

Effect of Protective Compounds on the Survival, Electrolyte Leakage, and Lipid Degradation of Freeze-Dried *Weissella paramesenteroides* LC11 During Storage

Yao, Amenan A.^{1*}, Bernard Wathelet², and Philippe Thonart^{1,3}

¹Wallon Center for Industrial Biology, Microbial Technology Unit, University of Liège, Sart-Tilman B40, B-4000 Liège, Belgium

²Industrial Biological Chemistry Unit, Gembloux Agricultural University, Passage des déportés 2, B-5030 Gembloux, Belgium

³Wallon Center for Industrial Biology, Bio-Industry Unit, Gembloux Agricultural University, Passage des déportés 2, B-5030 Gembloux, Belgium

Received: September 28, 2008 / Revised: December 2, 2008 / Accepted: December 11, 2008

The effect of cryoprotectants (maltodextrin+glycerol) and cryoprotectants+antioxidant [ascorbic acid and/or butylated hydroxytoluene (BHT)] mixtures on the survival, electrolyte leakage, and lipid degradation of freeze-dried *Weissella paramesenteroides* LC11 during storage was investigated and compared with that of the control (cells without additives) over a 90-day storage period at 4 or 20°C in glass tubes with water activity (a_w) of 0.23. The survival, electrolyte leakage, and lipid degradation were evaluated through colony counts, electrical conductivity, and thiobarbituric acid reactive substances (TBARS) content, respectively. The fatty acids composition was determined by gas chromatography, in both the total lipid extract and the polar lipid fraction, and compared with that of the control after the 90-day storage period. As the storage proceeded, increases in leakage value and TBARS content, as well as a decrease in viability, were observed. After 90 days of storage, the major fatty acids found in both the total lipid extract and the polar lipid fraction were palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids. The survival, leakage value, TBARS content and 18:2/16:0 or 18:3/16:0 ratio were the greatest for the protected strain held at 4°C. Cells with the cryoprotectants+BHT mixture showed the highest percentage of survival and 18:2/16:0 or 18:3/16:0 ratio in both lipid extracts, as well as the lowest leakage value and TBARS content after the 90-day storage period. Drying cells with the cryoprotectants+BHT mixture considerably slowed down polar lipid degradation and loss of membrane integrity, resulting in improved viability during storage.

Keywords: Antioxidant, cryoprotectants, electrolyte leakage, lactic acid bacteria, lipid oxidation, starter culture

Lactic acid bacteria (LAB) for the food industry are commonly freeze-dried for long-term storage. It is now well established that technical parameters such as freezing conditions, rehydration conditions, and the presence of protective compounds affect cell survival [27]. Many other factors, such as temperature, atmosphere, exposure to light, and relative humidity, affect the stability of freeze-dried bacteria during storage. These factors alone or in combination might lead to an unacceptably high loss of viable cells, owing to deteriorative chemical reactions. Membrane lipid oxidation was suggested as the cause of cell death during the storage of *Lactobacillus bulgaricus* in the presence of air, high relative humidity, and high storage temperature [2, 3]. Oxygen radicals could damage the polyunsaturated fatty acids (PUFAs) of cell membrane phospholipids [6]. Recently, a relationship between loss of viability and change in linoleic/palmitic (18:2/16:0) or linolenic/palmitic (18:3/16:0) ratio of freeze-dried LAB during storage was reported [5, 25]. It was previously reported that a free-radical chain reaction, which took place during PUFAs oxidation, might lead to the formation of lipid hydroperoxides that might be decomposed in many secondary products, such as malonic dialdehyde (MDA) [16]. MDA has been identified as the product of PUFAs oxidation that reacts with thiobarbituric acid (TBA) to produce red species collectively termed “thiobarbituric acid reactive substances” (TBARS) [17]. Apart from chemical damage, cell membranes were also prone to physical damage: changes in phospholipids composition had been related to an increase in membrane

*Corresponding author

Phone: +32-4-366-28-61; Fax: +32-4-366-28-62;

E-mail: AmenanAnastasia.Yao@student.ulg.ac.be

permeability, affecting membrane integrity [22]. Thus, UV-absorbing materials, membrane lipids, and ions might have leaked from the cells during rehydration [10]. Previously, Martos *et al.* [15] used conductimetry to evaluate the performance of freeze-dried *Lactobacillus delbrueckii* ssp. *bulgaricus* during rehydration in milk.

