Fermentation pH and Temperature Influence the Cryotolerance of *Lactobacillus acidophilus* RD758

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ABSTRACT

The effects of 3 fermentation temperatures (30, 37, and 42°C) and 3 fermentation pH (4.5, 5, and 6) on the cryotolerance of *Lactobacillus acidophilus* RD758 were studied in relation to their fatty acid composition. Cryotolerance was defined as the ability of the cells to recover their acidification activity after freezing and frozen storage at –20°C. Better cryotolerance was obtained in cells grown at 30°C or at pH 5; these cells showed no loss in acidification activity during freezing and a low rate of loss in acidification activity during frozen storage. On the other hand, cells grown at 42°C or at pH 4.5 displayed poor cryotolerance. The membrane fatty acid composition was analyzed and related to the cryotolerance using principal component analysis. The improved cryotolerance observed during the freezing step was associated with a high ratio of unsaturated to saturated fatty acids, a low C18:0 content, and high C16:0 and cyclic C19:0 relative concentrations. High resistance during frozen storage was related to a high cycC19:0 concentration. Finally, the low cryotolerance observed after fermentation at pH 4.5 was explained by a low C18:2 content.

(Key words: lactic acid bacteria, temperature, pH, fatty acid composition)

Abbreviation key: **dtf** = difference between the acidification activities measured before and after freezing; **k** = rate of loss in acidification activity; **tc** = initial acidification activity before freezing; **tpH5.5** = acidification activity (time necessary to reach pH 5.5); **ts** = storage time; **U/S** = ratio of unsaturated to saturated fatty acid concentrations.

INTRODUCTION

Lactic acid bacteria are important starters used in the food and pharmaceutical industries. Their preser-

vation is generally obtained by freezing or freeze-drying (To and Etzel, 1997) to maintain the viability and main technological properties of the bacteria: acidification activity, aroma production, texture formation, and probiotic properties (Fonseca et al., 2003). However, some strains are sensitive to freezing and freeze-drying, both of which lower the performance of starter cultures (Foschino et al., 1996). This sensitivity is related to the deterioration of the cell physiological state, caused by various stresses appearing during starter production and preservation. Cold stress, which takes place during the cooling and freezing steps and during frozen storage, is the main cause of loss of bacterial activity. Other unfavorable conditions such as heat stress (Desmond et al., 2002), acid stress (De Angelis et al., 2001), starvation (Maus and Ingham, 2003), osmotic stress (Guerzoni et al., 2001), and oxidative stress (Aubert et al., 2002) affect the performance of starters.

Adaptive responses to stress in lactic acid bacteria vary with bacterial species and stress conditions, but 2 main responses are observed. The first one consists of the synthesis of some specific proteins, which have been observed under various stress conditions, especially cold or heat stress and acid stress. A set of 7-kDa proteins, named cold shock proteins (CspA–CspI), was strongly induced in response to a sudden drop of temperature, by *Streptococcus thermophilus* (Wouters et al., 1999) and *Lactococcus lactis* (Wouters et al., 2001). In *Lc. lactis*, the heat shock response was characterized by an enhanced synthesis of heat shock proteins, such as GroEL, DnaK, DnaJ, and GrpE (Broadbent et al., 1997). The drop in final culture pH to 4.5 results in synthesis of 9 proteins (14.1 to 56.2 kDa) in *Lactobacillus acidophilus* (Lorca and Font de Valdez, 2001) and of some heat shock proteins in *Lc. lactis* (Frees et al., 2003). Finally, an acid shock at pH 4.75 also induced expression of 3 heat shock proteins (GroES, GroEL, and DnaK) in *Lactobacillus bulgaricus* (Lim et al., 2000).

