Resistance to freezing and to frozen storage of Streptococcus themophilus is related to membrane fatty acid composition

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Resistance to Freezing and Frozen Storage of *Streptococcus thermophilus* Is Related to Membrane Fatty Acid Composition

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ABSTRACT

The resistance to freezing and frozen storage of *Streptococcus thermophilus* was related to the fatty acid composition of the cell membrane. The effects of four experimental factors were investigated on the fatty acid concentrations and on the recovery of acidification activity of *S. thermophilus* stored at –20°C by using a complete experimental design: incorporating oleic acid in the culture medium, fermentation pH, addition of glycerol as cryoprotective agent and duration of storage. The acidification activity decreased during the freezing and the frozen storage of *S. thermophilus*. The storage time slightly enhanced the unsaturated fatty acid concentrations. The addition of glycerol did not modify the fatty acid composition but increased the resistance to frozen storage. The addition of oleic acid and the decrease of the fermentation pH enhanced the ratio unsaturated:saturated fatty acids and improved the recovery of the acidification activity. These results indicate that the resistance to frozen storage was closely related to the membrane fatty acid composition. We interpreted this as an adaptation of *S. thermophilus* to the addition of oleic acid and the unfavorable growth conditions that corresponded to a low fermentation pH.
(**Key words:** lactic acid bacteria, acidification activity, frozen storage, fatty acid composition)

Abbreviation key: CFA = cyclopropane fatty acid, **FAME** = fatty acid methyl ester, **k** = rate of loss in acidification activity (in minutes/day), **tm** = time necessary to reach the maximal acidification rate (in minutes), **tm0** = time necessary to reach the maximal acidification rate at the beginning of the storage (in minutes), **ts** = storage time (in days), **U/S** = ratio between unsaturated and saturated fatty acids.

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INTRODUCTION

Production and conservation of lactic acid starters are necessary for research and industrial uses, but up to now, their quality has not been well managed. Both their viability and their physiological state can define the quality of lactic acid starters (Monnet et al., 1996). According to Fonseca et al. (2000), the quality can be quantified by a measurement of the acidification activity of the bacteria.

The acidification activity of lactic acid bacteria at the different steps of their production (fermentation, cooling, concentration, cryoprotection, freezing, or freeze-drying) and during storage differs depending on the strain considered and on the operating conditions (Béal and Corrieu, 1994; Bozoglu et al., 1987; Gilliland and Rich, 1990; Simatos et al., 1994). The freezing step is especially critical as it negatively affects both viability and physiological state of the bacteria (Brashears and Gilliland, 1995; Foschino et al., 1996; Tsvetkov and Shishkova, 1982). The formation of ice crystals induces mechanical damage that leads to cellular death (McGann, 1978). In addition, the crystallization of the water leads to a cryo-concentration of the solutes, which induces some osmotic damage (Meryman, 1968). Adding cryoprotective agents (such as glycerol) attenuated the damaging effects of freezing, thus improving the bacterial resistance to freezing (Fonseca et al., 2000; Morice et al., 1992; Thunell et al., 1984). This protective effect was ascribed to interactions between glycerol and the membrane phospholipids during freezing (Anchordoguy et al., 1987).

Because the cell membrane is the first target to modification of the cell environment, its ability to adapt largely determines the survivability of the cell (Sajbidor, 1997). Lowering the temperature affects the structure and the properties of the cellular membrane. During freezing, the liquid phase moves to a liquid-crystalline phase, thus reducing membrane fluidity (Simatos et al., 1994). By considering the important role of fatty acid organization in membrane permeability, the

membrane viscosity (Sajbidor, 1997) and the membrane thickness (In't Veld et al., 1992) were ascribed to the unsaturation index of membrane fatty acids: the cell membrane adapts by increasing the proportion of unsaturated fatty acids (**CFA**) (Brennan et al., 1986; Teixeira et al., 1996). Unsaturated fatty acids and cyclopropane fatty acids (**CFA**) promote exchanges between extracellular and intracellular media by rigidifying the membrane and enhancing the membrane permeability: the dihydrosterculic acid ($\Delta C_{19:0}$–9) is responsible for the elasticity and the flexibility of the membrane (Smitte et al., 1974), and the lactobacillic acid ($\Delta C_{19:0}$–11) allows lateral mobility of cellular membrane (Nikkilä et al., 1996). The increased membrane permeability is related to the presence of the double bounds that tend to form less stable Van der Waals interactions with adjacent lipids (In't Veld, et al., 1992). As a consequence, altering the fatty acid composition of the membrane may improve membrane permeability at low temperature and then may allow the cell to adapt itself to freezing.

Relationships between the fatty acid composition and the viability of some lactic acid bacteria have been previously established. They considered either the concentration in some unsaturated fatty acids, or the ratio between unsaturated and saturated fatty acids (**U/S**). The viability of frozen cells of *Lactobacillus bulgaricus* increases with the dihydrosterculic acid concentration (Smittle et al., 1974). The U/S ratio enhances the resistance to freezing of *Lactococcus lactis* subsp. *lactis* and *Lactobacillus* sp. (Goldberg and Eschar, 1977). The viability of freeze-dried cultures of *Lb. bulgaricus* decreased with the relative concentration of unsaturated fatty acids (Castro et al., 1995). Finally, the viability of dried *Lb. bulgaricus* was related to the U/S ratio (Teixeira et al., 1996).

