

Survival of Freeze-dried *Leuconostoc mesenteroides* and *Lactobacillus plantarum* Related to Their Cellular Fatty Acids Composition during Storage

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Abstract Lactic acid bacteria strains *Lactobacillus plantarum* CWBI-B534 and *Leuconostoc* ssp. *mesenteroides* (*L. mesenteroides*) Kenya MRog2 were produced in bioreactor, concentrated, with or without cryoprotectants. In general, viable population did not change significantly after freeze-drying ($p > 0.05$). In most cases, viable population for cells added with cryoprotectants was significantly lower than those without ($p < 0.05$). Cellular fatty acids (CFAs) from the two strains in this study were analyzed before and after freeze-drying. Six CFAs were identified, namely, palmitic ($C_{16:0}$), palmitoleic ($C_{16:1}$), stearic ($C_{18:0}$), oleic ($C_{18:1}$), linoleic ($C_{18:2}$), and linolenic ($C_{18:3}$) acids were identified. Four of them, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, and $C_{18:1}$, make up more than 94% or 93% of the fatty acids in *L. mesenteroides* and *L. plantarum*, respectively, with another one, namely, $C_{18:3}$, making a smaller (on average 5–6%, respectively) contribution. The $C_{18:2}$ contributed very small percentages (on average $\leq 1\%$) to the total in each strain. $C_{16:0}$ had the highest proportion at most points relative to other fatty acids. Moisture content and water activity (a_w) increased significantly during the storage period. It was observed that $C_{16:1}/C_{16:0}$, $C_{18:0}/C_{16:0}$ and $C_{18:1}/C_{16:0}$ ratios for freeze-dried

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L. mesenteroides or *L. plantarum*, with or without cryoprotectants, did not change significantly during the storage period. According to the packaging mode and storage temperatures, $C_{18:2}/C_{16:0}$ and $C_{18:3}/C_{16:0}$ ratios for freeze-dried *L. mesenteroides* and *L. plantarum* with or without cryoprotectants decreased as the storage time increased. However, a higher $C_{18:2}/C_{16:0}$ or $C_{18:3}/C_{16:0}$ ratio for *L. mesenteroides* and *L. plantarum* was noted in the freeze-dried powder held at 4 °C or under vacuum and in dark than at 20 °C or in the presence of oxygen and light.

Keywords Viability · Oxidative stress · Heat stress · Freeze-drying · Cellular fatty acids · Lactic acid bacteria

Abbreviations

a_w	water activity
CFA	cellular fatty acid
K	Inactivation rate
k_a	acidification loss rate
<i>L</i>	<i>Leuconostoc</i>
LAB	Lactic acid bacteria
<i>Lb</i>	<i>Lactobacillus</i>
Me	Methyl esterified
MUFA	Monounsaturated fatty acids
P	cell without cryoprotectants
PC	cell with cryoprotectants
PUFAs	Polyunsaturated fatty acids
R^2	Determination coefficient
RH	Relative humidity
S	saturated fatty acids
Tg	Temperature of glass transition
Ts	Surface temperature of product
U	Unsaturated fatty acids

Introduction

The introduction of appropriate starter culture techniques may constitute one major step toward improved safety, quality, and security of traditional small-scale fermentation in Africa [1]. The industrial use of lactic starter cultures for the food industry depends on the concentration and preservation technologies employed, which are required to warrant long-term delivery of stable cultures in term of viability and functional activity [2]. Freeze-drying has commonly been used for this purpose; nevertheless freeze-dried cells are likely to lose their viability during storage [3–5]. Lipid oxidation of membrane fatty acids was deemed responsible for cell death during storage [6]. This is supported by indirect evidence presented in previous reports. For example, chemical injury in the form of free radical damage has been suggested by most researchers as one of the major culprits in desiccation injury [7]. Loss of water increases the ionic concentration (which can lead to the formation of reactive oxygen species [ROS]), and in the dry state, bio-molecules become more susceptible to the attack of oxygen. These species can damage proteins, modify bases and sugars in deoxyribonucleic acid (DNA) and cause lipid peroxidation [7, 8]. It is reported

that the presence of antioxidants increased the survival rate of dried bacteria during storage [6, 9]. There is a strong similarity between the loss of viability and the increase in free-radical concentration during storage of freeze-dried *Serratia marcescens* [10]. This hypothesis is supported by others authors, who found that the reactions between carbonyl compounds and cellular components are a major cause of mortality during storage of dried microorganisms [11]. However, to date no papers have been published identifying which bacterial cellular fatty acids are in fact oxidized or degraded during storage. In living organisms lipids, particularly polyunsaturated fatty acid (PUFA) components of cell membranes, are described as being extremely subject to environmental stress [12, 13]. Stresses such as decreases in a_w [14] or increases in temperature [15] are known to affect the fatty acid composition of bacteria. A number of factors, such as temperature, atmosphere, exposure to light and moisture influence the viability of freeze-dried cultures [16]. It is well established that cryoprotectants are almost indispensable when freezing and drying microorganisms, but the contribution of these compounds to stability of dried microorganisms during storage is discussed [17]. The disadvantages of dried cultures undermine their application, but advantages can outweigh disadvantages if the inactivation during storage can be more clearly understood and consequently reduced. Therefore, the purpose of this work was to study the influence of oxygen, humidity, and temperature on survival or cellular fatty acids of freeze-dried *L. mesenteroides* and *L. plantarum*, with or without cryoprotectants, during storage and, if possible, to show the interrelation between viability and polyunsaturated fatty acids degradation. We used whole-cell fatty acid methyl ester (FAME) analysis to examine the effect of storage conditions on fatty acid composition, as this approach reflects the fatty acid composition of phospholipids and lipopolysaccharides [18–20].