Membrane lipid degradation could be minimized by preventive measures, such as the use of antioxidants. Butylated hydroxytoluene (BHT) and ascorbic acid are currently used to control lipid oxidation in the food industry. Although BHT functions by interfering with the free-radical mechanism, ascorbic acid and its derivatives function as antioxidants by protecting double bonds and scavenging oxygen [19]. Teixeira *et al.* [21] found that ascorbic acid and monosodium glutamate provided significant protection to *Lactobacillus delbrueckii* ssp. *bulgaricus* when stored at 4°C. Another form of additives widely used to protect LAB during drying and storage are cryoprotectants. The beneficial effect of the addition of glycerol and maltodextrin either alone [4, 18] or in combination [5] has been clearly associated with high viability after freeze-drying rather than increased stability during storage. In such a case, the incorporation of an appropriate cryoprotectants-antioxidant mixture could protect the dried bacteria against the degradation that could occur during storage. To the best of our knowledge, no work has been reported that establishes the effect of cryoprotectants (glycerol, maltodextrin) in combination with antioxidants (ascorbic acid, BHT) on the viability, electrolyte leakage, and lipid degradation of dried bacteria during storage.

We have previously shown that the survival of freeze-dried *Weissella paramesenteroides* LC11 was the greatest during storage in aluminum foil (sealed under vacuum) or in glass tubes with water activity (a_w) of 0.11 compared with storage in glass tubes or glass tubes with $a_w=0.23$, respectively [25]. The aim of this work was to study the effect of cryoprotectants (glycerol, maltodextrin), alone or in combination with antioxidants (BHT and/or ascorbic acid), on the viability, electrolyte leakage, and lipid degradation of *W. paramesenteroides* stored at 4 or 20°C in glass tubes with $a_w=0.23$. The electrolyte leakage and lipid degradation were studied by measuring the electrical conductivity during rehydration and TBARS content, respectively. The effect of the mixtures after 90 days of storage on the 18:2/16:0 or 18:3/16:0 ratio in both the total lipid extract and the polar lipid fraction was determined and compared with that of the control. Previously, *W. paramesenteroides* LC11 had been isolated during cassava fermentation and was selected as a suitable starter culture for gari production [12, 13, 26].

MATERIALS AND METHODS

Microorganism

The LAB *W. paramesenteroides* LC11 was provided by the Federal Research Centre for Nutrition, Institute of Hygiene and Toxicology

(Karlsruhe, Germany). The strain was inoculated in MRS broth and incubated at 30°C for 18 h. Cells obtained after centrifugation (2,500 ×g, 20 min) were maintained in 50% (v/v) glycerol and frozen at -80°C.

Production, Treatment, and Storage

The strain was grown in a 500-l bioreactor containing MRS medium for 18 h, concentrated 20 times by centrifugation, and then the following mixtures were added: PC, cryoprotectants (maltodextrin [50 mg/g]+glycerol [20 mg/g]); PCA, cryoprotectants+ascorbic acid [0.2 mg/g]; PCB, cryoprotectants+BHT [0.075 mg/g]; PCAB, cryoprotectants+ascorbic acid [0.2 mg/g]+BHT [0.075 mg/g]. Cell suspensions without protective compounds were used as the control (P). The cell suspensions were freeze-dried in a Low freeze-drier (Leybold, Belgium) as described by Yao *et al.* [25]. Freeze-dried powders of about 2±0.5 g were stored in 20-ml glass tubes for 90 days at 4 and 20°C in a desiccator over potassium acetate (CH₃COOK) as a saturated salt solution with $a_w=0.23$.

Dry Cell Weight and Water Activity

The dry cell weight was determined after drying in a convection oven (105°C) until constant weight and the results were the mean of four determinations. The a_w of the saturated salt solution was confirmed using a Novasina (Novasina, Pfäffikon, Switzerland) water activity meter.

Survival

The viable counts were obtained using the plate count method after 48 h at 30°C. Percentage survival was calculated as $100 \times N/N_0$, where N is the CFU/g DW at a given time and N_0 is the CFU/g DW at the end of freeze-drying.

Electrolyte Leakage

For the analysis, 0.5 g of powder was soaked in Milli Q water (10 ml) for 4 h at room temperature. The electrical conductivity (ms/cm g DW) of the solution was measured using a ProfilLine Multi 197i (WTW GmbH, Weilheim, Germany) conductivity meter. Total conductivity was obtained after autoclaving the samples at 112°C for 20 min. The results were expressed as percentage of total conductivity.