The second response is related to changes in membrane fatty acid composition. The cellular adaptive mechanism induced by cold shock consists of an increase in the unsaturated fatty acid content of mem-

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brane phospholipids, which leads to a decrease in the solid-to-fluid transition temperature and thus, to an increase in membrane fluidity. Consequently, the ratio between unsaturated and saturated fatty acids (U/S) is inversely correlated with the growth temperature, as shown by Lonvaud-Funel and Desens (1990) with *Lactobacillus plantarum*, and by Suutari and Laakso (1992) with *Lactobacillus fermentum*. Béal et al. (2001) showed that a decrease in the fermentation pH increased the U/S ratio of *Strep. thermophilus* and improved the recovery of its acidification activity.

In addition, some specific fatty acids play an important role in stress response. Fernandez Murga et al. (2000) observed an increase of C16:0 and C18:2 fatty acids in *Lb. acidophilus* grown at low temperature (25°C). The C18:1 fatty acid concentration increased in response to low temperature in *Lb. plantarum* (Russell et al., 1995), to acid pH in *Strep. thermophilus* (Béal et al., 2001), and to osmotic stress in *Lc. lactis* (Guillot et al., 2000). On the contrary, C18:1 concentration decreased in response to freezing in lactic streptococci (Gilliland and Speck, 1974), and to spray-drying in *Lb. acidophilus* (Brennan et al., 1986). A high cycC19:0 concentration favored the cryotolerance of *Lb. bulgaricus*, *Lactobacillus helveticus*, and *Lb. acidophilus* (Gomez Zavaglia et al., 2000). The cycC19:0 also increased in response to acid stress (Béal et al., 2001), osmotic stress (Guillot et al., 2000), ethanol stress (Teixeira et al., 2002), and high age of culture (Drici-Cachon et al., 1996).

Different methods are presently proposed to improve the quality of lactic and probiotic starters: the use of effective cryoprotectants (Fonseca et al., 2003), the use of adequate freezing and storage conditions (Foschino et al., 1996), and the selection of more-resistant strains (Monnet et al., 2003). In addition, the application of special environmental conditions during fermentation has been proposed by some authors (Gilliland and Rich, 1990; Fernandez Murga et al., 2000; Palmfeldt and Hahn Hagerdal, 2000; Béal et al., 2001). Therefore, this work aims to adapt *Lb. acidophilus*, by applying different temperature and pH conditions during fermentation, to improve its resistance to freezing and frozen storage. The analysis of the membrane fatty acid composition of adapted cells should improve our understanding of the cellular adaptive response.

MATERIALS AND METHODS

Bacterial Strain and Media

Freeze-dried *Lb. acidophilus* RD758 (Danisco, Dangé-Saint-Romain, France) was stored at -20°C. It was thawed for 10 min at 37°C before inoculation, which was carried out at 3×10^6 cfu/mL.

For starter production, the culture medium was composed of 56.4 g/L of mild whey (BBA, Bonneuil-sur-Marne, France) that was heated to 110°C for 10 min. After centrifugation ($17,000 \times g$, 20 min, 15°C) and filtration (0.45 μ m), 20 g/L of lactose (Prolabo, Paris, France) and 5 g/L of yeast extract (Labosi, Oulchy-Le-Château, France) were added to the supernatant. The medium was sterilized in the fermentor at 110°C for 20 min.

For measuring acidification activity, the medium was composed of reconstituted dried skim milk (100 g/L; Elle & Vire, Condé sur Vire, France). It was pasteurized for 20 min at 110°C in 150-mL Erlenmeyer flasks.

Fermentation

Cultures were grown in a 2-L fermentor, at different temperatures and pH, with an agitation speed of 200 rpm. The pH was controlled by adding a 2 M NaOH solution that was continuously weighed. Absorbance measurements at 480 nm were used to characterize bacterial growth.

Growth was stopped at the beginning of the stationary phase. This was defined as the time at which the NaOH consumption rate, calculated in real time as the first time derivative of the NaOH weight decrease, started to decline. The cell suspension was then cooled to 15°C in the fermentor.