Materials and Methods

Microorganisms and Cultivation

The lactic acid bacteria *L. mesenteroides* ssp. *mesenteroides* Kenya MRog2, isolated and identified from cassava fermentation for gari production [21], was provided by the Federal Research Centre for Nutrition, Institute of Hygiene and Toxicology (Karlsruhe, Germany). The *L. plantarum* CWBI-B534 was isolated from poultry farms in the vicinity of Dakar (Senegal, West Africa). MRS broths inoculated with each strain were incubated at 30 °C for 18 h. The supernatants obtained after centrifugation (Sorvall RC2-B, Sorvall, USA) at 2,500×g for 20 min, were decanted. The cell pellets obtained were resuspended in 5 ml (50% v/v) glycerol and frozen at –80 °C.

Productions

The strains were grown in 500 L bioreactor containing MRS medium for 18 h and then concentrated 20 times by centrifugation. After centrifugation, cryoprotectants (2% (w/w) glycerol and 5% (w/w) maltodextrine) were added to the pellets (PC). Cell suspensions without additives were used as control (P). Cells were freeze-dried in a low freeze-drier (Leybold, Belgium) with a standard program by increasing the temperature gradually from –45 °C to 25 °C at 0.9 mbar pressure (30 h) followed by 15 h at 0.15 mbar. All fermentations were done in duplicate and average values reported.

Storage Conditions

Freeze-dried samples were stored during 90 days, in portion size (2 ± 0.5 g in each portion) at 20 °C (accelerated stability test, 30% RH) and at 4 °C (stability at refrigerated temperature, 70% RH). At each storage temperature, samples were stored, in open 20-ml white glass tubes and in aluminum foil packets which were vacuum-sealed. Samples were withdrawn for analysis each 30 days and kept at -20 °C (48–72 h) before analysis.

Dry Cell Weight and Water Content Determinations

The dry cell weight of 1 g of powder was determined at the end of freeze drying. The water content ($100 \text{ g dry weight}^{-1}$) of the freeze-dried samples during storage at 20 and 4 °C with aeration was determined each 15 days. The samples were dried in a convection oven until constant weight and results are mean of four determinations.

Water Activity Measurements

The water activity (a_w) of the freeze-dried samples was measured at 25 °C using a water activity meter Novasina (Novasina, Pfäffikon, Switzerland). Standard salt solutions (Novasina) of known water activity were used for calibration of the sensor at the measuring temperature. Readings were taken until four sequential readings gave the same results. Results obtained were the average of four determinations for each sample.

Survival Rate

Percentage survival of the strains after freeze-drying process was expressed as follows:

$$\text{Survival(\%)} = \frac{C_2 \times P_2}{C_1 \times P_1} \times 100,$$

where C_1 and C_2 are the CFU/g of the suspension before and after freeze-drying and P_1 and P_2 are amount of matter obtained before and after freeze-drying. Percentage survival after a 90-day storage was calculated as $100 \times N/N_0$, where N is the CFU/g of the freeze-dried sample at a given time and N_0 is the CFU/g of the sample at the end of freeze-drying.

Analysis of Cellular Fatty Acids

Cells wall fractions were obtained as described previously [22]. The lipids were extracted overnight from cell wall fractions (2.5 ml) and dried cells (1 g) with ethanol-ether (3:1 v/v) mixture according to an adaptation of the method of Ito (1969). Ethanol ether extracts were pooled, filtered, and then evaporated and concentrated under reduced pressure at 35 °C. Fatty acid methyl esters (FAME) were prepared from the concentrate with 14% (w/w) solution of boron trifluoride in methanol as reagent (Sigma, St Louis, MO, USA). After heating at 70 °C in a water bath for 90 min, 0.5 ml of saturated NaCl, 0.2 ml of sulfuric acid (10%) and 0.5 ml of *n*-hexane were added. The methylated fatty acids were extracted from the upper phase after decanting by means of a Pasteur pipette. Gas chromatographic analysis of the methyl esters was carried out on a HP 6890 (Hewlett Packard) gas chromatograph equipped with a flame ionization detector at 250 °C. A capillary column

(30×0.25 mm (i.d), film thickness 0.25 μm) was used. Helium was used as carrier gas (2.4 ml/min) and the injection volume was 1 μl . Injection was done at 250 °C in splitless mode for 1 min. The oven temperature was held at 50 °C for 1 min, increased by 30 °C/min to 150 °C, and then from 150 °C to 240 °C at 4 °C/min with a final hold of 10 min at 240 °C. Fatty acids methyl esters were identified by comparing their retention times with standard mixtures FAME MIX 47885U (Supelco, Bellefonte, USA). The relative fatty acid content was estimated as a percentage of the total peak area using a DP 700 integrator (Spectra physics). The relative content (%) of each fatty acid was normalized by expressing it as a ratio of the relative content (%) of palmitic (C16:0) acid for two reasons: [1] C16:0 had the highest proportion at most points relative to other fatty acids, [2] C16:0 did not change significantly during storage ($p>0.05$)

Statistical Analysis

Productions in bioreactor were done in duplicate. The mean values and the standard deviation were obtained with four determinations. These data were then compared by Turkey's honestly significant difference (Statistica 7.1, StatSoft Inc. 2005). The level of significance was determined at $p<0.05$. (CFU counts were transformed to their base 10 logarithms.)

Results and Discussion

Survival of Lactic Acid Bacteria after Freeze-drying

Lactic acid bacteria *L. mesenteroides* and *L. plantarum* were produced in bioreactor; the cells were harvested, added or not with cryoprotectants (5% maltodextrine, 2% glycerol) and freeze-dried. Table 1 shows viable counts during the process and survival after freeze-drying. A viable population of $11.93\pm0.02 \log_{10}$ CFU/g or $11.65\pm0.04 \log_{10}$ CFU/g and $11.88\pm0.03 \log_{10}$ CFU/g or $11.65\pm0.04 \log_{10}$ CFU/g for *L. mesenteroides* and *L. plantarum*, added or not with cryoprotectants, were obtained after the freeze-drying process. Different survival rates were obtained immediately after freeze-drying. *L. plantarum* successfully survived or recovered more than 90% of population values, whereas *L. mesenteroides* behaved with low biomass yield (<11%). The effect of the additives is not significant. Moisture content and a_w were determined every 15 days during

Table 1 Viability of *L. mesenteroides* and *L. plantarum* before and after freeze-drying.