Lipid Oxidation

Lipid oxidation was estimated as TBARS content, following the method described by Raharjo *et al.* [17], and modified as described here. Freeze-dried samples (1 g of powder), 25 µl of 3 mM BHT, and 40 ml of 5% (w/v) aqueous trichloroacetic acid (TCA) were homogenized in a blender for 1 min. The supernatant obtained after centrifugation (2,500 ×g, 10 min) was filtered into a 50-ml volumetric flask. The volume of the filtrate was adjusted to 50 ml with 5% (w/v) TCA. A 2 ml portion of the filtrate was mixed with 2 ml of 80 mM TBA at 96°C in a water bath for 60 min. The samples were then put on ice to stop the reaction. After cooling, 150 µl of 37% HCl and 2 ml *n*-butanol were added. The TBA-MDA complex was extracted from the upper phase after decanting, using a Pasteur-pipette, and the absorbance was recorded at 530 nm. The TBARS content was expressed as mg eq. MDA/g DW using an extinction coefficient of $1.48 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ [20].

Total Lipids Extraction and Fractionation

The total lipids were extracted overnight from dried cells (1 g) with an ethanol-ether (3:1 v/v) mixture, as described by Yao *et al.* [25].

Ethanol–ether extracts were pooled, filtered, and then evaporated and concentrated under reduced pressure at 35°C. The polar lipid fraction was obtained from the total lipid extract, using the acetone precipitation method described by Vandana *et al.* [23] and modified as follows: total lipid extract was dissolved in 2 ml of acetone, mixed for 1 min, and left on ice for 1 h; the acetone layer containing nonpolar lipids was decanted, and then the same procedure was repeated three times and acetone was removed under reduced pressure at 35°C. Thereafter, the acetone-insoluble precipitate, enriched in phospholipids, was dried under vacuum. The phospholipid extract (polar lipid fraction) was dissolved in an ethanol–ether (3:1 v/v) mixture and then stored at –80°C until further analysis.

Analysis of Fatty Acids

The fatty acids composition, in both the total lipid extract and the polar lipid fraction, was determined after the 90-day storage period. Fatty acid methyl esters (FAMES) were prepared from the lipid extracts and identified using a gas chromatographic analysis as described by Yao *et al.* [25]. The fatty acid relative content (%) was estimated as a percentage of the total peak area. The percentages of linoleic (18:2) and linolenic (18:3) acids were normalized by expressing as a ratio of the percentage of palmitic (16:0) acid [25].

Data Analysis

Production in the bioreactor was done in duplicate. In order to estimate the impact of storage conditions on the electrolyte leakage or lipid oxidation (TBARS content), only the newly released electrolyte or produced MDA–TBA complex was taken into account and expressed in relative terms as $(EC_t - EC_0)/g$ or $(MDA_t - MDA_0)/g$ DW, where EC_0 or MDA_0 and EC_t or MDA_t represent electrical

conductivity or TBARS content at the end of freeze-drying and at each storage time, respectively. The data were compared using Tukey's honest significant difference (Statistica 7.1, StatSoft Inc., 2005). The level of significance was determined at $P < 0.05$.

RESULTS

Effects of Protective Compounds on Survival

The LAB *W. paramesenteroides* was produced in a bioreactor, added with different mixtures as protective compounds, and then freeze-dried and held at 4 or 20°C in glass tubes with $a_w = 0.23$ for 90 days. The effects of the cryoprotectants (maltodextrin+glycerol) and cryoprotectants+antioxidant (ascorbic acid and/or BHT) mixtures on the survival were compared with those of the control (cells without protective compounds). It was found that, regardless of storage temperatures and treatments, the survival of the treated strain decreased as the storage time increased (Fig. 1). However, after the 90-day storage period, a significantly higher viable population was noted in dried samples held at 4°C, compared with those stored at 20°C ($P < 0.05$) (Table 1). Survival rates were significantly higher for PCB compared with those of PC, PCA, and PCAB after 90 days of storage. For example, the viable population for PCB was reduced from an initial population of 6.0×10^{11} CFU/g to 2.6×10^{11} or 1.1×10^{11} CFU/g with a survival of 48% or 20% after 90 days at 4°C or 20°C, respectively. After 90 days at

Table 1. Effects of protective compounds on the survival, electrolyte leakage, TBARS content of freeze-dried *W. paramesenteroides*, and water content of dried samples after 90-day storage.