Concentration and Preservation

Cells were harvested at 15°C and concentrated by centrifugation ($17,000 \times g$, 30 min at 4°C). Concentrated cells were resuspended at 4°C in 3 $\times$ their weight of supernatant. Aliquots of 1 mL of concentrated cells were frozen at 0.75°C/min and stored at -20°C for 3 mo. They were thawed at 37°C for 5 min before acidification activity measurements.

Acidification Activity Measurement

The Cinac system (Corrieu et al., 1988) was used to measure the acidification activity of the suspensions of *Lb. acidophilus* RD758. Acidification was measured at 37°C and triplicated. The pH of inoculated milk samples was continuously measured and the time necessary to reach pH 5.5 (tpH5.5; in min) was used to characterize the acidification activity of the bacterial suspensions. The higher the tpH5.5, the longer the latency phase, and thus the lower the acidification activity.

The acidification activity was measured before and after freezing, and during 90 d of frozen storage at -20°C. The loss in acidification activity as a function of storage time (ts; in d) was modeled according to linear regressions as proposed by Fonseca et al. (2000):

$$\text{tpH5.5} = \text{tc} + \text{dtf} + k \times \text{ts}$$

The parameter **tc** (in min) represents the initial acidification activity, measured before freezing. The parameter **dtf** (in min) displays the difference between the acidification activities measured before and after freezing. It corresponds to the loss in acidification activity during the freezing step. The parameter **k** (in min/d) is the slope of the regression line and represents the rate of loss in acidification activity during frozen storage.

Fatty Acid Analysis

The membrane fatty acid composition of the bacteria was determined using gas chromatography as described by Rozes et al., (1993) and adapted by Béal et al. (2001). Concentrated cells were washed in 0.05 M Tris solution. Methylation and extraction were performed simultaneously at 4°C by adding 1.5 mL of sodium methoxide (1 M in methanol) (Sigma Aldrich, Steinheim, Germany) and shaking for 1.5 min. Fatty acid methyl esters were extracted with 1 mL of hexane. One milliliter of undecanoic acid methyl ester (0.1 mg/mL in hexane) (Sigma Aldrich) was added as an internal standard for gas-liquid chromatography. After decanting for 5 min, the upper phase was removed and stored at -80°C in an airtight glass bottle until analysis.

The analyses were performed on a gas chromatographer (HP 6890, Hewlett Packard, Avondale, PA) equipped with a mass selective detector (Agilent 5973, Hewlett Packard). A capillary column (BPX 70, 60 m × 0.25 mm, SGE, Victoria, Australia) was used. Helium was used as carrier gas (1.2 mL/min), and the injection volume was 2 µL. Injection was done in splitless mode for 2 min. The oven temperature was increased from 65 to 230°C at 5°C/min, and maintained for 10 min at 230°C. Injection and detection temperatures were 230°C.

Results were expressed as relative percentages of each fatty acid, which were calculated as the ratio of the surface area of the considered peak to the total area of all peaks. The ratio of unsaturated to saturated fatty acids (U/S) was determined. The cycC19:0 fatty acid was considered unsaturated. Analyses were made in triplicate.

Identification of the Major Peaks

The fatty acid methyl esters were first identified by comparing their retention times with those of known standards (Sigma Aldrich). Their identification was confirmed using the mass selective detector. The electron impact energy was set at 70 eV and data were collected in the range of 30 to 400 atomic mass units.

The identities of the fatty acid methyl esters (carbon number, position of the double bounds, and existence of a cyclopropane) were confirmed by comparing their mass spectra with the data bank (NBS75K and WILEY 275.L, Hewlett Packard). The *cis-trans* isomery of the double bounds or the cyclopropane was not established by this method.

Experimental Design and Statistical Analyses

The fermentation conditions were designed around a reference point set at 37°C and pH 6. The reference point was duplicated. The experimental design made it possible to determine the effects of the fermentation pH (4.5, 5, and 6), during cultures at 37°C and of the fermentation temperature (30, 37, and 42°C), during cultures at pH 6. An additional fermentation was carried out at 30°C and pH 5.

