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ORIGINAL PAPER

Impact of fermentation pH and temperature on freeze-drying survival and membrane lipid composition of *Lactobacillus coryniformis* Si3

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Abstract During the industrial stabilization process, lactic acid bacteria are subjected to several stressful conditions. Tolerance to dehydration differs among lactic acid bacteria and the determining factors remain largely unknown. *Lactobacillus coryniformis* Si3 prevents spoilage by mold due to production of acids and specific antifungal compounds. This strain could be added as a biopreservative in feed systems, e.g. silage. We studied the survival of *Lb. coryniformis* Si3 after freeze-drying in a 10% skim milk and 5% sucrose formulation following different fermentation pH values and temperatures. Initially, a response surface methodology was employed to optimize final cell density and growth rate. At optimal pH and temperature (pH 5.5 and 34 °C), the freeze-drying survival of *Lb. coryniformis* Si3 was 67% (±6%). The influence of temperature or pH stress in late logarithmic phase was dependent upon the nature of the stress applied. Heat stress (42 °C) did not influence freeze-drying survival, whereas mild cold- (26 °C), base- (pH 6.5), and acid- (pH 4.5) stress significantly reduced survival. Freeze-drying survival rates varied fourfold, with the lowest survival following mild cold stress (26 °C) prior to freeze-drying and the highest survival after optimal growth or after mild heat (42 °C) stress. Levels of different membrane fatty acids were analyzed to determine the adaptive response in this strain. Fatty acids changed with altered fermentation conditions and the degree of membrane lipid saturation decreased when the cells were subjected to stress. This study shows the importance of selecting appropriate fermentation conditions to maximize freeze-drying viability of *Lb. coryniformis* as well as the effects of various unfavorable conditions during growth on freeze-drying survival.

Keywords Lactic acid bacteria · Fermentation stress · Freeze-drying survival · Lipid composition

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Introduction

Lactobacillus coryniformis Si3 originates from grass silage and inhibits molds effectively [20, 21]. Future industrial uses of *Lb. coryniformis* Si3 rely on effective and robust fermentation and formulation procedures, with high storage stability. Industrial stabilization of starter cultures involves many stressful conditions and even well documented microorganisms might fail to reach the market due to losses in viability during stabilization. Freeze-drying is a commonly used stabilization technique for lactic acid bacteria, yielding satisfactory survival rates for many strains. The formulation and the freeze-drying process are closely related, and are of importance for the structural appearance and ease of use of the final product, as well as the microbial viability therein [16, 28]. Lyoprotective agents are of indisputable importance for survival, and act through preservation of lipid bilayer integrity by water replacement, vitrification (glass formation), and depression of membrane transition temperatures (T_m), as reviewed extensively by Crowe et al. [6–8].

Several reports have suggested that improved stress tolerance can be achieved if cells are mildly stressed

during growth [29, 36], and correlations between membrane lipid compositions of different *Lactobacillus* spp. and their freeze tolerance (cryotolerance) have been demonstrated [1, 35]. However, knowledge of the effects of stressing lactic acid bacteria prior to freeze-drying is limited. Any environmental change resulting in a biochemical and/or physiological adaptive response, such as altering membrane lipid composition is known as stress. Changing growth conditions have been shown to change the membrane properties of lactic acid bacteria [5, 32]; however, this family of bacteria is very heterogeneous and responses to stress vary among strains [23, 34]. Modifying the physical properties of the membrane is a crucial strategy for cell survival in unfavourable environmental conditions [4, 22, 32]. In response to stress, the lipid content is modified by the cell in order to maintain the fluidity of the membrane, a phenomenon known as homeoviscous adaptation [30]. This is done by changing the relative amounts of long and short chain fatty acids, straight and branched fatty acids, changed saturation levels, as well as by shifting *cis*- to *trans*-fatty acid configurations [4, 15, 18, 33]. In lactic acid bacteria, the proportion of unsaturated and cyclopropane fatty acids have been reported to be important for membrane adaptation [1, 13, 25].