The U/S ratio depends on the medium and environmental conditions in which the cells are cultivated and stored. Concerning lactic acid bacteria, the addition of oleic acid (or Tween 80) in the culture medium enhances the concentration in dihydrosterculic acid and the U/S ratio (Goldberg and Eschar, 1977; Smittle et al., 1974). The biosynthesis of unsaturated $C_{18:1}$ fatty acids by some lactic acid bacteria is stimulated by the addition of ethanol and leads to an increase of the U/S ratio (Lonvaud-Funel and Desens, 1990). The pH of the culture influences the total fatty acid concentration: the lactobacillic acid concentration is appreciably reduced at low pH (Nikkilä et al., 1996; Palmfeldt and Hahn-Hägerdal, 2000; Schmitt et al., 1989), and the U/S ratio and the amount of cyclopropane fatty acids are inversely related to the pH (Drici-Cachon et al., 1996). This was explained by Nikkilä et al. (1996), who showed that the cyclopropane fatty acid synthase, an enzyme that is involved in the methylation of vaccenic and oleic acids ($C_{18:1}$–11 and $C_{18:1}$–9), is activated at low pH. The incubation temperature strongly affects the U/S ratio: decreasing the temperature increases the U/S ratio by accumulation of vaccenic acid (Lonvaud-Funel and Desens, 1990) and the degree of cyclization (Suutari and Laakso, 1992), thus corresponding to an adaptation phenomenon to low temperature. The fatty acid distribution also depends on the age of the culture. A shift from vaccenic acid to lactobacillic acid (Lonvaud-Funel and Desens, 1990) or from oleic acid to dihydrosterculic acid (Suutari and Laakso, 1992) was observed with the augmentation of the age of the culture. Finally, the fatty acid composition evolves during storage. Castro et al. (1996) observed two phases: a first increase of the U/S ratio, which is explained by lipolysis reactions, followed by a decrease. Teixeira et al. (1996) showed that U/S ratio is stable within 49 d of storage and then decreases. This decrease is linked to the oxidation of unsaturated fatty acids that are very sensitive to oxygen (Castro et al., 1996) and is accentuated by an increase in the residual relative humidity that activates the oxidation processes (Castro et al., 1995).

From these data, it is clear that acting on the membrane fatty acid composition can modulate the U/S ratio. This was achieved by using appropriate operating conditions and led to a better recovery of cellular viability after freezing and frozen storage. Nevertheless, as viability measurements are insufficient to express both viability and physiological states of lactic acid bacteria, these have to be proved by considering the acidification activity of lactic acid bacteria.

This work aimed to characterize the resistance to freezing and to frozen storage of a *Streptococcus thermophilus* strain in relation to its fatty acid composition. Fatty acid composition was modulated by performing different operating conditions, such as medium composition, pH of the culture and addition of glycerol as cryoprotective agent. The resistance to freezing and to storage was determined as the cellular ability to recover its acidification activity.

MATERIALS AND METHODS

Bacterial Strain and Media

Streptococcus thermophilus CFS2 (INRA, Thiverval-Grignon, France) was stored at −75°C. It was thawed for 5 min at 30°C before inoculation, which was carried out at 10^7 cfu ml^{-1}.

For starter production, the culture medium was composed of 60 g L^{-1} of mild whey (BBA, Bourgbarré, France) that was heated at 110°C for 10 min. After centrifugation (17,000 × *g*, 15 min, 4°C) and filtration (0.45 μm), 20 g L^{-1} of lactose (Prolabo, Paris, France),

5 g L^{-1} of yeast extract (Difco, Detroit, MI) and 1 ml L^{-1} of antifoam (Rhodorsil 426R, Prolabo) were added to the supernatant. When necessary, 1 g L^{-1} of oleic acid (in the form of Tween 80: polyoxyethylene sorbitan mono-oleate) (Prolabo) was added. The medium was sterilized in the fermentor at 110°C for 20 min.

For acidification activity measurement, the medium was composed of reconstituted dried skim milk (100 g L^{-1}) (Elle & Vire, Condé sur Vire, France). It was pasteurized for 1 h at 95°C in 250-ml Erlenmeyer flasks.

Fermentation

Cultures were performed in a 2-L fermenter, at 42°C, with an agitation speed of 200 rpm. The pH was controlled at 6.0, by adding a 14% NH_4OH solution, which was continuously weighted. Absorbance measurements at 480 nm were used to characterize bacterial growth.

Cultures were stopped at the beginning of the stationary phase. This was defined by the time at which NH_4OH consumption rate, calculated in real time, as the first time derivative of the NH_4OH weight decrease, started to decline. Cell suspension was then cooled to 15°C in the fermentor.

Concentration and Preservation

Cells were harvested at 15°C and concentrated 30 times by centrifugation (14,000 × *g*, 30 min, 4°C). Concentrated cells were resuspended in the same weight of supernatant, at 4°C. To half of the samples, glycerol (50 g L^{-1}) was added as cryoprotective agent. Five hundred microliters of cell concentrates was frozen at 0.75°C/min and stored at –20°C for 8 wk. The concentrates were thawed at 30°C for 15 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 lactic acid bacteria before and after freezing and during storage. Acidification was performed at 42°C. Each measurement was performed in triplicate. The pH of inoculated milk samples was continuously measured, which allowed the calculation of its first-time derivative, representing the acidification rate. For each sample, the time necessary to reach the maximum acidification rate in milk, **tm** (in min), was used to characterize the acidification activity of the bacterial suspensions. The higher the tm, the longer the latency phase and then, the lower the acidification activity.