To relate the acidification activity variables to the fatty acid composition of *Lb. acidophilus*, principal component analysis (Statbox) was performed for all the fermentation conditions studied. Three acidification variables (tc, dtf, and k) and 6 composition variables (U/S ratio and C16:0, C18:0, C18:1, C18:2, and cycC19:0 relative concentrations) were considered.

RESULTS AND DISCUSSION

Influence of Fermentation Temperature on the Cryotolerance of *Lb. acidophilus* RD758

The effect of the fermentation temperature on the cryotolerance of *Lb. acidophilus* RD758 was determined by measuring the acidification activity after fermentation and concentration, after freezing, and during frozen storage. The initial acidification activity (tc, in min), which represents the cellular activity before freezing, varied with the fermentation temperature (Figure 1A). The highest initial acidification activity (i.e., the lowest tc) was obtained at 37°C. By considering the confidence intervals, no significant difference was observed when fermentations were performed at 30 or 42°C. As a consequence, the initial acidification activity was directly related to the fermentation temperature, which was optimal for growth at 37°C (results not shown).

According to Fonseca et al. (2000), the difference (dtf, in min) between the initial acidification activity and the acidification activity measured just after freezing at -20°C represents the loss in acidification activity during freezing. From Figure 1A, dtf increased with fermentation temperature: at 30°C, no loss in acidification activity was observed (dtf = 0 min), whereas dtf rose to 168 min at 42°C. This indicates that a low fermentation temperature dramatically improved the cellular resistance to freezing.

Figure 1. Effect of fermentation temperature on the acidification activity of *Lactobacillus acidophilus* RD758 at different steps of production. Fermentations were conducted at pH 6; tc (◆) = initial acidification activity measured before freezing (min); dtf (■) = difference of acidification activity before and after freezing (min); k (●) = rate of loss in acidification activity during storage (min/d); t90 (▲) and t365 (*) = residual acidification activity after 90 and 365 d of frozen storage (in min).

During frozen storage, the acidification activity decreased linearly with storage time (Béal et al., 2001). This decrease was directly related to the loss in cellular viability (Figure 2). The rate of loss in the acidification activity of *Lb. acidophilus* RD758 during frozen storage (k ; in min/d) was between 1.2 and 2.7 min/d (Figure 1B), depending on fermentation temperatures. The k values were similar at 30 and 37°C, but increased significantly at 42°C. This high fermentation temperature induced a more rapid decrease of the acidification activity, whereas, lower fermentation temperatures enabled better resistance of *Lb. acidophilus* RD758 to frozen storage.

The residual acidification activity was measured after 3 mo (t90) and calculated after 1 yr (t365) of frozen storage at -20°C (Figure 1B). These variables both displayed a higher acidification activity of the cells grown at 37 or 30°C. These observations were related to the good initial acidification activity and to the excellent resistance during freezing after fermentation at these temperatures, respectively.

Figure 2. Linear relationship between the acidification activity (tpH5.5, in min) and the microbial count (X , in cfu/mL) enumerated on MRS agar incubated at 37°C for 48 h; each value is the mean of 3 replicates. $\text{tpH5.5} = 878 - 29.7 \times \text{Ln}(X)$ ($R^2 = 0.9964$).

Finally, better cryoresistance of *Lb. acidophilus* RD758 was observed when the cells were cultivated at a low fermentation temperature, between 30 and 37°C. This is in agreement with previous studies, which showed an adaptation of *Lb. acidophilus* to freezing when the culture was conducted at low temperature (Fernandez Murga et al., 2000). Nevertheless, these authors demonstrated that lower temperatures (22 or 25°C) were necessary to induce cryoadaptation. The diversity of the strains used and the different fermentation conditions (noncontrolled pH) may explain these different values.