We have previously published a study on the influence of formulation factors, such as lyoprotectant concentration, cell density and ice formation on freeze-drying survival and product appearance of *Lb. coryniformis* Si3 [28]. The objectives of this study were to (1) establish an fermentation regime for *Lb. coryniformis* Si3 using a response surface methodology, (2) determine the freeze-drying survival in a 10% skim milk and 5% sucrose formulation after optimization of the fermentation and (3) evaluate the effect of different mild stress during fermentation on freeze-drying survival of *Lb. coryniformis* Si3.

Materials and methods

Design of experiments to standardize fermentation

The experimental design software MODDE version 6 (Umetrics AB, Umeå, Sweden) was used to study the impact of pH and temperature on growth rate and cell density of *Lb. coryniformis* Si3 in commercial de Man, Rogosa, Sharpe broth (MRS, Oxoid Ltd., Basingstoke, England). Fermentation conditions were evaluated by varying pH from 5.0 to 6.0 and temperatures from 30 to 44 °C. A full factorial study on three levels with 12 independent runs was conducted and a multiple linear regression (MLR) model was fitted to the data. Evaluation of optimal conditions was performed with regard to both growth rate and final cell density. The proportion of the total variation explained (r^2), and predicted variation after cross-validation (q^2) were calculated. A positive correlation indicated that the response value increase with the factor in question.

Fermentation conditions

Lb. coryniformis Si3 was identified as previously described [28]. Long-term storage was at −70 °C in a glycerol-based freezing medium (15 w/v % glycerol salt solution; 0.82 g L^{-1} of K_2HPO_4, 0.18 g L^{-1} of KH_2PO_4, 0.59 g L^{-1} of Na-citrate, and 0.25 g L^{-1} $MgSO_4 \bullet 7H_2O$). An E-flask with 200 mL MRS was inoculated with cells from the frozen stock, incubated overnight at 30 °C and used as inoculum in the fermentor. Fermentation was performed in a 2 L vessel (Belach, Stockholm, Sweden) with a total culture volume of 1.8 L MRS and 10 mL inoculum. Tween 80 is present at 1 g L^{-1} in the commercially available MRS media. The fermentor has the shape of a glass cylinder with an inner diameter of 0.10 m and a height of 0.26 m and is equipped with impellers of the Rushton type. Media were sterilized in the fermentor at 121 °C for 17 min. The pH was adjusted by addition of 4 M NaOH and 3 M K_2PO_4. Growth kinetics were monitored as change in optical density at 600 nm (OD 600). Growth rate (μ, in h^{-1}) in the initial phase when growth was unrestricted was calculated according to Eq. (1), where N is the approximate cell number in OD units and t is time in hours.

$$\mu_{\max} = \frac{\ln N_{t2} - \ln N_{t1}}{(t_2 - t_1)} \quad (1)$$

Two growth strategies were selected to define stress regimes; either growing the cells optimally to late log-phase (12 h) followed by a change in temperature or pH for 6 h, or by growing the cells at the non-optimal condition of pH 4.5 and temperature of 30 °C throughout the fermentation (Table 1). Cells were harvested in the stationary phase and samples were withdrawn for freeze-drying and lipid membrane fatty acid analysis. Cells grown using the optimised standard fermentation regime determined by the multivariate approach were used as the control.

Extraction of lipids and trans-esterification

Lipids were extracted with chloroform/methanol/water as described by Bligh and Dyer [3]. Fatty acid methyl esters (FAME) were prepared by incubation for 15 min at 80 °C in boron trifluoride/methanol, applying the method of Morrison and Smith [24]. FAME samples were extracted with hexane.