Population (log CFU/g)	Treatment ^a	<i>L. mesenteroides</i>	<i>L. plantarum</i>
Before freeze-drying	P	$11.8\pm0.03 \text{ a}^1$	$11.8\pm0.05 \text{ ab}$
	PC	$11.7\pm0.08 \text{ b}$	$11.7\pm0.05 \text{ a}$
After freeze-drying	P	$11.9\pm0.02 \text{ a}$	$11.9\pm0.03 \text{ b}$
	PC	$11.6\pm0.04 \text{ b}$	$11.6\pm0.04 \text{ a}$
Survival (%) ^b	P	13 ± 2	94 ± 4
	PC	18 ± 1	97 ± 5

Values are means \pm standard deviation (SD) ($n=4$)

^a P, cells without cryoprotectants; PC, cells with cryoprotectants (5% maltodextrine, 2% glycerol)

^b Values represent percentage survival of the strain after freeze-drying process

¹ Cell count values in the same column with different letters are significantly different ($p<0.05$)

storage at 4 °C (70% RH) and 20 °C (30% RH) in open white glass tube containing 2 ± 0.2 g of dried powder. The relationship between a_w and moisture content ($100 \text{ g dry weight}^{-1}$) for freeze-dried strains during storage at 4 or 20 °C was established for storage in open white glass tube. The water contents were stable in vacuum-sealed aluminum foil at the same temperature. Moisture content and a_w increased significantly during the storage period in these conditions. At the end of freeze-drying, moisture content for freeze-dried *L. mesenteroides* or *L. plantarum* P and PC was $3.6 \pm 0.7\%$ and $4.8 \pm 0.5\%$ or $3.1 \pm 0.3\%$ and $4.7 \pm 0.7\%$ with a_w 0.1 ± 0.01 for each batch type. After a 90-day storage, water content for freeze-dried *L. mesenteroides* or *L. plantarum* P and PC increased to $20.2 \pm 0.4\%$ and $21.6 \pm 0.3\%$ or $19.4 \pm 0.8\%$ and $21.8 \pm 0.2\%$ at 4 °C, to $11.4 \pm 0.7\%$ and $16.9 \pm 1.1\%$ or $12.1 \pm 1.1\%$ and $15.4 \pm 0.4\%$ at 20 °C, respectively (Tables 4, 5, and 6). During the same time, a_w increases to 0.6 ± 0.01 and 0.6 ± 0.01 or 0.6 ± 0.01 and 0.6 ± 0.01 at 4 °C, to 0.4 ± 0.01 and 0.4 ± 0.01 or 0.4 ± 0.01 and 0.4 ± 0.01 at 20 °C, respectively.

The rate of dry matter in a lyophilized powder, which is nearly 94%, and a water activity less than 0.2 would be necessary to ensure a level of storage stability at room temperature. These conditions are consistent with research undertaken by French's Patent FR No. 2829147 [23], which stipulates that in a dried product the following parameters: very low water activity (<0.2), rate of dry matter around 96% and a $T_g = T_s - 25 \text{ °C} > 20 \text{ °C}$, contribute to the stability of the product for an extended period during the storage

Survival of Lactic *L. mesenteroides* and *L. plantarum* during Storage

These authors [23, 24] showed that stability of dried lactic acid bacteria upon storage was better in the 0.1–0.2 a_w zone. In this study, the water activity of strains after freeze drying have some a_w less than 0.170 at 25 °C (data are not shown). This result shows that the lactic acid bacteria are well lyophilized. Figures 1 and 2 show the survival of freeze-dried *L. mesenteroides* and *L. plantarum* during the storage period, respectively. In vacuum-

Fig. 1 Survival of freeze-dried *L. mesenteroides* during storage at 4 °C or 20 °C. Symbols: open circle, freeze-dried strain without cryoprotectants (P); filled circle, freeze-dried strain with cryoprotectants (5% maltodextrine + 2% glycerol) (PC). Dotted lines for storage in vacuum-sealed aluminum foil, continuous lines for storage in opened white glass tube. Values represent means \pm SD ($n=4$)