Storage temperature (°C)	Treatment	Moisture content (%)	Survival		Electrolyte leakage		TBARS (mg/kg DW) [‡]
			CFU/g DW	(%)*	Electrical conductivity (ms/cm g DW)	(%) [†]	
4	P	11.4±0.6 ^a	4.4×10^9	0.1 ^a	98.0±3.0	13.9±0.9 ^{ac}	2.0±0.4 ^a
	PC	12.3±0.3 ^a	4.1×10^9	0.8±0.2 ^a	81.3±4.6	13.5±1.5 ^{ac}	2.1±0.3 ^{ac}
	PCA	12.2±0.8 ^a	2.7×10^{10}	3.7±0.6 ^a	72.6±1.4	13.4±2.1 ^{ac}	4.0±0.7 ^b
	PCB	12.7±0.3 ^a	2.6×10^{11}	48.1±4.5 ^b	70.9±3.2	7.5±0.9 ^a	0.8±0.3 ^c
	PCAB	12.4±0.7 ^a	3.2×10^{11}	39.4±3.3 ^c	69.3±3.9	9.5±1.2 ^a	4.2±0.9 ^b
20	P	11.2±0.3 ^a	5.3×10^7	<0.01 ^a	101.9±5.2	32.2±2.7 ^b	2.8±0.6 ^a
	PC	12.5±0.4 ^a	4.5×10^7	≤0.01 ^a	83.5±3.0	22.6±2.0 ^c	2.8±0.5 ^{ab}
	PCA	12.3±0.8 ^a	1.9×10^8	≤0.03 ^a	78.5±4.7	28.8±3.3 ^{bc}	6.1±0.9 ^d
	PCB	12.5±0.9 ^a	1.1×10^{11}	20.2±4.6 ^d	75.3±4.0	9.7±0.8 ^a	1.4±0.3 ^{ac}
	PCAB	11.8±0.8 ^a	1.0×10^{11}	12.2±1.5 ^d	76.4±1.9	20.4±3.1 ^{ac}	6.3±1.1 ^d

CFU, colony-forming units.

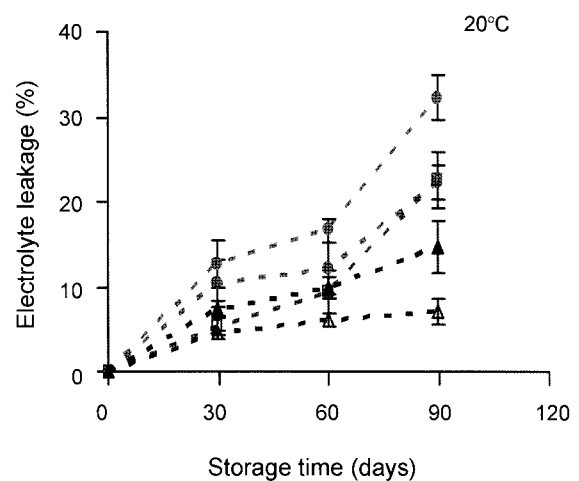
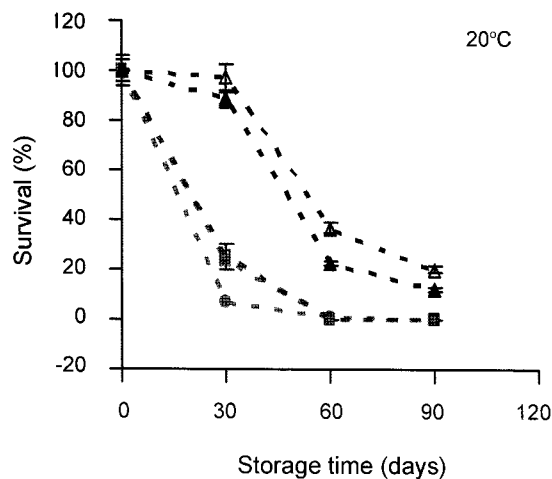
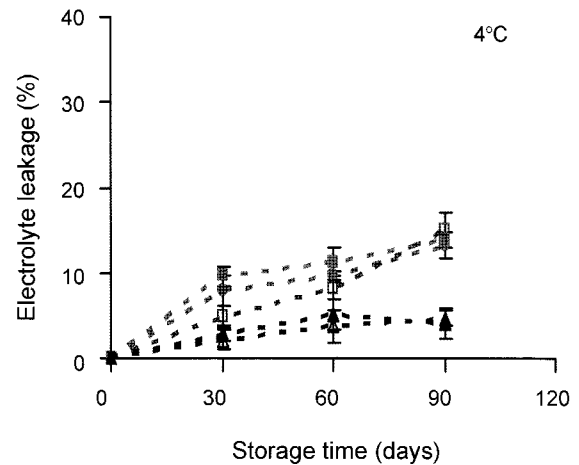
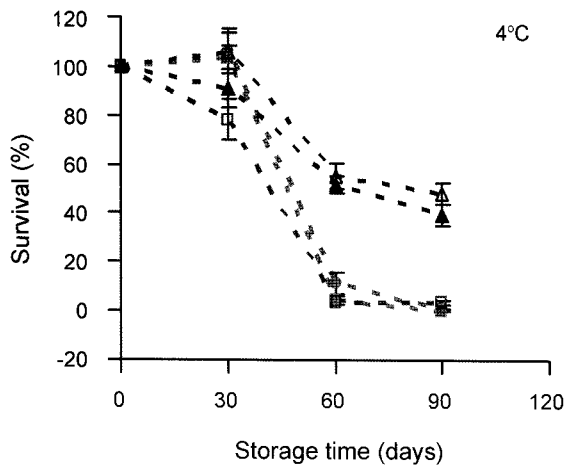
a–d, Values not sharing the same superscript letter within a column are significantly different, $P < 0.05$ (Tukey HSD test, $n = 4$).