Table 1 The growth conditions selected in this study

	First 12 h	+6 h, then harvest	+38 h, then harvest
Optimal	pH 5.5, 34 °C	pH 5.5, 34 °C	n.a
Cold	pH 5.5, 34 °C	pH 5.5, 26 °C	n.a
Heat	pH 5.5, 34 °C	pH 5.5, 42 °C	n.a
Acid	pH 5.5, 34 °C	pH 4.5, 34 °C	n.a
Base	pH 5.5, 34 °C	pH 6.5, 34 °C	n.a
Acid/cold	pH 4.5, 30 °C	n.a	pH 4.5, 30 °C

Cold, heat, acid and base stress were determined by using optimal pH ± 1 unit and optimal temperature ±8 °C. Harvesting was done in stationary phase, as determined by growth curves. The base supply (e.g. acid production) showed that the cells were still growing at 12 h

n.a. not applicable

Analysis of fatty acid composition by GC-FID

Analysis of FAME in hexane was performed using a quadruple GC System (HP5890, Hewlett & Packard, Palo Alto, USA) equipped with a split/splitless injector. A CP-Sil 88 capillary column (Chrompack, Middelburg, The Netherlands; length, 50 m; inner diameter, 0.25 mm; 0.25 μm film) was used for the separation of the FAME. GC conditions were: injector temperature was held at 240 °C, detector temperature was held at 270 °C. The injection was splitless, carrier gas was He at a flow of 2 mL/min. The temperature programme was: 40 °C, 2 min isothermal; 8 °C/min to 220 °C; 15 min isothermal at 220 °C. The pressure programme was: 27.7 psi (=186.15 kPa), 2 min isobaric; 0.82 psi/min (5.65 kPa/min) to the final pressure 45.7 psi; 15.55 min isobaric at 45.7 psi (310.26 kPa). The relative amounts of carboxylic acids were calculated based on peak areas of the total ion chromatograms (TIC). Fatty acids were identified by GC and co-injection of authentic reference compounds obtained from Supelco (Bellefonte, USA). Degree of saturation of the membrane fatty acids was defined as the ratio between the three saturated fatty acids (SFA) (14:0, 16:0, 18:0) and the unsaturated fatty acids (UFA) (16:1Δ9*cis*, 18:1Δ9*cis*, 18:1Δ11*cis*) present in these bacteria.

Sample preparation for freeze-drying

After harvest, cells were centrifuged at 4,000×*g* for 30 min at room temperature (20–30 °C) and washed once in peptone water. The washed cells were concentrated to approximately 2×10^{10} CFU mL^{-1} in bacteriological peptone water. The cellular input in the final formulation was calculated by plate counts on MRS agar. A formulation stock for freeze-drying, composed of 20% (w/v) skimmed milk (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and 10% (w/v) sucrose (Merck KGaA, Darmstadt, Germany) in autoclaved water, was mixed in a 1:1 volume ratio with the cell suspension, creating a final water-based formulation of approximately 1×10^{10} CFU mL^{-1} in 10% skimmed milk, 5% sucrose, 0.1% bacteriological peptones (Oxoid Ltd., Basingstoke, England) and approximately 0.01 g L^{-1} Tween 80 (Merck Schuchardt OHG, Hohenbrunn, Germany).

Freeze-drying protocol

Freeze-drying was performed in a pilot plant freeze-dryer (Lyostar II, FTS kinetics, NY, USA). We used 7.0 mL vials (PharmaPack, Sweden) with a final fill volume of 2 mL. Freezing was performed in dryer to a shelf temperature of −40 °C at 5°C min^{-1}. The vacuum was decreased to 13.3 Pa and shelf temperature increased to −20 °C where the sublimation (primary drying) occurred below the collapse temperature (T_c). Secondary drying was performed in a stepwise manner up to 25 °C for a total of 18 h. After freeze-drying, the samples were sealed in the dryer under vacuum (13.3 Pa) and analyzed the same day. No visible structural collapse was detected in any of the vials and the dried material was of highly porous structure, capable of instant reconstitution upon addition of water.

After drying, the formulation was rehydrated in autoclaved ultra filtrated water with a temperature of 25 °C to the same volume as before drying. Dilutions were made in peptone water with Tween 80 to minimize cell aggregation. In this study we define culturability and viability as operational synonyms [17]. Cell viability, determined by cell counts, was determined by serial tenfold dilutions in peptone water and spotting of 10 μL onto MRS agar plates. The plates were incubated, wrapped in parafilm at 30 °C for 48 h, and 10–30 CFU in 7–9 replicates were counted. Survivals are reported as the ratio between cell counts before drying and cell counts after drying, and given as % values.

