic membrane (Murata, 1989). The low value of lipid phase transition temperature observed for cells grown in MRS broth can be originated from the high content of UFA observed in their membrane. Unsaturated fatty acids have low melting points, thus lowering the T_m of cell lipid membranes. In addition, cyclic FA ($\Delta C19:0$), which was observed in high content for cells grown in MRS broth, may also contribute to lower the T_m value of cells. Perly et al. (1985) showed that the introduction of a cyclopropane ring in the membrane

model led to a reduction in T_m values. Conversely, for cells grown in mild whey, a lipid membrane phase transition at high positive temperatures was observed ($T_m = +33^\circ\text{C}$ during thawing), confirming previous work on *Lb. delbrueckii* ssp. *bulgaricus* CFL1 (Oldenhof et al., 2005). The presence of a high content of SFA could, thus, explain the high value of lipid phase transition temperature obtained in the current study.

The freezing resistance of *Lb. delbrueckii* ssp. *bulgaricus* CFL1 grown in MRS broth could, thus, be ascribed to a lipid phase transition occurring at subzero temperatures and overlapping with ice nucleation, which is induced by a high content of unsaturated and cyclic FA in the cell membrane. However, focusing only on the temperature value of the membrane lipid phase transition is not enough for complete characterization of the membrane biophysical behavior during freezing. Furthermore, the in situ and in-real-time FTIR study of the freezing event allows the proposal of 2 major biophysical mechanisms for explaining cell membrane permeabilization observed following freezing: cell dehydration and membrane lipid lateral phase separation.

We have already showed that *Lb. delbrueckii* ssp. *bulgaricus* CFL1 survival during freezing and thawing is highly sensitive to the cellular biophysical event of cell dehydration (Fonseca et al., 2006). Extracellular ice formation occurring after ice nucleation exposes cells to high solute concentrations (osmotic stress) and leads to cell dehydration and volume contraction of cells (mechanical stress; Mazur, 1984). Although similar nucleation ice temperatures were observed for cells grown in MRS broth and in mild whey medium, the biophysical state of their membrane was completely different when the nucleation event took place. In the case of cells grown in MRS broth, the ice formation and melting overlapped with the membrane phase transition. Consequently, when ice nucleated and cells started to dehydrate, the cell membranes were in a fluid and flexible state. Water could leave cells freely and cell volume reduction could occur easily. Moreover, the high content of UFA and SFA resulted in high membrane fluidity, facilitating cell responses to environmental changes all over the freeze-thaw process. Conversely, the membrane of cells grown in mild whey medium had reached a rigid and ordered state when ice nucleation occurred. Cells were, thus, subjected to important mechanical constraints following water escape from the cell and the resulting cell shrinkage. Membrane leakage could then take place for facilitating water flux escape from cell. Actually, this explanation was supported by the high loss of membrane integrity of cells grown in mild whey.

In addition to cell dehydration, the cell membrane undergoes successive phase transitions from the liquid

crystalline to the gel states during freezing and from the gel to the liquid crystalline states during thawing. Following lipid membrane transition, the presence of several FA within the lipid membrane generally results in local transitions, generating in turn, lateral lipid phase separation with packing effects and changes in lipid-protein interactions (Beney and Gervais, 2001; Tymczynsyn et al., 2005). This phenomenon of lateral phase separation could lead to membrane permeabilization and leakage. The membrane phase transition of cells grown in mild whey medium occurred over a broader temperature range (around 50°C) than cells grown in MRS broth (21°C) during freezing. The presence within the membrane of cells grown in mild whey medium of 5 major FA (80% of total FA), exhibiting different successive lipid phase transition temperature values, could be responsible for promoting important lateral phase separation and, thus, lengthening the lipid membrane phase transition and shifting T_m to higher values upon thawing. This is consistent with the higher membrane permeabilization observed following freezing. The lower resistance to freeze-thawing of *Lb. delbrueckii* ssp. *bulgaricus* cells grown in whey-based medium can, thus, be attributed to a combination of membrane deformations caused by volumetric contractions and structural changes in membrane lipids during a long phase transition, leading to packing effects and loss of membrane integrity.

CONCLUSIONS

The present study reported the biophysical characterization by FTIR spectroscopy of LAB cell membrane behavior following freezing and thawing. It was demonstrated that the modulation of the membrane lipid composition has substantial influence on membrane biophysical properties. The differences observed in cell resistance to freezing were related to the lipid phase transition. The best resistance to freezing of *Lb. delbrueckii* ssp. *bulgaricus* CFL1 was mainly associated with the presence of unsaturated and cyclic FA in the membrane, which in turn, lowered the lipid membrane phase transition temperature and consequently made it possible to maintain high membrane fluidity all along the freeze-thawing process.

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