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):

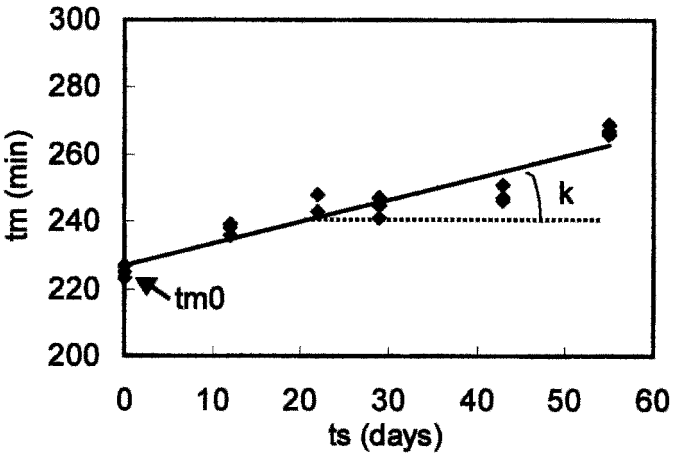

Figure 1. Acidification activity (tm) as a function of storage time (ts) of *Streptococcus thermophilus* CFS2 stored at –20°C with glycerol (50 g/L) as cryoprotective agent. Fermentation was conducted at pH 6.5 without oleic acid in the culture medium. tm = 226.9 (± 4.1) + 0.65 (± 0.11) × ts (r2 = 0.889).

$$tm = tm0 + k \times ts,$$

where **k** is the slope of the regression line (in min/d) and **tm0** is the intercept (in min). As an example, Figure 1 illustrates the loss in acidification activity of *S. thermophilus* CFS2 during 8 wk of storage at –20°C. The slope k represents the rate of loss in acidification activity during storage. A higher slope indicated a faster decrease of the acidification activity and then a lower resistance to frozen storage. The parameter tm0 represents the acidification activity of the cell suspension at the beginning of the storage. In the case of *S. thermophilus* CFS2, it is similar to the tm obtained before freezing, thus indicating that freezing did not affect the acidification activity of this strain (Fonseca et al., 2000).

Fatty Acids Analyses

At the end of the fermentation, before and after freezing and during storage, the membrane lipid composition was determined by using the method described by Rozès et al. (1993). Concentrated cells were washed twice in a solution of 1 g L^{-1} of bactopeptone. Methylation and extraction were performed simultaneously at 4°C by adding 1.5 ml of sodium methoxide (1 *M* in methanol) and shaking for 1 min. Fatty acid methyl esters (**FAME**) were extracted with 0.9 ml of hexane, and 0.1 ml of decanoic acid methyl ester (0.6 mg ml^{-1} in hexane) (Sigma, Saint-Quentin-Fallavier, France) was added as internal standard for gas-liquid chromatography. After decanting for 2 min, the upper phase was removed and stored at 4°C in an airtight glass bottle until analysis.

Gas chromatography was performed on a Chrompack cp9001 (Middlebur, The Netherlands) equipped with a flame-ionization detector and connected to a Winapex integrator (SRA Instrument, Marcy l'Etoile, France). A capillary column packed with polyethylene glycol (FFAP, Hewlett Packard, Avondale, PA) was employed. Helium at 1 ml min^{-1} was used as carrier gas, and injection volume was 1 μl. Injection was done splitless for 2 min. Oven temperature was raised from 100 to 240°C at 5°C min^{-1}, held 30 min at 240°C. Injection and detection temperatures were 230°C.

Results were expressed as relative percentages of each fatty acid that were calculated as the ratio of the surface area of the considered peak on the total area of all peaks. The ratio between unsaturated and saturated fatty acids (U/S) was determined. Samples were analyzed twice.

It was verified that the culture medium did not contain any fatty acids, except when oleic acid was added. In this last case, only $C_{18:1}$ was identified.

Identification of the Major Peaks

The fatty acid methyl esters were first identified by comparison of their retention times with those of known standards (Sigma).

Identification of FAME was confirmed by using a mass selective detector. A Hewlett Packard mass spectrometer (HP 68910A) was equipped with a nonpolar capillary column (HP 5 MS; 30 m × 0.25 mm; film thickness 0.25 μm) packed with diphenyl (5%) and dimethyl polysiloxane (95%). Carrier gas was helium at 1.6 ml min^{-1}. Injection (1 μl) was done splitless at 250°C. The oven temperature increase was programmed from 40 to 250°C at 10°C min^{-1}. The electron impact energy was set at 70 eV, and data were collected in the range of 29 to 300 atomic mass units at a scan rate of 1.68 scan s^{-1}.

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

Experimental Design

A complete experimental design, including four factors, was performed. It allowed the determination of the effects of the fermentation pH (5.5, 6.0, and 6.5), the addition of oleic acid (0 and 1 g L^{-1}) in the culture medium, the addition of glycerol (0 and 50 g L^{-1}) as cryoprotective agent, and the length of storage at –20°C (0, 2, 4, 6, and 8 wk). These factors have been tested on the acidification activity and on the fatty acid composition.

Statistical Analysis

A four-factor analysis of variance with two-factor interactions (Statgraphics *Plus 3*) was performed to determine the effects of the fermentation pH, the addition of oleic acid and glycerol, and the length of storage. The Neuman-Keuls multiple comparison procedure was used to discriminate among the means for significant differences at the 5% confidence level.

RESULTS AND DISCUSSION

Fatty Acid Composition of *S. thermophilus* CFS2

A total of nine fatty acids made up the membrane of *S. thermophilus* CFS2 cultivated at pH 6.0, in absence of oleic acid in the culture medium (Table 1). When oleic acid was present, seven fatty acids were identified. The peaks were identified as tetradecanoic (myristic) acid ($C_{14:0}$), hexadecanoic (palmitic) acid ($C_{16:0}$), hexadecenoic (palmitoleic) acid ($C_{16:1}$), octadecanoic (stearic) acid ($C_{18:0}$), cis-9-octadecenoic (oleic) acid ($C_{18:1}$–9), cis-11-octadecenoic (vaccenic) acid ($C_{18:1}$–11), methyleneoctadecenoic (dihydrosterculic) acid ($\Delta C_{19:0}$–9), eicosanoic (arachidic) acid ($C_{20:0}$), and eicosenoic acid ($C_{20:1}$). The cyclopropane fatty acid $\Delta C_{19:0}$–9 is produced by methylation of oleic acid, and is considered as unsaturated (Goldberg and Eschar, 1977).