Statistics

The optimal pH and temperature for growth of strain *Lb coryniformis* Si3 were evaluated by the MODDE software as previously described [9]. Fermentations, freeze-drying survival and lipid composition analysis were performed with independent triplicate samples, i.e. separate fermentations. Student's *t* test ($n = 3$, two-sided, equal variance) was used to assess differences in survival after drying and in lipid compositions. The significance levels are designated in the graphs as follows; $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***.

Results and discussion

Experimental model of the impact of pH and temperature on growth

Optimal pH and temperature during fermentation of a bacterial strain are primarily determined with a one-variable-at-a-time (OVAT) approach or with multivariate design. The latter is preferred with regard to quicker and cost-effective analyses, and improved accuracy, especially if factors interact. In this study, we have established an optimal fermentation protocol for *Lb. coryniformis* Si3 by an experimental design approach. The growth kinetics of 12 independent fermentations with different pH and temperature were analyzed to assess maximal logarithmic growth rate and stationary phase cell density. The models for *Lb. coryniformis* Si3 describing the effects of pH and temperature on growth rate had an r^2 value of 0.94 ($q^2 = 0.80$) and on cell density, an $r^2 = 0.98$ ($q^2 = 0.77$), thus were deemed satisfactory.

Interactions between the parameters pH and temperature were found and the models used to describe a fermentation optimum for growth rate and for cell density included three terms; linear, interaction and quadratic. For *Lb. coryniformis* Si3, the measured maximal growth rate (μ_{max}) varied between 0.15 and 0.61 h^{-1}, and the maximal OD_{600} in stationary phase varied between 0.8 and 5.7 (Fig. 1). An optimum pH and temperature for *Lb. coryniformis* Si3, with regard to both density and growth rate was predicted at pH 5.5 and 34 °C. When experimentally verified, the settings resulted in a maximal growth rate of >0.53 h^{-1} and an OD_{600} of >5.5, corresponding to cell numbers of 10^9–10^{10} CFU mL^{-1}. This is in agreement with previously reported growth optima for members of the genus *Lactobacillus*, i.e. pH for mesophilic lactic between 5.4 and 5.8 [27] and a temperature optimum between 30 and 40 °C acid bacteria [2].

Freeze-drying survival and membrane fatty acid composition

Previously, we have studied the freeze drying survival of *Lb. coryniformis* Si3 in different concentrations of sucrose, where survival of *Lb. coryniformis* Si3 varied from less than 6% to over 70%, depending on cell density, sucrose concentration and freezing-rate [28]. The cellular inputs used in this study were carefully controlled (10.0 and 10.2 log CFU mL^{-1}; Figs. 2, 3) and the formulation and freeze-drying procedure were kept constant. Freeze-drying survival of cells harvested in stationary phase, following optimal growth was 67% (±6%) (Fig. 2). This survival agrees fairly well with other reports on freeze-drying survival of lactic acid bacteria. The maximum viability after freeze-drying of *Lb. brevis* and *O. oeni* in yeast extract and sodium glutamate was 68 and 54%, respectively [37]. *Lb. reuteri* survived to 80% after freeze-drying in skim milk [26]. *Lb. bulgaricus* ATCC 11842 reached a viability of 78% with 0.04 M cysteine [11]. However, many fermentation, formulation and freeze-drying factors, as well as strain, affect survival, and meaningful comparisons are difficult.