P, control; PC, cryoprotectants (maltodextrin+glycerol); PCA, cryoprotectants+ascorbic acid; PCB, cryoprotectants+BHT; PCAB, cryoprotectants+ascorbic acid+BHT.

*Percentage compared with those obtained after freeze-drying. Initially, P, PC, PCA, PCB, or PCAB with a water content of 3.6, 4.3, 4.0, 3.9, and 3.7 g H₂O/100 g DW contained 8.6×10^{11} , 5.6×10^{11} , 7.7×10^{11} , 6.0×10^{11} , or 9.0×10^{11} CFU/g of *W. paramesenteroides*, respectively.

[†]Values compared with those obtained after freeze-drying. Initially, the electrical conductivity for P, PC, PCA, PCB, and PCAB was 80.2, 66.2, 67.9, 67.2, and 67.5 ms/cm g DW and represented 84.3, 83.1, 89.4, 76.0, and 84.7% of total electrolyte (after 20 min at 112°C), respectively.

[‡]Values compared with initial values. TBARS value at the end of freeze-drying for P, PC, PCA, PCB, and PCAB was 0.07, 0.09, 0.08, 0.09, and 0.12 mg/kg DW, respectively.



■ - P ● - PC ▲ - PCA ◆ - PCB ▼ - PCAB

■ - P ● - PC ▲ - PCA ◆ - PCB ▼ - PCAB

Fig. 1. Effects of protective compounds on the survival of freeze-dried *W. paramesenteroides* stored for 90 days in a glass tube with $a_w=0.23$.

P, control; PC, cells with cryoprotectants (maltodextrin+glycerol); PCA, cells with cryoprotectants+ascorbic acid; PCB, cells with cryoprotectants+BHT; PCAB, cells with cryoprotectants+ascorbic acid+BHT. Values are presented as means \pm SD ($n=4$).

Fig. 2. Effects of protective compounds on the electrolyte leakage of freeze-dried *W. paramesenteroides* stored for 90 days in a glass tube with $a_w=0.23$.

P, control; PC, cells with cryoprotectants (maltodextrin+glycerol); PCA, cells with cryoprotectants+ascorbic acid; PCB, cells with cryoprotectants+BHT; PCAB, cells with cryoprotectants+ascorbic acid+BHT. The electrolyte leakage values represent the difference compared with initial values. Values are presented as means \pm SD ($n=4$).

4°C or 20°C, the survival rates for PC and PCA were statistically the same and did not differ significantly from that of the control ($P>0.05$). Among the mixtures tested, the one that resulted in the highest survival was the association of cryoprotectants with BHT. At the end of freeze-drying, the water content for P, PC, PCA, PCB, and PCAB was 3.6 ± 0.6 , 4.3 ± 0.4 , 4.0 ± 0.3 , 3.9 ± 0.5 , and 3.9 ± 0.5 g H₂O/100 g DW with $a_w=0.09\pm 0.01$, respectively. After the 90-day storage period at 4°C and 20°C, the water content did not differ significantly among the samples ($P>0.05$) (Table 1).