In the absence of oleic acid, the major peaks were $C_{16:0}$, $C_{18:0}$, $C_{18:1}$–9, $C_{18:1}$–11, and $C_{20:1}$. These fatty acids represented more than 90% of the total fatty acids. As U/S ratio was equal to 1.02, saturated and unsaturated fatty acids were well balanced. When oleic acid was added in the culture medium, the U/S ratio was higher (1.43), thus indicating a shift from saturated to unsaturated fatty acids in the membrane composition. This was due to a decrease in $C_{14:0}$, $C_{16:0}$, and $C_{18:0}$ and an increase in $C_{18:1}$–9 and $C_{20:1}$ fatty acids. As confidence intervals overlapped, $C_{18:1}$–11, $\Delta C_{19:0}$, and $C_{20:0}$ did not show any significant difference, whether or not oleic acid was present.

Except for the $C_{20:1}$, most of these fatty acids have been identified in other species of lactic acid bacteria: *L. lactis* subsp. *lactis* and *Lactobacillus* sp. (Goldberg and Eschar, 1977), *Lb. bulgaricus* (Castro et al., 1995, 1996; Smittle et al., 1974; Thunell et al., 1984), *Lb. plantarum* (Rozès et al., 1993), *Lb. acidophilus* (Brennan et al., 1986), *Lb. büchneri* and *Lb. fermentum* (Nikkilä et al., 1996), *Lactobacillus* sp. (Dionisi et al., 1999), *Leuconostoc oenos* (Drici-Cachon et al., 1996; Lonvaud-Funel and Desens, 1990; Rozès et al., 1993; Tracey and Britz, 1989) and *Leuconostoc mesenteroides* (Schmitt et al., 1989). As no data are available for *S. thermophilus*, the occurrence of $C_{20:1}$ could characterize this strain.

Table 1. Relative fatty acid composition of *Streptococcus thermophilus* CFS2 in presence or in absence of oleic acid (1 g/L) in the culture medium.[1]

		No oleic acid		Oleic acid 1 g/L	
Fatty acid	RT	Mean*	ci	Mean*	ci
$C_{14:0}$	20.0	1.62	0.108	nd	
$C_{16:0}$	25.0	33.68	0.040	31.63	0.023
$C_{16:1}$	25.7	3.53	0.058	nd	
$C_{18:0}$	28.9	12.36	0.364	8.21	0.106
$C_{18:1-9}$	29.3	5.72	0.300	14.45	1.324
$C_{18:1-11}$	29.5	24.83	1.240	21.22	2.664
$\Delta C_{19:0}$	31.7	1.34	0.044	1.50	0.022
$C_{20:0}$	33.4	1.80	0.038	1.30	0.178
$C_{20:1}$	34.3	15.13	0.004	21.68	0.694
U/S		1.02	0.010	1.43	0.004

[1]RT = Retention time; ci = 95% confidence interval; nd = not detected; U/S = ratio between unsaturated and saturated fatty acids.

*The mean corresponded to three repetitions.

Combined Effects of pH, Oleic acid, Glycerol, and Storage Time on Membrane Fatty Acid Composition of *S. thermophilus* CFS2

The effect of the storage time, the fermentation pH, and the addition of oleic acid in the culture medium and of glycerol as cryoprotective agent on membrane fatty acid composition was studied. Analyses of variance were done to determine the significance level of the effect of each factor. The ratio between unsaturated and saturated fatty acids was affected by the fermentation pH, the addition of oleic acid in the culture medium and the storage time, while the addition of glycerol had no effect (Table 2). Relative fatty acid concentrations were diversely influenced by these first three factors. Two kinds of interactions were observed and will be discussed.

The addition of glycerol as cryoprotective agent did not modify the fatty acid composition of the membrane because this molecule was added at 4°C after the concentration step, immediately before freezing. At this stage of production, cells were still in a quiet physiological state. Moreover, at this temperature, the membrane permeability is low (McGann, 1978) and the passive diffusion of the glycerol from extracellular to intracellular medium was slowed down. Consequently, glycerol probably acted mainly as an extracellular cryoprotective agent.

As expected, the addition of oleic acid increased the concentration of unsaturated fatty acids $C_{18:1}$–9 and $C_{20:1}$, and decreased the concentration of saturated fatty acids $C_{16:0}$ and $C_{18:0}$ ($P < 0.001$), while proportions of $C_{18:1}$–11 and $\Delta C_{19:0}$ were not modified (Table 3). The U/S ratio increased from 0.85 to 1.60 in the presence of oleic acid was. These results agree with those reported by Smittle et al. (1974) and Goldberg and Eschar (1977) who showed that the U/S ratio is strongly dependent on the presence of oleic acid in the medium. This indicated that oleic acid, when incorporated in the in-

Table 2. Effects of the addition of oleic acid (OA) in the culture medium, the fermentation pH, the addition of glycerol (G) and the storage time (ST) on the fatty acid composition of *Streptococcus thermophilus* CFS2.[1]