Studies on *Lb. acidophilus* RD758 have indicated improved freeze-tolerance after combining low temperature (30 °C) and low pH (pH 5, i.e. one pH unit from the optimal pH) [35]. When *Lb. coryniformis* Si3 was grown at a lower temperature in combination with lower pH throughout fermentation (30 °C and pH 4.5), the freeze-drying survival dropped to 38% (±7) (Fig. 3). However, when *Lb. acidophilus* RD758 was grown in even more acidic environments (pH 4.5), freeze tolerance was lower [35]. Our results showed a negative effect on freeze-drying tolerance of *Lb. coryniformis* Si3 following growth in both acidic and low temperature conditions, as well as after acid stress applied in late exponential phase. Base- (pH 6.5), acid- (pH 4.5) and cold- (26 °C) stress induction decreased

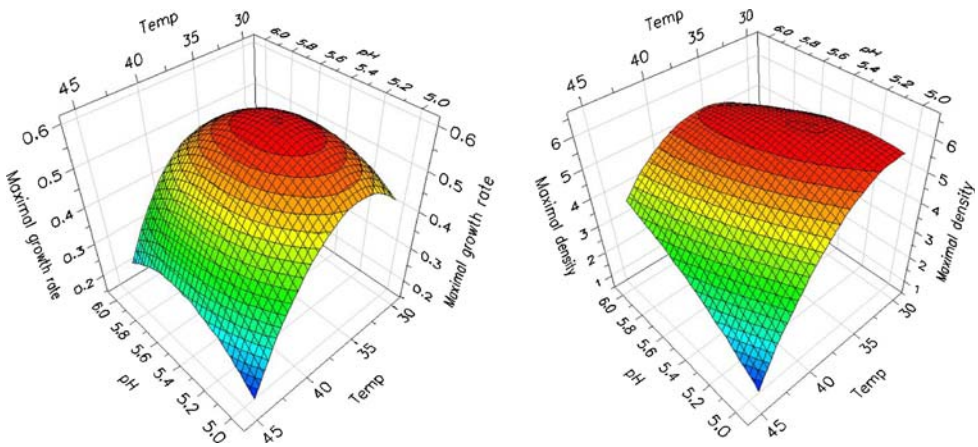

Fig. 1 Response surface plots showing the influence of temperature and pH on *Lb. coryniformis* Si3 growth rates (*left*, h^{-1}) and cell density (*right*, OD_{600})

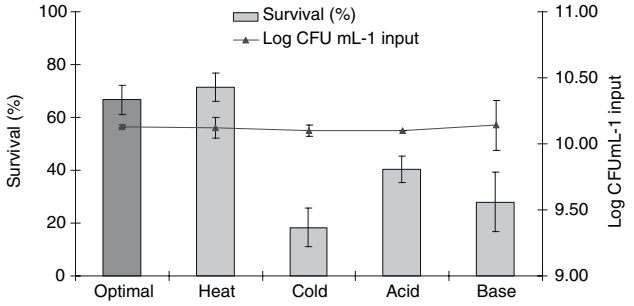

Fig. 2 Freeze-drying survival (%) of *Lb. coryniformis* Si3 grown at pH 5.5 and 34 °C for 18 h (control), and exposed to a heat (42 °C), cold (26 °C), acid (pH 4.5) or base (pH 6.5) shift in the logarithmic phase. The log CFU mL^{-1} input is displayed as *triangles* on a secondary axis and all *error bars* show standard deviation of three independent replicates. $p < 0.05$ = *, $p < 0.01$ = ** and $p < 0.001$ = ***

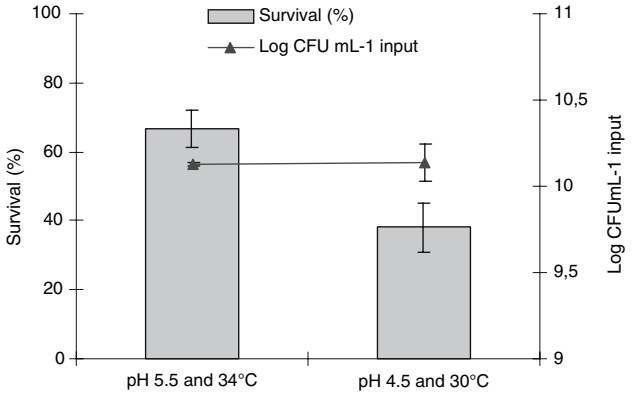

Fig. 3 Freeze-drying survival (%) of *Lb. coryniformis* Si3 grown at pH 5.5 and 34 °C or at pH 4.5 and 30 °C. The log CFU mL^{-1} input is displayed as *triangles* on the secondary *y*-axis. The *error bars* show standard deviation of three independent replicates. Students *t* test, equal variance, two sided test give $p < 0.01$ = **