Influence of Fermentation pH on the Cryotolerance of *Lb. acidophilus* RD758

Cultures of *Lb. acidophilus* RD758 were conducted at 3 fermentation pH levels to demonstrate the effect of this factor on the initial acidification activity (t_c , in min), and on the cryoresistance of this strain. From Figure 3A, the cells grown at pH 6 displayed the higher initial acidification activity, as t_c was about 175 min lower than at other pH values. This indicates that the physiological state of cells grown in acidic conditions (pH 4.5 and 5) deteriorated more at the end of the culture than at pH 6. This result may be related to the optimal pH value for growth, which was pH 6 for *Lb. acidophilus* (Taillandier et al., 1996).

The loss of acidification activity during freezing (dtf, in min) of cells grown at different pH is shown in Figure 3A. Cells grown at acidic pH (pH 4.5 and 5) did not lose their acidification activity during freezing (dtf = 0 min), unlike at pH 6 (dtf = 90 min). However, this loss of acidification activity observed at pH 6 only partially counterbalanced the initial advantage observed with t_c (175 min).

Figure 3. Effect of fermentation pH on the acidification activity of *Lb. acidophilus* RD758 at different steps of its production. Fermentations were conducted at 37°C (closed symbols) or 30°C (open symbols); tc (◆) = initial acidification activity measured before freezing (in min); dtf (■) = difference of acidification activity before and after freezing (in min); k (●) = rate of loss in acidification activity during storage (in min/d); t90 (▲) and t365 (*) = residual acidification activity after 90 and 365 d of frozen storage (in min), respectively.

During frozen storage, the rates of loss of acidification activity (k , in min/d) were between 0.83 and 1.9 min/d, depending on the fermentation pH (Figure 3B). They were higher at pH 4.5, whereas no significant difference was observed between pH 5 and 6. This result indicates that very acidic conditions (pH 4.5) during fermentation were detrimental to bacterial resistance during frozen storage.

After 3 or 12 mo of frozen storage at -20°C , the residual acidification activity was highest (t_{90} and t_{365} values were the lowest) at pH values between pH 5 and 6 (Figure 3B). The good performance observed at pH 5 was mainly due to the low loss in acidification activity during freezing and storage. For cells grown at pH 6, the high initial acidification activity and relatively low rate of loss in acidification activity resulted in good residual activity. Finally, cells grown at pH 4.5 displayed the worst residual acidification activity, indicating that there was no adaptation at this fermentation pH for *Lb. acidophilus* RD758. This suggests that a low pH limit exists, under which the adaptation of *Lb. acidophilus* RD758 may not occur.

Our results are in agreement with previous works indicating that an acidic fermentation pH was better for the preservation of lactic acid bacteria: pH 5 for *Lb. acidophilus* (Gilliland and Rich, 1990) and *Lactobacillus reuteri* (Palmfeldt and Hahn Hagerdal, 2000), and pH 5.5 for *Strep. thermophilus* (Béal et al., 2001). Moreover, they pointed out that different events occurred with freezing or during frozen storage, thus leading to different bacterial resistance.

To combine the good cryotolerances observed at acidic pH and low temperature, an additional fermentation was conducted at pH 5 and 30°C . The initial acidification activity (t_c) and the loss in acidification activity during freezing (dtf) were equivalent to those observed after fermentation at pH 6 and 37°C (Figure 3A). However, the cells displayed a lower rate of loss in acidification activity during frozen storage ($k = 0.6$ min/d) and better residual acidification activity after 12 mo of storage (Figure 3B). This demonstrates that combining a low fermentation pH (pH 5) and a low fermentation temperature (30°C) was a good way to improve the cryotolerance of *Lb. acidophilus* RD758 during frozen storage.