	Factors				Interactions					
	OA	pH	G	ST	OA × pH	OA × G	OA × ST	pH × G	pH × ST	G × ST
$C_{16:0}$	0.00	0.00	37.89	0.18	0.00	98.35	15.62	74.29	43.98	78.84
$C_{18:0}$	0.00	0.00	58.92	12.40	1.31	86.40	16.55	91.55	15.69	84.81
$C_{18:1}$–9	0.00	0.00	96.34	36.12	0.00	67.98	10.41	86.61	91.14	53.57
$C_{18:1}$–11	6.59	0.00	59.84	0.08	0.00	63.64	6.26	93.10	15.97	81.24
$\Delta C_{19:0}$	73.51	0.00	46.57	1.72	0.01	26.68	5.17	5.60	25.57	86.65
$C_{20:1}$	0.00	0.00	48.27	12.78	0.00	63.12	71.06	19.24	80.68	21.37
ΣSFA	0.00	0.00	62.01	0.02	0.00	88.59	12.45	45.73	87.84	20.27
ΣUFA	0.00	0.00	77.80	0.01	0.00	67.33	3.35	42.69	10.90	52.95
U/S	0.00	0.00	64.35	0.20	0.00	64.87	4.71	73.03	33.47	56.73

[1]Probability levels (%) were associated with the ANOVA for two factor interactions. $C_{16:0}$ to $C_{20:1}$ are the fatty acids identified in *S. thermophilus* CFS2. ΣSFA = Sum of saturated fatty acid concentrations; ΣUFA = Sum of unsaturated fatty acid concentrations; U/S = ratio between unsaturated and saturated fatty acid concentrations.

Table 3. Means and Neuman-Keuls multiple comparison test for the fatty acid composition of *Streptococcus thermophilus* CFS2 showing significant effects of the main factors.[1]

	Oleic acid (g/L)		Fermentation pH			Storage time (days)				
Factors	0	1	5.5	6.0	6.5	0	14	28	42	56
$C_{16:0}$	36.15^{e}	29.37^{f}	27.52^{g}	31.45^{f}	39.31^{e}	33.99^{c}	33.80^{c}	31.48^{d}	32.40^{d}	32.15^{d}
$C_{18:0}$	15.77^{e}	8.15^{f}	11.47^{f}	13.37^{e}	11.05^{f}	ns	ns	ns	ns	ns
$C_{18:1}$–9	4.36^{f}	13.78^{e}	10.18^{e}	9.04^{f}	7.99^{g}	ns	ns	ns	ns	ns
$C_{18:1}$–11	ns	ns	20.02^{f}	21.44^{e}	22.04^{e}	20.31^{f}	21.16^{ef}	20.85^{ef}	21.85^{e}	21.67^{e}
$\Delta C_{19:0}$	ns	ns	3.77^{e}	1.62^{f}	1.25^{f}	2.02^{b}	2.06^{b}	2.38^{a}	2.16^{ab}	2.43^{a}
$C_{20:1}$	13.34^{f}	23.21^{e}	21.32^{e}	19.60^{f}	13.90^{g}	ns	ns	ns	ns	ns
ΣSFA	51.92^{e}	37.53^{f}	38.99^{g}	44.82^{f}	50.36^{e}	46.15^{e}	45.30^{e}	44.68^{ef}	43.84^{ef}	43.65^{f}
ΣUFA	41.33^{f}	60.11^{e}	55.29^{e}	51.70^{f}	45.18^{g}	49.30^{f}	49.99^{ef}	50.74^{ef}	51.68^{e}	51.90^{e}
U/S	0.85^{f}	1.60^{e}	1.45^{e}	1.18^{f}	1.05^{g}	1.18^{d}	1.19^{d}	1.21^{cd}	1.28^{c}	1.27^{c}

[1] ΣSFA = Sum of saturated fatty acid concentrations; ΣUFA = sum of unsaturated fatty acid concentrations; U/S = ratio between unsaturated and saturated fatty acid concentrations. Different letters account for means significantly different at a 5% level (a, b), 1% level (c, d), 0.1% level (e, f, g).

ns = No significant difference at a 5% level.

tracellular medium, gives oleic acid and eicosenoic acid, thus unbalancing the fatty acid composition. As the concentration of $\Delta C_{19:0}$ is strain dependent (Goldberg and Eschar, 1977; Smittle et al., 1974) and was not affected by oleic acid in this study, it can be considered that this result is specific to the strain CFS2.

Decreasing the pH from 6.5 to 5.5 led to a highly significant effect on all fatty acids ($P < 0.001$). Figure 2 shows the effect of the fermentation pH on the U/S ratio determined in the absence of oleic acid. The relative concentrations in $C_{16:0}$ decreased, whereas those in $C_{18:1}$–9, $\Delta C_{19:0}$, and $C_{20:1}$ increased. Consequently, the U/S ratio changed from 1.05 to 1.45 (Table 3). The unsaturated fatty acids that were in a minority (45%) at pH 6.5 became predominant under pH 6.0 (52 to 55%). These results are in accordance with other reported previously (Drici-Cachon et al., 1996; Schmitt et al., 1989). In this study, the specific growth rates were determined. They were lower at pH 5.5 (0.52 ± 0.05 h^{-1}) than at pH 6.0 (1.23 ± 0.20 h^{-1}) and pH 6.5 (1.24 ± 0.24 h^{-1}), whether or not oleic acid was added. Thus, it can be proposed that the intracellular accumulation of unsaturated fatty acids was a response to the unfavorable environmental conditions caused by the low pH. This can be ascribed to a homeostatic mechanism that allows bacterial strains to maintain the permeability of the membrane (Krulwich and Guffanti, 1983) at low pH. Sajbidor (1997) confirmed this and indicated that the pH affects the transbilayer movements of phospholipids.