survival to 28% (±11%), 40% (±5%), and 18% (±7%), respectively. However, heat stress for 6 h at 42 °C in late logarithmic phase did not affect freeze-drying survival, with survival rates of 72% (±5%) compared to cells grown at optimal temperature; 67% (±6%). Obviously, heat-induced modifications of membrane fatty acids in *Lb. coryniformis* Si3 do not directly affect freeze-drying tolerance; therefore, other cellular factors must be involved in the maintenance of freeze-drying tolerance following heat stress.

We determined the fatty acid compositions of *Lb. coryniformis* Si3 after the different fermentation conditions studied (Table 2). The lipid membrane of *Lb. coryniformis* Si3 contained three SFA (14:0, 16:0, 18:0) and the UFA (16:1Δ9*cis*, 18:1Δ9*cis*, 18:1Δ11*cis*), as well as lactobacillic acid (cyclo 19:0). These fatty acids are commonly found in other lactic acid bacteria strains [1, 10, 12, 19, 31, 32, 35]. The optimal setting of pH 5.5 and a temperature of 34 °C yielded the highest saturated to unsaturated fatty acid ratio, indicating a low stress level. In *Lb coryniformis* Si3, major changes as a response to different stresses were observed in the relative content of oleic acid (18:1Δ9*cis*). Alterations of the oleic acid contents led to changes in important sum parameters expressing membrane fluidity, namely, the ratio of SFA to UFA, also known as the degree of saturation, and the ratio between cyclopropane fatty acids (Cyclo) and UFA (Table 2). Generally, the degree of saturation decreased when the cells were subjected to a stress during fermentation. The fatty acid compositions of *Lb. coryniformis* Si3 grown in different conditions are shown in Table 2, together with corresponding freeze-drying survival rates.

For the stresses tested, a decrease in the environmental pH led to the highest increase in C18:1Δ9*cis* (Table 2), but also to a loss of freeze-drying tolerance of *Lb. coryniformis* Si3. The cryotolerance of *S. thermophilus* showed an increase following growth in acidic environments that was related to an increase in C18:1Δ9*cis* [1]. However, in the case of *S. thermophilus*, the saturation ratio was only affected in the absence of oleic acids in the fermentation media [1]. For *Lb. coryniformis* Si3, a significant decrease in saturation was observed when the pH was lowered from 5.5 to 4.5 during the late exponential phase. Upon fermentation of *Lb. coryniformis* Si3 at a slightly lower temperature (30 °C) and low pH 4.5, the effects on the degree of saturation were large (Table 2.) With these fermentation settings, 50 h was required for the culture to reach stationary phase, allowing the cells to adjust to the hostile environment. However, this also led to reduced freeze-drying tolerance (Fig. 3).

When *Lb. coryniformis* Si3 was subjected to cold temperature (26 °C) for 6 h initiated at late logarithmic growth, a decrease in saturation level and in relative amounts of cyclo 19:0 was observed, resulting in loss of freeze-drying tolerance. Similarly, a decrease in cyclo 19:0 has been shown for *Lb. acidophilus* following growth at 25 °C [10]. However, when *Lb. acidophilus* was subjected to this cold stress, it showed increased resistance to freeze-thaw and salt stress. In other reports, *Lb. acidophilus* RD758 showed improved cryotolerance when cells were grown at 30 °C or at pH 5, compared to cells grown at 42 °C or at pH 4.5. This enhanced cryotolerance was thought to be associated with a high content of UFA [35]. Cultures of *Lb. reuteri* pre-stressed with bile salt, with a lower ratio saturated to unsaturated membrane fatty acids, were shown to tolerate freezing to a lesser extent than control cells [31].