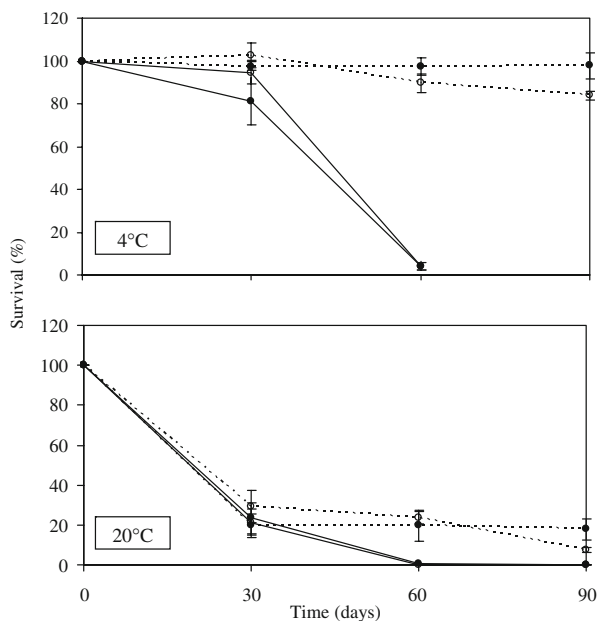
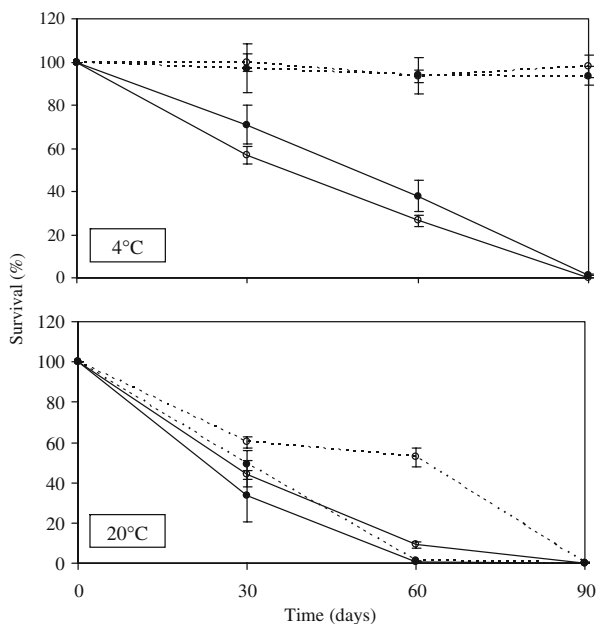


Fig. 2 Survival of freeze-dried *L. plantarum* during storage at 4 °C or 20 °C. Symbols: open circle, freeze-dried strain without cryoprotectants (P); filled circle, freeze-dried strain with cryoprotectants (5% malto-dextrine + 2% glycerol) (PC). Dotted lines for storage in vacuum-sealed aluminum foil, continuous lines for storage in opened white glass tube. Values represent means \pm SD ($n=4$)



sealed aluminum foil and open white glass tube at 4 °C and 20 °C. Table 4 shows a summary of the survival of test organisms and water content of the freeze-dried powder under different packaging and storage temperature after a 90-day storage. It was found that regardless of packaging mode and storage temperatures, the viable cells of *L. mesenteroides* (Fig. 1) and *L. plantarum* (Fig. 2), added or not with cryoprotectants, decreased as the storage time increased. However, a higher viable population of *L. mesenteroides* and *L. plantarum* was noted in the freeze-dried powder held at 4 °C or under vacuum and in the dark than at 20 °C or in presence of air and light. For example, the viable population of *L. plantarum* with cryoprotectants held in vacuum-sealed aluminum foil or open white glass tube was reduced from an initial population of 11.65 to 11.72 log CFU/g or 9.88 log CFU/g with no population reduction or a reduction of only 1.77 log CFU/g and a survival percentage of 93.5% or 1.4% after a 90-day storage at 4 °C (Fig. 2, Table 4) compared to a larger population reduction of 2.64 log CFU/g or 4.68 log CFU/g and a less survival of 0.19% or less than 0.1% at 20°C, respectively. As shown in Figs. 1 and 2 and Table 4, it was noted that for either *L. mesenteroides* or *L. plantarum* there was no significant effect of cryoprotectants on survival after a 90-day storage.

The powder produced from *L. plantarum* and *L. mesenteroides* meet all these conditions and yet conservation is not optimal during storage except that the survival rate of *L. plantarum* is slightly higher than *L. mesenteroides*. This difference between the two freeze-dried bacteria during storage finds its origin in the structure of the cells. Apart from this, freeze-dried powders meet the above-mentioned criteria and yet the survival rate is not high during storage. According to the work of [26], a number of factors can influence the percentage of survival in function of time. With regard to the other predominant factors to this loss of viability, one could cite (the water activity), probably taken into contained pure water powders, the effect of temperature and light for hermetically sealed bags (under vacuum) containing 2–3 g of powder. Loss of viability was also noted that at this level find its origin by the combined effect of temperature and oxidation of cellular constituents as

Table 2 Cellular fatty acid composition of *L. mesenteroides* before and after freeze-drying.

Treatment ^a		Relative content (%) ^b					
		C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Before freeze-drying	P	49.7±0.7 ab ¹	7.4±0.1 a	8.5±0.1 a	28.9±0.8 a	1.0±0.1 a	4.5±0.1 a
	PC	50.3±0.1 ab	6.3±0.7 a	7.9±0.1 a	30.0±0.9 a	1.0±0.2 a	4.5±0.3 a
After freeze-drying	P	48.6±1.3 a	7.9±0.7 a	8.7±1.6 a	29.3±1.6 a	1.1±0.1 a	4.5±0.2 a
	PC	53.1±1.1 b	6.8±0.1 a	7.7±0.2 a	26.9±1.3 a	0.8±0.1 a	4.7±0.3 a

The six main fatty acids are palmitic (C16:0), palmitoleic (C16:1), stearic (C 18:0), oleic (C 18:1), linoleic (C 18:2), linolenic (C 18:3)

^a P, cells without cryoprotectants; PC, cells with cryoprotectants (5% maltodextrine, 2% glycerol)

^b Values are means ± SD (*n*=4) and represent proportions of total fatty acids (%)

¹ Values in the same column with different letters are significantly different (*p*<0.05).

unsaturated fatty acids [34]. This oxidation finds its source by oxygen effect, lyophilized powders that create free radicals. These free radicals are toxic substances that lead to rupture the membrane and thus cellular cell death in the future. These primarily mitigate the adverse events and are especially responsible for significant losses during storage that biologists increasingly need antioxidant to stop or reduce the oxidation products at the level of freeze-dried powders. It is safe to say that based on our experiences and those of others, the loss of viability during storage is subject to a phenomenon of autooxidation, which should optimize the survival rate especially during storage at room temperature. The important and intrinsic parameters (dry cell matter, water activity) merit attention while optimizing for the increase and prolonged time storage of lyophilized powders of lactic acid bacteria. For this reason and many others, some authors argue that the cell membrane is the seat of the cell survival in the sense that it contains phospholipids that are probably modified by the oxidation [26].