Effects of Protective Compounds on Electrolyte Leakage

The electrolyte leakage for both treated samples and the control increased with the storage time (Fig. 2). Decreases in membrane integrity were observed, and this was reflected in the increased amount of electrolyte leaking from the cell during rehydration. However, after the 90-day storage period, a significantly lower leakage value was noted in dried samples held at 4°C or with BHT, compared with those stored at 20°C or the other protective compounds (cryoprotectants, ascorbic acid), respectively ($P<0.05$) (Table 1). For example, the leakage

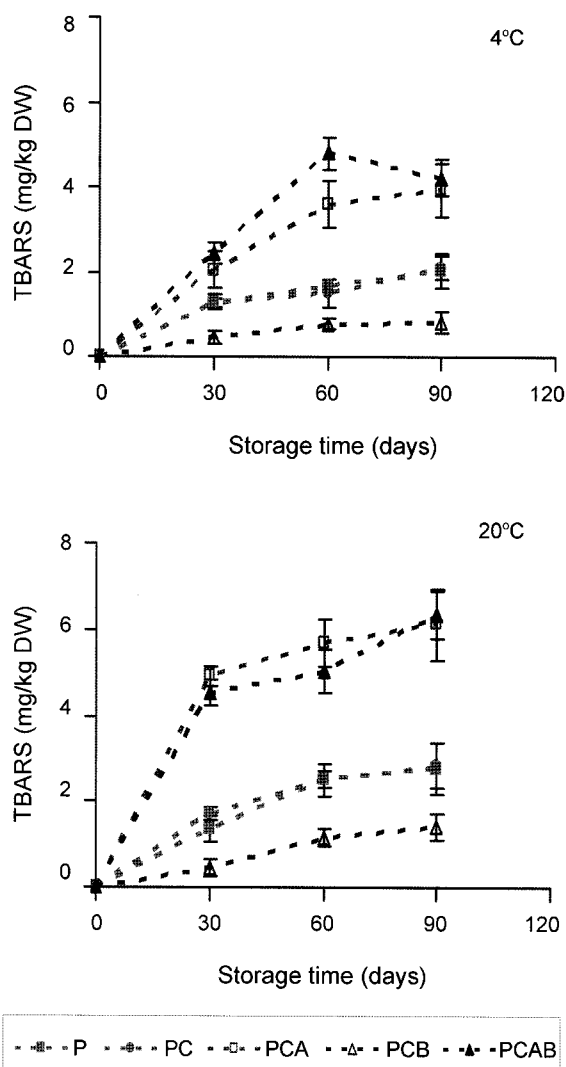


Fig. 3. Effects of protective compounds on the TBARS content of freeze-dried *W. paramesenteroides* stored for 90 days in a glass tube with $a_w=0.23$.

P, control; PC, cells with cryoprotectants (maltodextrin+glycerol); PCA, cells with cryoprotectants+ascorbic acid; PCB, cells with cryoprotectants+BHT; PCAB, cells with cryoprotectants+ascorbic acid+BHT. The TBARS values represent the difference compared with initial values. Values are presented as means \pm SD ($n=4$).

value for PCB increased from the original value by 7.5% with an electrical conductivity of only 70.9 ms/cm g DW at 4°C, compared with a larger increase from the original value by 9.7% with an electrical conductivity of 75.3 ms/cm g DW at 20°C after 90 days at 4°C or 20°C, respectively (Fig. 2 and Table 1). After the 90-day storage period at 4 or 20°C, the leakage value for PCB was approximately 6% or 22% lower than that of the control, respectively. In the same conditions, the leakage values for PC and PCA were statistically the same and did not differ significantly from that of the control ($P>0.05$). The lowest leakage value after 90 days of storage was obtained with the cryoprotectants+BHT mixture.

Table 2. Effects of the cryoprotectants+BHT mixture on fatty acids composition of the total lipid extract after 90-day storage.

Fatty acid (s)	Storage temperature (°C)			
	4		20	
	P	PCB	P	PCB
	Percentage of total*			
16:0	54.2 \pm 1.9	54.3 \pm 0.5	53.3 \pm 1.6	54.0 \pm 1.1
16:1	9.1 \pm 0.9	5.2 \pm 0.3	7.9 \pm 1.5	5.1 \pm 1.7
18:0	9.5 \pm 0.7	12.2 \pm 1.4	9.8 \pm 0.5	12.0 \pm 1.3
18:1	24.8 \pm 1.2	22.5 \pm 0.8	27.8 \pm 2.3	24.9 \pm 1.1
18:2	0.4 \pm 0.0	2.1 \pm 0.4	0.3 \pm 0.1	1.6 \pm 0.6
18:3	2.0 \pm 0.3	3.7 \pm 0.7	0.9 \pm 0.1	2.3 \pm 0.4
Σ	100.0	100.0	100.0	100.0
	100 \times Ratio [†]			
18:2/16:0	0.8	3.9	0.6	2.9
18:3/16:0	3.7	6.9	1.7	4.2
	Percentage of initial ratio [‡]			
18:2/16:0	51.5 \pm 3.7 ^a	83.2 \pm 4.7 ^b	37.2 \pm 5.6 ^a	61.9 \pm 6.4 ^c
18:3/16:0	38.5 \pm 4.9 ^a	73.8 \pm 3.5 ^b	18.0 \pm 2.2 ^c	45.0 \pm 5.2 ^a

P, control; PCB, cells with cryoprotectants+BHT.

Palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids.

a–c, Values not sharing the same superscript letter within a horizontal line are significantly different, $P<0.05$ (Tukey HSD test, $n=4$).

*Percentage of total fatty acids content.

[†]Values represent ratio between each fatty acid and the 16:0.

[‡]Percentage compared with those obtained after freeze-drying. Initially, 18:2/16:0 and 18:3/16:0 acid ratios for P and PCB were 0.02 and 0.10, and 0.05 and 0.10, respectively.

Effects of Protective Compounds on Lipid Oxidation

The effects of the four mixtures on lipid oxidation (TBARS content) during the storage of freeze-dried *W. paramesenteroides* is shown in Fig. 3 and Table 1. The TBARS content increased during the storage period; however, the level of lipid oxidation for samples stored at 4°C or PCB mixture was significantly lower compared with those stored at 20°C or the other samples (P, PCA, PCAB), respectively ($P<0.05$). After the 90-day storage period at 4 and 20°C, the TBARS content for PCB was approximately 1.2% and 1.4% lower than that of the control, respectively. On the contrary, the TBARS content for PCA and PCAB were 2.0% and 3.4%, and 2.2% and 3.6% higher than that of the control, respectively (Table 1). The TBARS content did not change significantly between PC and the control ($P>0.05$). The highest protection against lipid oxidation during storage was obtained with the cryoprotectants+BHT mixture.

Effects of Cryoprotectants+BHT Mixture on Fatty Acids Composition

The protective effects of the cryoprotectants+BHT mixture on the fatty acids composition of the total lipid extract and the polar lipid fraction was compared with that of the

Table 3. Effects of cryoprotectants+BHT mixture on fatty acids composition of the polar lipid fraction after 90-day storage.

Fatty acid (s)	Storage temperature (°C)			
	4		20	
	P	PCB	P	PCB
	Percentage of total*			
16:0	51.1±2.3	53.6±1.1	54.3±1.0	53.0±1.0
16:1	9.5±1.4	6.3±0.8	8.4±0.9	6.5±1.1
18:0	11.5±1.1	11.3±0.9	11.8±0.8	12.5±0.9
18:1	26.6±2.1	23.6±1.2	24.7±1.7	25.1±0.8
18:2	0.6±0.2	2.2±0.7	0.4±0.1	1.1±0.2
18:3	1.1±0.3	2.8±0.4	0.7±0.2	1.8±0.5
Σ	100.0	100.0	100.0	100.0
	100×Ratio [†]			
18:2/16:0	1.2	4.2	0.7	2.0
18:3/16:0	2.2	5.3	1.2	3.3
	Percentage of initial ratio [‡]			
18:2/16:0	44.6±5.6 ^{ac}	82.6±5.8 ^b	24.1±5.0 ^a	40.0±3.9 ^c
18:3/16:0	27.4±1.7 ^a	61.7±2.8 ^c	15.0±3.2 ^c	38.7±4.4 ^d

P, control; PCB, cells with cryoprotectants+BHT.

Palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids.

a–d, Values not sharing the same superscript letter within a horizontal line are significantly different, $P < 0.05$ (Tukey HSD test, $n = 4$).

*Percentage of total fatty acids content.

[†]Values represent ratio between each fatty acid and the 16:0.

[‡]Percentage compared with those obtained after freeze-drying. Initially, 18:2/16:0 and 18:3/16:0 acid ratios for P and PCB were 0.03 and 0.09, and 0.06 and 0.09, respectively.

control after the 90-day storage period (Tables 2 and 3). In both the total lipid extract and polar lipid fraction, the major fatty acids found were palmitic (16:0), oleic (18:1), stearic (18:0), palmitoleic (16:1), linolenic (18:3), and linoleic (18:2) acids. The percentage of 16:0 remained high and did not change significantly during storage, as described earlier under Materials and Methods. Therefore, the percentages of the other fatty acids were expressed as a ratio between each fatty acid and the 16:0. The 16:1/16:0, 18:0/16:0, and 18:1/16:0 ratios did not change significantly during the storage period (data not shown). The main modification observed was a decrease in the 18:2/16:0 and 18:3/16:0 ratios (data not shown). After the 90-day storage period, the 18:2/16:0 and 18:3/16:0 ratios for samples stored at 4°C were significantly higher than for those stored at 20°C ($P < 0.05$) (Tables 2 and 3). For example, the 18:3/16:0 ratio in the total lipid extract and the polar lipid fraction of PCB decreased from the original value by 26% and 55%, with a ratio reduction of only 0.03 and 0.04 at 4°C compared with the larger decrease from the original value by 38% and 61% with a ratio reduction of 0.06 at 20°C, respectively. After 90 days at 4 and 20°C, the 18:2/16:0 and 18:3/16:0 ratios in the total lipid extract of PCB were 32% and 25% and 35% and 27% higher than those of