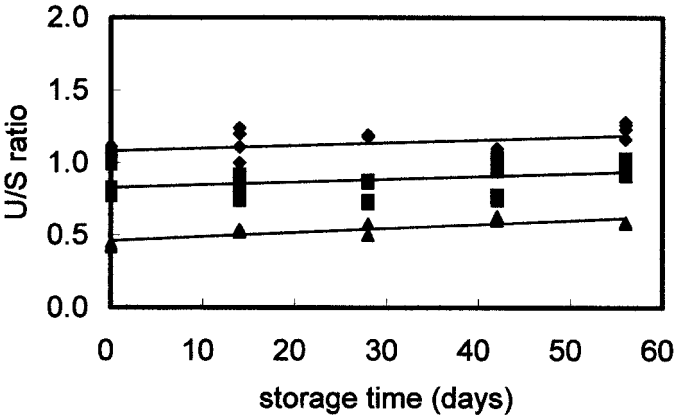

Figure 2. Influence of the pH of the fermentation on the evolution of the ratio between unsaturated and saturated fatty acids (U/S ratio) during frozen storage of *Streptococcus thermophilus* CFS2 at –20°C, in presence or absence of 50 g/L of glycerol as cryoprotective agent. Fermentations were conducted with no added oleic acid and at pH 6.5 (▲), pH 6.0 (■), or pH 5.5 (♦).

An interaction between the pH of the fermentation and the oleic acid was observed for all the considered variables (Table 2). The fatty acid relative concentrations and the U/S ratio were significantly affected by the pH only in the absence of oleic acid (Figure 3). By considering that the pH effect was a response of the membrane to disadvantageous conditions, and as addition of oleic acid led to an improvement of the cellular response to these conditions, it can be proposed that the influence of the pH was not revealed when good environmental conditions were combined.

From Table 2, the storage time of the frozen bacteria slightly affected the fatty acid composition ($P < 0.05$). By increasing the storage time from 0 to 56 d, the $C_{16:0}$ concentration decreased, whereas the $C_{18:1}$–11 and $\Delta C_{19:0}$ concentrations increased (Table 3). Nevertheless, the differences remained slight. From Figure 2, the U/S ratio increased with storage time and according to Table 3, it raised from 1.18 to 1.27. The effect observed on this ratio was more significant as it combined the negative effects that were discerned on the saturated fatty acids and the positive effects noticed on the unsaturated fatty acids. These results agree with those

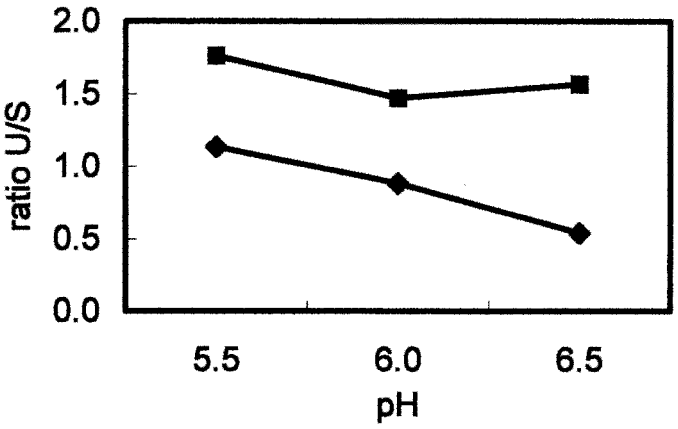

Figure 3. Interaction plot between fermentation pH and oleic acid addition for the ratio between unsaturated and saturated fatty acids (U/S ratio) measured during frozen storage of *Streptococcus thermophilus* CFS2 at –20°C. Culture medium was added (■) or not (♦) with 1 g/L of oleic acid.

of Castro et al. (1996) who showed that the U/S ratio increases during 4 wk of storage of freeze-dried *Lb. bulgaricus*. This was ascribed to lipolysis reactions that altered saturated fatty acid concentrations. Nevertheless, in this work the U/S ratio never decreased as it was shown for freeze-dried or spray-dried *Lb. bulgaricus* (Castro et al., 1995, 1996; Teixeira et al., 1996). The oxidation phenomena that were described by these authors were not active in the membrane of *S. thermophilus*. As in this study, cells were frozen and not dehydrated, they were less sensitive to oxidation reactions.

An interaction was observed between oleic acid and storage time on the U/S ratio, but it was slightly significant ($P < 0.05$). It could be attributed to a more important effect of the time of storage in the presence of oleic acid in the culture medium than when it was absent.

Combined Effects of pH, Oleic Acid, and Glycerol on the Resistance to Frozen Storage of *S. thermophilus* CFS2

The acidification activity of *S. thermophilus* CFS2 was not significantly different before and after the freezing step. The mean value of tm varied from 212.2 (±8.9) min to 215.7 (±13.4) min, regardless the experimental conditions. This indicates that the strain was not sensitive to freezing, as was shown previously (Fonseca et al., 2000).

During frozen storage, the acidification activity decreased, as evidenced by the tm increase in all environmental conditions. The mean value of tm rose from 215.7 (±13.4) min at the beginning of the storage to a value ranging from 219.5 to 338 min, after 8 wk of storage at –20°C. This phenomenon was modeled by linear relationships that allowed the quantification of the rate of loss in acidification activity (k) as well as the acidification activity (tm0) at the beginning of the storage (Fonseca et al., 2000). The variance of tm0 and k was calculated to determine the significance level of the influence of the three environmental factors on these parameters (Table 4). The parameter tm0 was affected by none of the factors. As the freezing step did not influence the acidification activity of *S. thermophilus* CFS2, it was concluded that this strain showed a good resistance to this operation. The resistance to frozen storage was influenced by the fermentation pH ($P < 0.001$), the addition of oleic acid in the culture medium ($P < 0.01$) and the addition of glycerol as cryoprotectant ($P < 0.01$).