Cellular Fatty Acid Relative Contents after Freeze-Drying and Changes in Fatty Acid Composition Due to Storage

Cellular fatty acids (CFAs) from the two strains were analyzed before and after freeze-drying. The mean relative contents of CFAs from *L. mesenteroides* and *L. plantarum* are

Table 3 Cellular fatty acid composition of *L. plantarum* before and after freeze-drying.

Treatment ^a		Relative content (%) ^b					
		C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Before freeze-drying	P	55.0±0.7 a ¹	7.4±0.1 a	7.9±0.6 a	22.7±0.8 a	1.1±0.2 a	6.0±0.9 a
	PC	54.3±0.9 a	7.9±1.0 a	8.4±0.4 a	22.7±0.5 a	1.0±0.1 a	5.8±0.7 a
After freeze-drying	P	55.2±1.3 a	6.2±0.1 a	8.1±0.9 a	23.9±0.6 a	0.8±0.1 a	5.8±0.3 a
	PC	53.6±0.7 a	7.0±1.2 a	9.7±0.3 a	23.2±0.1 a	0.8±0.1 a	5.7±0.2 a

The six main fatty acids are palmitic (C16:0), palmitoleic (C16:1), stearic (C 18:0), oleic (C 18:1), linoleic (C 18:2), linolenic (C 18:3)

^a P, cells without cryoprotectants; PC, cells with cryoprotectants (5% maltodextrine, 2% glycerol)

^b Values are means ± SD (*n*=4) and represent proportions of total fatty acids (%)

¹ Values in the same column with the same letter are not significantly different (*p*>0.05).

presented in Tables 2 and 3, respectively. Six CFAs, namely palmitic ($C_{16:0}$), palmitoleic ($C_{16:1}$), stearic ($C_{18:0}$), oleic ($C_{18:1}$), linoleic ($C_{18:2}$), and linolenic ($C_{18:3}$) acids were identified. Four of the CFAs, namely $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, and $C_{18:1}$, make up more than 94% or 93% of the fatty acids in *L. mesenteroides* and *L. plantarum* respectively, with another one, namely, $C_{18:3}$ making a smaller (on average 5–6%, respectively) contribution. The

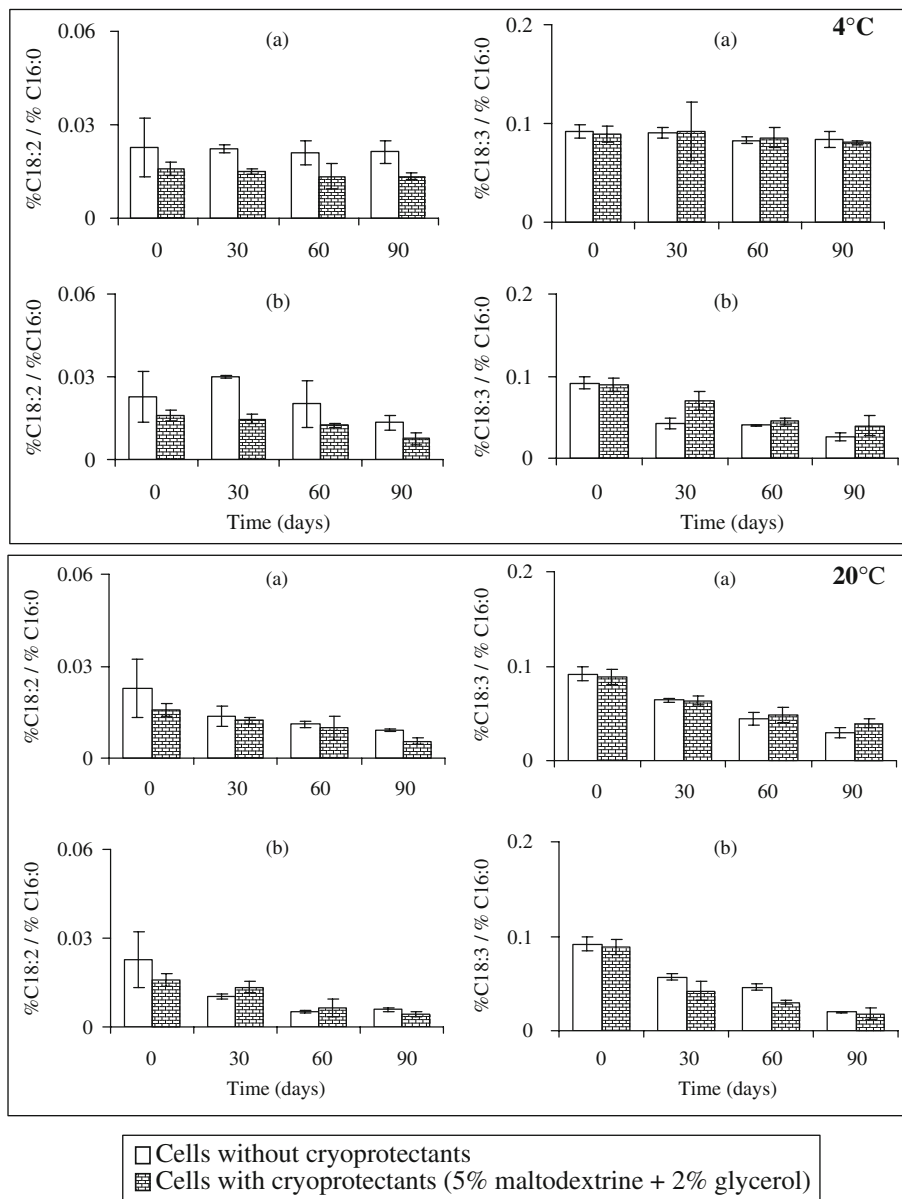


Fig. 3 C18:2/C16:0 and C18:3/C16:0 ratios of freeze-dried *L. mesenteroides* during 90-day storage at: 4 °C (A) or 20 °C (B) in vacuum-sealed aluminum foil (a) or in open white glass tube (b). Bars are means \pm SD ($n=4$) and represent the ratio between each fatty acid and the palmitic ($C_{16:0}$) acid

C_{18:2} represent very small percentages (on mean $\leq 1\%$) to the total in each strain. C_{16:0} had the highest proportion. There was no significant modification in CFAs of *L. mesenteroides* and *L. plantarum* in response to dehydration ($p>0.05$). Moreover, the addition of glycerol and maltodextrine before freeze drying did not modify significantly the CFAs of the two strains.