In accordance with previously reported data [13, 35], the lipid membrane of *Lb. coryniformis* Si3 shifted to a lower degree of saturation when subjected to temperature or pH stress, indicating that the pH and temperature determined

Table 2 Effect of stress conditions on freeze-drying survival and membrane lipid fatty acid composition of *Lb. coryniformis* Si3

	Optimal	Cold	Heat	Acid/cold[a]	Acid	Base
	34 °C, pH 5.5	26 °C, pH 5.5	42 °C, pH 5.5	30 °C, pH 4.5	34 °C, pH 4.5	34 °C, pH 6,5
Fatty acid (s)	Percentage of total[b]					
14:0	7.64 (0.54)	5.89 (0.79)	6.29 (1.02)	2.40 (0.00)	4.88 (1.38)	7.63 (0.76)
16:0	23.52 (1.10)	20.18 (0.74)	20.35 (1.11)	14.63 (1.69)	18.23 (1.02)	20.14 (0.57)
16:1*cis*	3.22 (0.10)	3.87 (0.26)	2.52 (0.25)	5.43 (6.39)	2.84 (0.55)	3.91 (0.36)
18:0	2.43 (0.02)	2.90 (0.38)	3.18 (0.42)	2.39 (0.29)	2.52 (0.22)	2.53 (0.35)
18:1Δ9*cis*	22.24 (2.86)	28.77 (0.50)	32.44 (2.01)	43.28 (3.46)	36.61 (7.56)	29.98 (2.74)
18:1Δ11*cis*	13.52 (0.53)	16.34 (2.76)	13.27 (2.00)	10.44 (2.82)	11.91 (0.51)	11.24 (0.97)
Cyclo 19:0	27.43 (0.85)	22.05 (1.68)	21.94 (4.24)	21.41 (1.07)	23.01 (4.32)	24.57 (3.52)
Σ	100.00	100.00	99.99	99.98	100.00	100.00
	Ratio[c]					
SFA/UFA	0.86	0.59**	0.62*	0.33***	0.51*	0.62*
Cyclo 19:0/UFA	0.77	0.49**	0.49*	0.40***	0.49*	0.60
	Percentage of freeze-drying survival[b,c]					
	67 (6)	18 (7)***	72 (5)	38 (7)**	40 (5)**	28 (11)**

Tween 80 (sorbitan monooleate, C18:1) is present at 1 g L^{-1} in the growth media used in this article

SFA saturated fatty acids, *UFA* unsaturated fatty acids

[a] In this experiments, the cells were grown at constant low temperature and pH until stationary phase, 50 h in total

[b] Standard deviations of independent triplicate samples are shown in brackets

[c] Significance, in comparison with the control (optimal), is reported as follows: $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***

as optimal induced a lower stress on the cells than the selected deviations. However, no direct correlations between changes in fatty acid composition and freeze-drying survival rates were observed in our investigation. The highest growth rate during fermentation, a high degree of fatty acid saturation, and good survival after freeze-drying of *Lb. coryniformis* Si3 was observed after growth at 34 °C and pH 5.5. Interestingly, there was no significant effect on freeze-drying survival after subjecting *Lb. coryniformis* Si3 cells to a mild heat stress (42 °C), even though the lipid composition changed in a similar fashion as for the other stresses tested. All other mild stress conditions, either applied throughout the fermentation or applied as a stress in late exponential growth, resulted in a decrease in the degree of fatty acid saturation and a significant negative effect on freeze-drying survival. Decreased degree of saturation leading to a more fluid membrane can result in a higher permeability and perhaps leakage of important metabolites [14]. Our results showed that for *Lb. coryniformis* Si3, exposure to a mild stress before freeze-drying had no significant or negative effect on survival rates. To our knowledge, this is the first report on the effects of preconditioning lactic acid bacteria prior to freeze-drying. Freeze-drying and rehydration induce complex changes on the lipid layers and the cells also adjust to unfavourable conditions by altering different stress proteins. These changes might be of importance for understanding why survival differs among strains and in various growth conditions. These will be objectives of further research on *Lb. coryniformis* Si3.

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