Cellular Fatty Acid Composition of *Lb. acidophilus* RD758

To understand the physiological modifications induced by the previously observed responses, the membrane fatty acid composition of *Lb. acidophilus* RD758 was characterized. Thirteen fatty acids were observed in the membrane of *Lb. acidophilus* RD758 cultivated at pH 6 and 37°C . The 7 main peaks were identified as tetradecanoic (myristic) acid (C14:0), pentadecanoic acid (C15:0), hexadecanoic (palmitic) acid (C16:0), octadecanoic (stearic) acid (C18:0), octadecenoic (oleic or vaccenic) acids (C18:1), octadecadienoic (linoleic) acid (C18:2), and methylenoctadecenoic (dihydrosterculic or lactobacillic) acids (cycC19:0). Their relative percentages were between 1 and 48%, corresponding to more than 90% of all fatty acids. Six minor fatty acids were also detected, at lower relative concentrations: decanoic acid (C10:0), dodecanoic acid (C12:0), tridecanoic acid (C13:0), hexadecenoic (palmitoleic) acid (C16:1), heptadecanoic acid (C17:0), and methylenhexadecanoic acid (cycC17:0). All these fatty acids, encountered in the membrane of *Lb. acidophilus* RD758, have been identified previously in *Lb. acidophilus* (Brennan et al., 1986; Fernandez Murga et al., 2000).

The ratio between unsaturated and saturated fatty acids was equal to 0.23 for the cells grown at 37°C and pH 6. It was low compared with the values obtained by Fernandez Murga et al. (1999) (2.88) and Gomez Zavaglia et al. (2000) (0.86) with *Lb. acidophilus*. This

Figure 4. Influence of fermentation temperature on the membrane fatty acid composition of *Lb. acidophilus* RD758. Fermentations were conducted at pH 6 and at 30 (■), 37 (▒), and 42°C (□).

may be explained by the different conditions used by these authors, and mainly by the presence of Tween 80 in the media used in the 2 earlier studies.

Influence of Fermentation Temperature on Membrane Fatty Acid Composition of *Lb. acidophilus* RD758

The fermentation temperature influenced the concentration of the main membrane fatty acids of *Lb. acidophilus* RD758. The highest U/S ratio (0.34) was found in cells grown at the lowest temperature (30°C), whereas values between 0.23 and 0.24 were observed at 37 and 42°C. When considering the confidence intervals (± 0.12), there was no significant difference between these 2 temperatures. Consequently, a membrane adaptation of *Lb. acidophilus* RD758, corresponding to an increase in the U/S ratio, was observed at low fermentation temperature. This result was in agreement with those of Lonvaud-Funel and Desens (1990) with *Lb. plantarum*, Suutari and Laakso (1992) with *Lb. fermentum*, and Fernandez Murga et al. (2000) with *Lb. acidophilus*.

The changes in the relative percentages of 5 predominant fatty acids (C14:0, C16:0, C18:0, C18:1, and cycC19:0), measured after fermentations at different temperatures, are shown in Figure 4. The fermentation temperatures did not significantly affect the C14:0, C15:0, and C18:2 relative concentrations that were 13, 13, and 2%, respectively. This result differed from that of Suutari and Laakso (1992), obtained with *Lb. fermentum*, probably because of the different bacterial species tested. The C16:0 and C18:1 fatty acids demonstrated similar distribution, with a low value at 37°C, as compared with 30 and 42°C. They both influenced

the U/S ratio in the same way. This result partially agreed with those reported in the literature: the C16:0 concentration in *Lb. acidophilus* (Fernandez Murga et al., 2000) and the C18:1 concentration in *Lb. plantarum* (Russell et al., 1995) increased in response to low culture temperatures. The relative percentage of the C18:0 fatty acid was low (7%) at 30°C but increased significantly at higher temperatures (19%). This large difference (12%) greatly influenced the U/S ratio. A low C18:0 concentration in *Lb. acidophilus* grown at 30°C was found by Fernandez Murga et al. (2000), even though they also obtained a low value at 37°C. Finally, a high relative concentration of the cycC19:0 fatty acid was observed at 30°C. This result was in agreement with that of Suutari and Laakso (1992) but differed from that of Fernandez Murga et al. (1999). These differences may be related to the different strains and culture media used.