Cellular fatty acids freeze-dried of *L. mesenteroides* and *L. plantarum* packaged in vacuum-sealed aluminum foil or opened white glass tube and held at 20 or 4 °C during

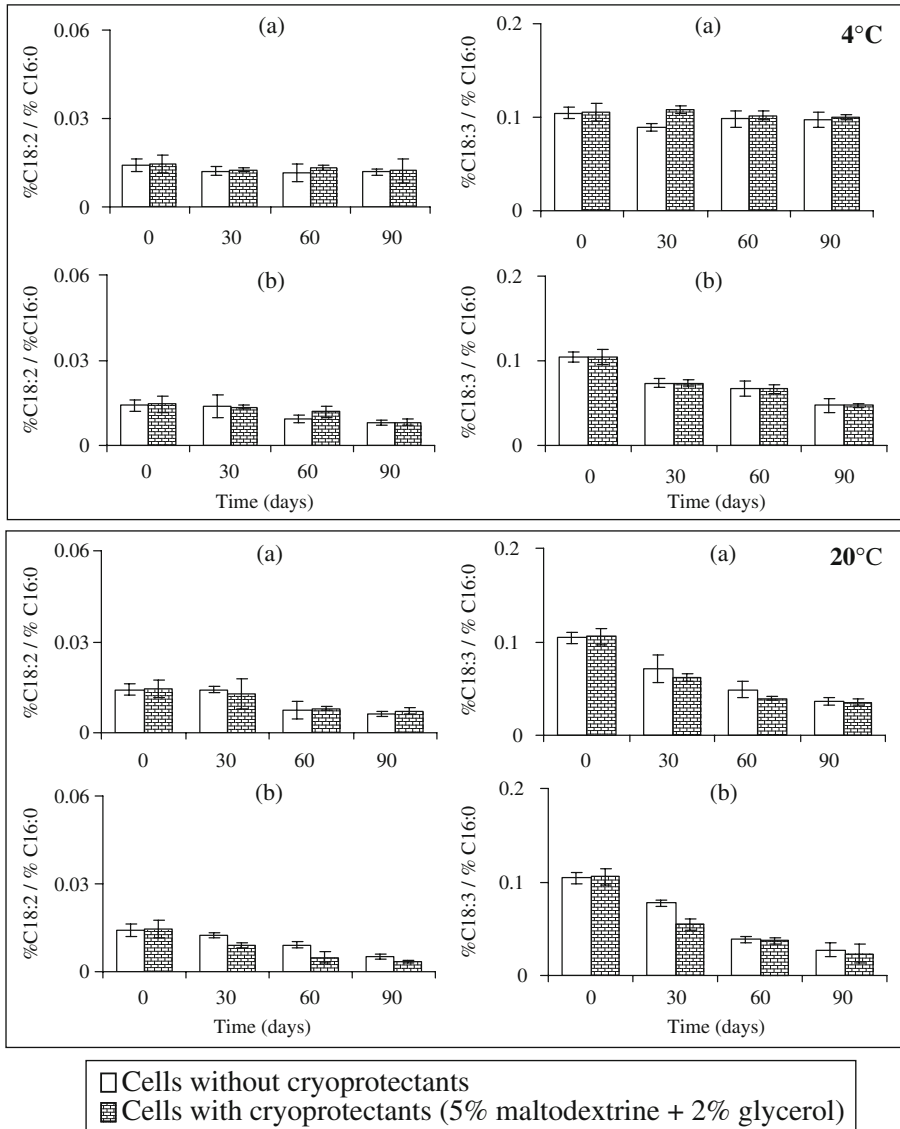


Fig. 4 C_{18:2}/C_{16:0} and C_{18:3}/C_{16:0} ratios of freeze-dried *L. plantarum* during 90-day storage at: 4 °C (A) or 20 °C (B) in vacuum-sealed aluminum foil (a) or in open white glass tube (b). Bars are means \pm SD ($n=4$) and represent the ratio between each fatty acid and the palmitic (C_{16:0}) acid

Table 4 Moisture content and survival of freeze-dried *L. mesenteroides* and *L. plantarum* after 90-day storage.

Storage Temperature (°C)	Container	Treatment ^a	<i>L. mesenteroides</i> ^b			<i>L. plantarum</i> ^b		
			Moisture content (%)	Population (log CFU/g)	Survival ^c (%)	Moisture content (%)	Population (log CFU/g)	Survival (%)
4	Vacuum-sealed aluminum foil	P	3.6±0.7 a ¹	11.9±0.03	83.9±1.9 a	3.1±0.3 a	11.9±0.02	98.0±5.2 a
		PC	4.8±0.5 a	12.0±0.04	97.8±5.9 b	4.7±0.7 a	11.7±0.07	93.5±4.3 a
	Opened white glass tube	P	20.2±0.4 b	nd	nd	19.4±0.8 b	8.9±0.03	0.1±0.01 b
		PC	21.6±0.3 b	nd	nd	21.8±0.2 c	9.9±0.19	1.4±0.6 b
20	Vacuum-sealed aluminum foil	P	3.6±0.7 a	10.8±0.08	7.2±1.3 c	3.1±0.3 a	9.4±0.02	0.3±0.02 b
		PC	4.8±0.5 a	11.2±0.14	17.9±5.2 d	4.7±0.7 a	9.0±0.05	0.2±0.04 b
	Opened white glass tube	P	13.4±0.7 c	6.7±0.03	<0.1 c	12.1±1.1 d	7.0±0.01	<0.1 b
		PC	16.9±1.1 d	6.6±0.10	<0.1 c	15.4±0.4 e	7.0±0.02	<0.1 b