From Table 4, the addition of oleic acid in the culture medium showed a significant effect on the rate of loss of acidification activity ($P < 0.05$). The recovery of the acidification activity was improved by the addition of oleic acid in the culture medium (Figure 4). These results are in agreement with those of Smittle et al. (1974) who showed that the cellular death of *Lb. bulgaricus* was linearly correlated with the proportion in $\Delta C_{19:0}$, and with those of Goldberg and Eschar (1977), who indicated that *Lactobacillus* sp. and *L. lactis* viability was improved by adding oleic acid in the culture medium. This could be related to the changes in the fatty acid composition of the cellular membrane that were observed in the first part of this work.

Decreasing the fermentation pH led to a highly significant decrease ($P < 0.001$) of the slope k, i.e., of the rate of loss in acidification activity (Figure 5). From a Neuman-Keuls multiple comparison test, k values varied from 0.96 (pH 6.5) to 0.38 (pH 6.0) and to 0.185 min/d (pH 5.5). This indicates that decreasing the pH of the fermentation improves the resistance of *S. thermophilus* to frozen storage. These results are in agreement with those of Gilliland and Rich (1990), who showed that *Lb. acidophilus* survived better when it was grown at pH 5.0 instead of pH 5.5 to 7.0.

As expected, the addition of glycerol as a cryoprotective agent considerably improved the resistance to frozen storage of *S. thermophilus* (Figure 4). The mean values of the slope k, determined from a Neuman-Keuls multiple comparison test, decreased from 0.75 min/d in the absence of glycerol to 0.27 min/d when glycerol was added. The rate of loss in acidification activity was three times lower when glycerol was added. This result confirmed the findings of Fonseca et al. (2000). Moreover, the glycerol effect was revealed whatever the other experimental conditions were (presence of oleic acid in the culture medium or fermentation pH). This indicated

Table 4. Effects of the addition of oleic acid (OA) in the culture medium, the fermentation pH, the addition of glycerol (G) and the storage time (ST) on the parameters characterizing the loss in acidification activity during freezing and frozen storage of *Streptococcus thermophilus* CFS2.[1]

	Factors			Interactions		
	OA	pH	G	OA × pH	OA × G	pH × G
k	0.33*	0.09*	0.12*	10.89	38.41	1.27*
tm0	88.34	11.57	54.68	78.79	93.56	67.09

[1]Probability levels (%) were associated with the ANOVA for two factor interactions. k = Slope of the regression line established between the acidification activity and the storage time (in min/d); tm0 = constant of the regression line (in min).

* = Significant probabilities at a 5% level.

that the glycerol displayed an additional effect that can be combined with other beneficial effects.

The interaction between glycerol and pH ($P < 0.05$) showed that the k value was more affected by the pH in the absence of glycerol, i.e., in the worst conditions for the recovery of acidification activity (Figure 6). This result can be ascribed to an adaptation phenomenon of the cells to unfavorable pH conditions that can be related to the fatty acid composition as was previously exhibited.

Relationship Between the Resistance to Frozen Storage of *S. thermophilus* CFS2 and its Membrane Fatty Acid Composition

The fatty acid composition of *S. thermophilus* CFS2 and the ability of the cells to restore their acidification activity were closely related. The higher the ratio between unsaturated and saturated fatty acids, the better is the resistance to frozen storage. This was observed in all the operating conditions used during the fermentation and the cryoprotection. Furthermore, the effects were synergistic, except in the case of the existence of interactions.

A better resistance to frozen storage was achieved by adding oleic acid in the culture medium that led to higher U/S ratios. The positive effect of oleic acid was detected regardless of the fermentation pH and whether or not glycerol was added. Adding oleic acid increased the permeability for water of the membrane, thus favoring transport across the membrane (In't Veld et al., 1992). As a consequence, the cryo-syneresis was enhanced, thus leading to a reduction of the cellular volume by removal of free water from the cytoplasm. The cells were then more adapted to suffer the intracellular ice crystallization during freezing and the water mobility during storage. Furthermore, the membrane lipids interact with protein interfaces, either by maintaining the protein structure and activity, or by inhibiting or activating protein functions, such as carrier proteins, which mediate solute transport (McIntosh, 1999). For example, the activity of the membrane bound

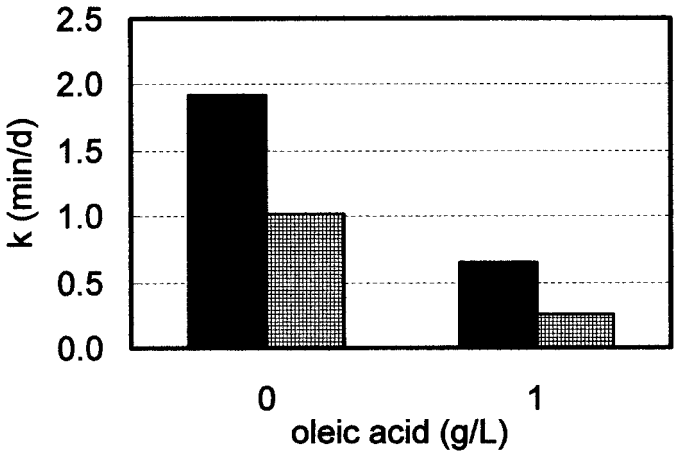

Figure 4. Combined effect of the addition of oleic acid in the culture medium and glycerol as cryoprotective agent on the rate of loss in acidification activity (k) of *Streptococcus thermophilus* CFS2 stored at –20°C. No added glycerol (hatched bar) or with 50 g/L glycerol (black bar).