Finally, our results showed that a low fermentation temperature induced membrane adaptations in *Lb. acidophilus* RD758. It was related to high C16:0 and cycC19:0 relative concentrations, to a low C18:0 content and to a high U/S ratio.

Influence of Fermentation pH on Membrane Fatty Acid Composition of *Lb. acidophilus* RD758

The U/S ratios of *Lb. acidophilus* RD758 membranes grown at different fermentation pH were calculated. They were higher for the cells grown at acidic pH (0.30 to 0.35) compared with the cells grown at pH 6 (0.23). This phenomenon has been observed in *Lb. reuteri* (Palmfeldt and Hahn Hagerdal, 2000), *Oenococcus oeni* (Bastianini et al., 2000), and *Strep. thermophilus* (Béal et al., 2001). The lowest U/S ratio, found in the cells grown at pH 6, can be related to the lowest acid stress under these conditions.

The relative concentrations of 5 fatty acids (C14:0, C16:0, C18:0, C18:1, and cycC19:0) were influenced by the fermentation pH. According to Figure 5, the changes in the relative concentrations of C16:0, C18:0, and cycC19:0 fatty acids were large (4.5 to 10%), whereas the variations of C14:0 and C18:1 were low (2.5%). The relative concentrations of C15:0 and C18:2 were not affected by fermentation pH. A high C16:0 concentration was observed at acidic pH (45%) compared with pH 6 (38%). This was not consistent with previous studies. According to Drici-Cachon et al. (1996) with *Oe. oeni*, and Béal et al. (2001) with *Strep. thermophilus*, the relative C16:0 concentration was low at pH 2.9 and 5.5, respectively. This difference may be related to the use of different bacterial species by these authors. The cycC19:0 concentration increased when lowering the fermentation pH. It was high (12%) at pH 4.5 and 5,

Figure 5. Influence of fermentation pH on the membrane fatty acid composition of *Lactobacillus acidophilus* RD758. The fermentations were conducted at 37°C and at pH 4.5 (■), pH 5 (▒), and pH 6 (□).

instead of 6% at pH 6. This result is in agreement with previous studies (Drici-Cachon et al., 1996; Bastianini et al., 2000; Béal et al., 2001). Unlike the C16:0 and cycC19:0 fatty acids, the C18:0 relative content was lower at acidic pH, as already shown in *Strep. thermophilus* (Béal et al., 2001). This highly affected the U/S ratio because of the large difference observed (10%).

Consequently, a membrane adaptation of *Lb. acidophilus* RD758 was observed at low fermentation pH, as shown by an increase in the U/S ratio and in the C16:0 and cycC19:0 relative concentrations, and by a decrease in the C18:0 relative concentration. This adaptation was similar to that observed previously at low fermentation temperature.

Relationship Between the Membrane Fatty Acid Composition and the Cryotolerance of *Lb. acidophilus* RD758 Grown at Different pH and Temperatures

Our results showed some relation between the cryotolerance and the membrane fatty acid composition of *Lb. acidophilus* RD758, cultivated at different fermentation pH and temperatures. As a consequence, a principal component analysis was performed to evaluate these relationships, for the whole range of culture conditions (Figure 6A).

The first 2 dimensions accounted for 83% of the data variance. The fermentation conditions were discriminated along the first axis according to the C18:0, C16:0, and cycC19:0 relative concentrations, the U/S ratio, and the loss of acidification activity during freezing (dtf). Principal component 2 was determined by the rate of loss in acidification activity during frozen storage at -20°C (k) and, to a lesser extent, by the initial acidifica-

Figure 6. Principal component analysis of the 3 acidification variables (tc, dtf, and k) and the 6 fatty acid composition variables (unsaturated/saturated ratio, C16:0, C18:0, C18:1, C18:2, and cycC19:0 relative concentrations) (A), showing the distribution of the samples obtained after fermentations at different pH and temperatures (B). tc = Initial acidification activity measured before freezing; dtf = difference in acidification activity before and after freezing; k = rate of loss in acidification activity during storage.

tion activity (tc) and the C18:1 content. The third axis accounted for an additional variance of 13% and was controlled by the C18:2 relative concentration.