Values are means ± SD (*n*=4)

nd: not determined

^a P, cells without cryoprotectants; PC, cells with cryoprotectants (5% maltodextrine, 2% glycerol)^b Initially, freeze-dried *L. mesenteroides* or *L. plantarum* P and PC with a moisture content of 3.6% and 4.8% or 3.1% and 4.7% contained 11.9 log CFU/ml and 11.6 log CFU/ml or 11.9 log CFU/ml and 11.6 log CFU/ml, respectively.^c Values represent percentage in cell reduction compared with those obtained immediately after freeze-drying¹ Values in the same column with different letters are significantly different (*p*<0.05)

90 days were analyzed. The palmitic acid ($C_{16:0}$) relative content remained high and changed only little during storage. Therefore, the content of the others fatty acids was expressed as a ratio between each fatty acid and the $C_{16:0}$. It was observed that $C_{16:1}/C_{16:0}$, $C_{18:0}/C_{16:0}$ and $C_{18:1}/C_{16:0}$ ratios for freeze-dried *L. mesenteroides* or *L. plantarum*, added or not with cryoprotectants did not change significantly during the storage period. Figures 3 and 4 show linoleic/palmitic ($C_{18:2}/C_{16:0}$) or linolenic/ palmitic ($C_{18:3}/C_{16:0}$) ratios for freeze-dried *L. mesenteroides* and *L. plantarum* during the storage period, respectively. Tables 5 and 6 show a summary of linoleic/palmitic ($C_{18:2}/C_{16:0}$) and linolenic/palmitic ($C_{18:3}/C_{16:0}$) ratios for freeze-dried organisms under different packaging and storage temperature after a 90-day storage, respectively. It was found that regardless of packaging mode and storage temperatures, $C_{18:2}/C_{16:0}$ and $C_{18:3}/C_{16:0}$ ratios for freeze-dried *L. mesenteroides* (Fig. 3) and *L. plantarum* (Fig. 4) added or not with cryoprotectants decreased in function of time. However, a higher $C_{18:2}/C_{16:0}$ or $C_{18:3}/C_{16:0}$ ratio for *L. mesenteroides* and *L. plantarum* was noted in the freeze-dried powder held at 4 °C or under vacuum and in the dark than at 20 °C or in the presence of air and light (Table 4). For example, $C_{18:2}/C_{16:0}$ and $C_{18:3}/C_{16:0}$ ratios were 0.01 and 0.11 for freeze-dried *L. plantarum* with cryoprotectants at the end of freeze-drying, respectively (Tables 5 and 6). After a 90-day storage in vacuum-sealed aluminum foil or open white glass tube, $C_{18:2}/C_{16:0}$ ratio decreased from the original value by 0% or 19.0% with a ratio reduction of only 0 or 0.002

Table 5 Moisture content and C18:2/C16:0 ratio for freeze-dried *L. mesenteroides* and *L. plantarum* after 90-day storage.

Storage		Treatment ^a	<i>L. mesenteroides</i> ^c			<i>L. plantarum</i> ^c		
Temperature (°C)	Container		Moisture content (%)	Ratio ^b (%)	(%) ^d	Moisture content (%)	Ratio ^b (%)	(%)
4	Vacuum-sealed aluminum foil	P	3.6±0.7 a ¹	2.13	86.4±1.3	3.1±0.3 a	1.19	83.9±5.7
		PC	4.8±0.5 a	1.35	85.3±0.8	4.7±0.7 a	1.22	122.0±8.3
	Opened white glass tube	P	20.2±0.4 b	1.35	57.8±1.8	19.4±0.8 b	0.81	56.9±1.4
		PC	21.6±0.3 b	0.75	47.5±0.9	21.8±0.2 c	0.81	81.0±7.5
20	Vacuum-sealed aluminum foil	P	3.6±0.7 a	0.90	37.8±2.2	3.1±0.3 a	0.63	44.7±2.4
		PC	4.8±0.5 a	0.54	34.4±0.4	4.7±0.7 a	0.72	71.9±3.3
	Opened white glass tube	P	13.4±0.7 c	0.59	23.9±5.9	12.1±1.1 d	0.53	37.1±0.7
		PC	16.9±1.1 d	0.43	27.0±4.0	15.4±0.4 e	0.35	35.3±3.4

Values are means ± SD ($n=4$).

^a P, cell without cryoprotectants; PC, cell with cryoprotectants (5% maltodextrine, 2% glycerol)

^b Values represent ratio between each fatty acid and the palmitic acid

^c Initially, freeze-dried *L. mesenteroides* or *L. plantarum* P and PC with a moisture content of 3.6% and 4.8% or 3.1% and 4.7% contained 0.03 of C16:0 and 0.02 of C16:0 or 0.01 of C16:0 and 0.01 of C16:0, respectively

^d Values represent percentage compared to the initial ratio

¹ Values in the same column with different letters are significantly different ($p<0.05$)

Table 6 Moisture content and C18:3/C16:0 ratio for freeze-dried *L. mesenteroides* and *L. plantarum* after 90-day storage.