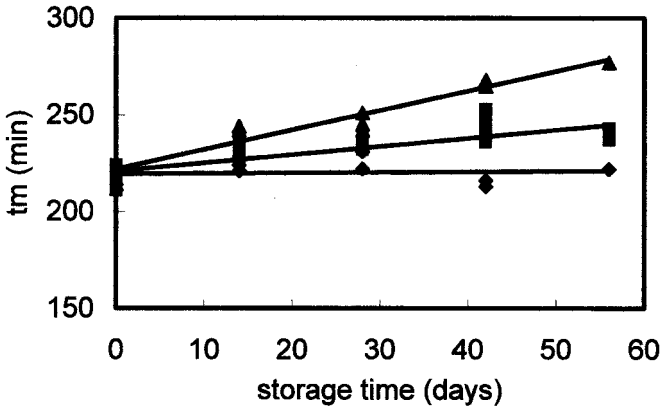

Figure 5. Influence of the fermentation pH on the acidification activity (tm) during frozen storage of *Streptococcus thermophilus* CFS2 at –20°C, added with 50 g/L of glycerol as cryoprotective agent. Fermentations were conduced at pH 6.5 (▲), pH 6.0 (■), or pH 5.5 (♦).

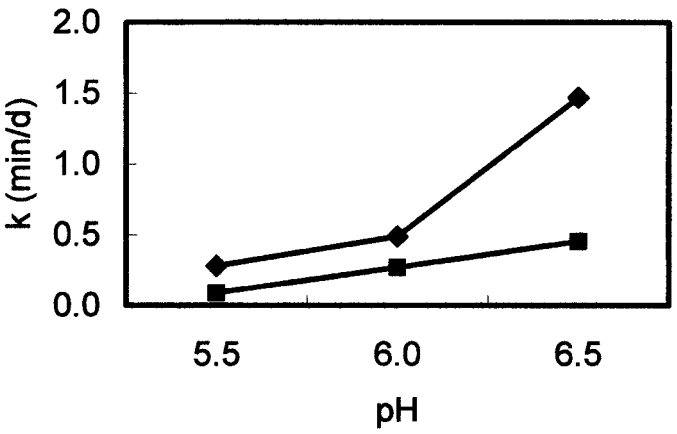

Figure 6. Interaction plot between fermentation pH and glycerol addition for the rate of loss in acidification activity (k) measured during frozen storage of *Streptococcus thermophilus* CFS2 at –20°C. In absence (♦) or presence (■) of 50 g/L of glycerol.

enzyme Na-K-ATPase was shown to be regulated by the lipid portion of the membrane (Suutari and Laakso, 1992).

The effect of the fermentation pH appears to be more complex, as was revealed the interactions, including this factor. Lowering the pH reduced the rate of loss in acidification activity and increased the ratio between unsaturated and saturated fatty acids, only when oleic acid was present. When oleic acid was added, the effect of the fermentation pH on the rate of loss in acidification activity could be explained by an influence on some enzymatic activities that may modify the protein composition and content of the cell. This is corroborated by the results of Rallu et al. (2000) who reported, in the case of *L. lactis*, an increase in the concentration of cold-shock proteins when the cells suffered an acidic stress. In the absence of oleic acid, however, the pH decrease enhanced the U/S ratio. This indicated that pH might act directly on the biosynthesis of the unsaturated fatty acids, even if no information has been found about the pH effect on the enzymes involved in fatty acid biosynthesis and desaturation. The appearance of unsaturated fatty acids can then be linked to disadvantageous growth conditions. Furthermore, the previous hypothesis, which proposed a pH effect on some enzymatic activities, could also be considered when no oleic acid was added.

The positive effect of glycerol on the resistance to frozen storage is independent of the U/S ratio, which was not affected by this factor. This indicates that two different mechanisms accounted for the different rates of loss in acidification activity: first, a better cellular adaptation, related to the higher water permeability of the membrane, achieved with high U/S ratios; second, a cryoprotective effect of glycerol that took place in addition to the previous effects. This interpretation corroborated the previous hypothesis that this molecule probably acted as an extracellular cryoprotective agent.

The relationship between the fatty acid composition of *S. thermophilus* and its ability to recover acidification activity led to important consequences for performing starter production. From our results, the resistance to frozen storage was improved by increasing the U/S ratio that was obtained by applying unfavorable experimental conditions for growth (low fermentation pH) and by adding oleic acid in the culture medium. Consequently, performing the starter production with unfavorable pH conditions, i.e., with a lower productivity, enhanced the recovery of acidification activity. This concept can be broadened by relating the membrane fatty acid composition to disadvantageous growth conditions, such as low temperature (Lonvaud-Funel and Desens, 1990; Sajbidor, 1997; Suutari and Laakso, 1992), acid stress (Rallu et al., 2000), ethanol stress (Lonvaud-Funel and Desens, 1990), salt stress (Monteoliva-Sanchez et al., 1993), osmotic stress (Kets et al., 1996; Linders et al., 1998) or high age of the culture (Brashears and Gilliland, 1995; Drici-Cachon et al., 1996).

CONCLUSIONS

The resistance to freezing and frozen storage of lactic acid starters was defined by its ability to recover acidification activity after thawing. The rate of loss in acidification activity during frozen storage varied according to the conditions in which the cells were cultivated and cryoprotected. In this work, it was possible to improve the stability of a frozen starter of *S. thermophilus* CFS2: after 8 wk of storage of a starter produced in unfavorable culture conditions, the loss in acidification activity (tm) reached 100 min, whereas, in the favorable conditions, no loss was noticed.

The resistance to frozen storage was improved by growing at unfavorable (acid) pH and by adding oleic acid in the culture medium and glycerol as cryoprotective agent. This improvement was related to an increase of the membrane ratio between unsaturated and saturated fatty acids. Furthermore, though some interactions appeared, these environmental conditions may be combined to enhance the favorable effects.

The relationship between the U/S ratio and the addition of oleic acid was obvious, but the dependence of the U/S ratio to the pH remained still unclear. In the future, the behavior towards pH of the enzymes involved in the biosynthesis of the unsaturated fatty acids has to be specified. Moreover, the effects of other environmental factors must be quantified to generalize the conclusions of this work.

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