High correlations were observed between C18:0, C16:0, and cycC19:0 concentrations and the U/S ratio,

thus confirming the conclusions previously obtained. Moreover, the variable associated with the freezing step (dtf) exhibited a good correlation with these variables: a high tolerance to the freezing step, i.e., a low dtf, was associated with a high U/S ratio ($R = -0.874$), a low C18:0 content ($R = 0.934$), and high C16:0 ($R = -0.77$), and cycC19:0 ($R = -0.96$) relative concentrations. The rate of loss in acidification activity during frozen storage (k) was inversely correlated to the cycC19:0 relative concentration ($R = -0.725$): a high cycC19:0 induced a low k value and a high resistance during storage. Finally, the initial acidification activity correlated well with C18:1 ($R = 0.752$) and C16:0 ($R = 0.72$) concentrations.

Clustering was obtained according to the fermentation conditions (Figure 6B). The 2 fermentations conducted at pH 6 and 37°C are grouped in the lower right quadrant. They showed a high value of dtf, but low t_c and k values, associated with high C18:0 and cycC19:0 relative concentrations, high U/S ratio, and low C16:0 and C18:1 relative concentrations. The fermentation performed at pH 6 and 42°C is situated in the upper right quadrant. It is characterized by high C18:0 and C18:1 contents and low cyc C19:0 concentration, and by high values of dtf and k , thus indicating very poor cryotolerance. The fermentations conducted at low temperature (pH 6 and 30°C) or at low pH (37°C and pH 5 or 4.5) are grouped in the negative half of the first axis. They were distinguished by their high C16:0 and cycC19:0 relative concentrations and U/S ratio, and their low C18:0 relative concentration. These characteristics were associated with a low dtf value, i.e., a good resistance to the freezing step, which was counteracted by a high t_c value. Finally, the third axis made it possible to differentiate the fermentation performed at pH 4.5, which was characterized by a low C18:2 concentration and a relatively high k value.

From these results, strong relationships were observed between the resistance of *Lb. acidophilus* RD758 to the freezing step and the relative contents of C16:0, C18:0, and cycC19:0 fatty acids on the one hand, and between the resistance during the frozen storage and the cycC19:0 relative concentrations, on the other hand. Moreover, the poor cryotolerance observed at pH 4.5 was associated with a decreased content in C18:2 fatty acid. Therefore, good control of the concentration of these 4 key fatty acids may improve the cryotolerance of *Lb. acidophilus* RD758. Such control was obtained by applying specific fermentation conditions (low fermentation temperature and pH). This was confirmed by the fermentation conducted at pH 5 and 30°C, which showed high tolerance to the freezing step (dtf = 0 min) and to frozen storage ($k = 0.6$ min/d), and displayed

high contents in C16:0 and cycC19:0 fatty acids and a low C18:0 concentration.

CONCLUSIONS

The fermentation pH and temperature influenced the resistance to freezing and frozen storage of *Lb. acidophilus* RD758. The best initial acidification activity was obtained when the cells were cultivated at 37°C and pH 6, which corresponded to the optimal temperature and pH for growth. The loss in acidification activity during freezing and frozen storage increased with fermentation temperature and pH. The best tolerance was observed at 30°C or at pH 5. The increased resistance to freezing was linked to low C18:0 content, and to high C16:0 and cycC19:0 relative concentrations. The best resistance to frozen storage was explained by a high cycC19:0 content. Finally, a fermentation pH of 4.5 was detrimental to cryotolerance, which was related to the C18:2 content.

These results need to be complemented by an analysis of the proteomic responses of cells to different culture conditions in relation to their cryotolerance. This analysis may point out the increased synthesis of some stress proteins (Wouters et al., 1999; Lorca and Font de Valdez, 2001) and, particularly, of some enzymes involved in the synthesis of these specific fatty acids, such as desaturases.

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