Storage		Treatment ^a	<i>L. mesenteroides</i> ^c			<i>L. plantarum</i> ^c		
Temperature (°C)	Container		Moisture content (%)	Ratio ^b (%)	(%) ^d	Moisture content (%)	Ratio ^b (%)	(%)
4	Vacuum-sealed	P	3.6±0.7 a ¹	8.4	88.1±2.4	3.1±0.3 a	9.8	92.9±1.2
	aluminum foil	PC	4.8±0.5 a	8.1	93.1±2.6	4.7±0.7 a	10.0	95.2±6.9
	Opened	P	20.2±0.4 b	2.6	27.3±2.9	19.4±0.8 b	4.7	44.7±3.2
	white glass tube	PC	21.6±0.3 b	3.9	44.4±8.5	21.8±0.2 c	5.0	47.3±0.2
20	Vacuum-sealed	P	3.6±0.7 a	2.9	31.3±3.5	3.1±0.3 a	3.6	34.1±1.7
	aluminum foil	PC	4.8±0.5 a	3.9	44.2±6.8	4.7±0.7 a	3.5	33.9±5.1
	Opened white	P	13.4±0.7 c	2.0	21.7±2.7	12.1±1.1 d	2.7	25.9±0.7
	glass tube	PC	16.9±1.1 d	1.8	20.3±4.1	15.4±0.4 e	2.3	22.0±2.7

Values are means ± SD (*n*=4).

^a P, cell without cryoprotectants; PC, cell with cryoprotectants (5% maltodextrine, 2% glycerol)

^b Values represent ratio between each fatty acid and the palmitic acid

^c Initially, freeze-dried *L. mesenteroides* or *L. plantarum* P and PC with a moisture content of 3.6% and 4.8% or 3.1% and 4.7% contained 0.10 of C16:0 and 0.09 of C16:0 or 0.11 of C16:0 and 0.11 of C16:0 for P and PC, respectively

^d Values represent percentage compared to the initial ratio

¹ Values in the same column with different letters are significantly different (*p*<0.05)

at 4 °C (Fig. 3, Table 5) compared to a larger decreased from the original value by 28.1% or 64.71% with a ratio reduction of 0.003 or 0.007 at 20 °C, respectively. During the same time C_{18:3}/C_{16:0} ratio decreased from the original value by 4.8% or 52.7% with a ratio reduction of only 0.010 or 0.060 at 4 °C (Fig. 3, Table 6) compared to a larger decreased from the original value by 66.1% or 78.0% with a ratio reduction of only 0.075 or 0.087 at 20 °C, respectively. In most cases, after 90-day storage, the decreased in C_{18:2}/C_{16:0} ratio was less than that in C_{18:3}/C_{16:0} ratio (Tables 5 and 6). C_{18:2}/C_{16:0} and C_{18:3}/C_{16:0} ratios for freeze-dried strains added with or without cryoprotectants are not significantly different after 90-day storage (*p*>0.05).

Lipid oxidation and specially lipids of membrane fatty acid was probably deemed responsible for cell death during storage [9, 6]. Cellular fatty acids of *L. mesenteroides* and *L. plantarum* consisted of: palmitic, palmitoleic, stearic, oleic, linoleic, and linolenic. All these acids have been identified previously in cellular membrane of acetic acid bacteria and lactic acid bacteria [27, 28] ratios. Contrarily to their saturated and unsaturated analogs, polyunsaturated fatty acids (PUFAs) are much more susceptible to oxidation [25, 28]. After 90 days, C_{18:2}/C_{16:0} and C_{18:3}/C_{16:0} ratios were significantly lower for both freeze-dried strain held in open glass tube at 20 °C. The effect of temperature during storage on freeze-dried powder is harmful to microorganisms' survival. What concern light, its action is detrimental and leads to decrease the survival rate. Upon exposure to combined light, temperature, and oxygen, a cooperative, deleterious effect was noted resulting in an even higher loss in viability. A possible interpretation is that under these conditions linoleic and linolenic acids can be further rapidly converted to hydroperoxides [29–30]. This is supported by the fact that, during storage in opened white glass tube at 4 °C and in vacuum-sealed aluminum foil at 20 °C C_{18:2}/C_{16:0} and C_{18:3}/C_{16:0} ratios decrease more slowly. The occurrence of many of these compounds has been explained on the basis of the formation

and/or combination of free radicals resulting from homolytic cleavage of C–C linkages near the double bond. Thus, linoleic and linolenic acids degradation may not be dependant on the presence or absence of free molecular oxygen but on the presence of reactive free radicals in the dried matrix. Linolenic acid was degraded faster than linoleic acid and even in cell membrane, as previous studies have shown, an unsaturated fatty acid with more double bonds (and/or conjugated double bonds) would be more easily oxidized [31]. Our results strongly point toward the possibility that the loss of water during freeze-drying process generates an oxidative stress. Pereira et al., (2003) reported also that freeze-dried cells showed increase in oxidation during storage. However, the origin of free radicals during dehydration remains unknown [32]. The influence of temperature during storage of unsaturated fatty acids in the absence of oxygen, dimeric compounds and substances of lower molecular weight are more likely to be produced [33].

Conclusion

The results presented here provide experimental support to the hypothesis that storage in the presence of air, high moisture content, and high temperature is detrimental to freeze-dried powders. Low survival during storage was associated with a decrease in C18:2/C16:0 and C18:3/C16:0 ratios. Our results confirm the importance controlling temperature, atmosphere, and residual water activity of dried cultures to optimize survival. These data further support the view that PUFAs (linoleic and linolenic acids) play a key role in determining cellular susceptibility to oxidative and/or heat stress. The beneficial effect of the use of cryoprotectants is associated clearly with high viability after freeze-drying rather than increased stability during storage. Further attempts to stabilize freeze-dried cells during storage must be made, and the addition of compounds that interact with the cytoplasmic membrane could be explored. Detection of linoleic or linolenic acids degradation by-products will allow having a correct picture of lipid degradation proceeds.

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