the control, respectively. Similarly, the 18:2/16:0 and 18:3/16:0 ratios in the polar lipid fraction of PCB were 38% and 16%, and 34% and 24% higher than those of the control, respectively. After the 90-day storage period, the cryoprotectants, cryoprotectants+ascorbic acid, and cryoprotectants+ascorbic acid+BHT mixtures showed lower 18:2/16:0 and 18:3/16:0 ratios in both the total lipid extract and polar lipid fraction and there was no significant difference from those of the control (data not shown).

DISCUSSION

The effects of cryoprotectants (maltodextrin+glycerol) and cryoprotectants+antioxidant (ascorbic acid and/or BHT) mixtures on the viability, electrolyte leakage, and lipid degradation (TBARS content) of freeze-dried *W. paramesenteroides* stored in glass tubes were investigated and compared with that of the control. Significant alterations in the viable cell of the protected strain, but lower than that of the control, were observed during the storage period. However, differences in the viability of the protected strain were observed, indicating different protective abilities of the mixtures used. The cryoprotectants+BHT mixture was the best protectant after 90 days of storage at 4 and 20°C, giving a cell viability of about 48% and 20% compared with 0.1% and 0.01% for the control, respectively. Our results showed that the storage temperature and moisture were critical parameters affecting the survival of the strain. A better percentage of survival of freeze-dried bacteria during storage at low temperatures [24] and in the 0.1–0.2 a_w zone with a dry-matter rate of almost 96% [11] had been reported previously. Our results also showed that neither the cryoprotectants mixture nor the control significantly improved survival during storage. Coulibaly *et al.* [5] demonstrated that *Lactobacillus plantarum* CWBI-B534 cell recoveries ranged from 97% after freeze-drying, using a 5% maltodextrin+2% glycerol mixture. It is now well established that cryoprotectants are almost indispensable when freezing and drying microorganisms, and the contribution of these compounds to the stability of dried microorganisms during storage has been discussed [7]. After the 90-day storage period, cellular fatty acids in both the total lipid extract and the polar lipid fraction of *W. paramesenteroides* were palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids. All these acids had been identified previously in the cellular membrane of freeze-dried LAB [5]. As the storage proceeded, increases in leakage value and TBARS content were observed, with a corresponding decrease in viable cell. However, the lowest TBARS content and leakage value, as well as highest percentage survival and 18:2/16:0 or 18:3/16:0 ratio in the polar lipid fraction of PCB, were obtained after 90 days at 20°C. These results suggest that the

cryoprotectants+BHT mixture could increase oxidative tolerance by reducing membrane lipid damage. The protection bestowed by the cryoprotectants+BHT mixture could derive from a reaction between the peroxy or oxy-free radicals and the antioxidant (BHT). It was reported that antioxidants scavenge chain-carrying peroxy radicals, and reduce the formation of the radicals that increase membrane leakage [1]. After 90 days at 20°C, while TBARS content for PCA or PCAB (sample containing ascorbic acid) was approximately 3.4% or 3.6% higher than that of the control, the amount of electrolytes leaking from the cell during rehydration was 3.5% or 11.8% lower than that of the control, respectively. This could be because ascorbic acid was much more susceptible to degradation than BHT during storage. Our results are in complete agreement with those of Hayashi *et al.* [8] who reported that ascorbic acid–protein mixtures of low moisture content stored in aerobic conditions became red, resulting from an amino-carbonyl reaction of oxidized ascorbic acid. Furthermore, the significance and specificity of the TBA reaction has been previously discussed [14]. A full comparison of the data from the present study with those from previous studies is difficult. Some sources of discrepancy between our results and the data in the literature arise from the different microorganisms and storage conditions, as well as from the nature and concentrations of protective compounds used [4, 21].

We could suggest that a large reduction in the 18:2/16:0 or 18:3/16:0 ratio in the polar lipid fraction of *W. paramesenteroides* after 90-day storage at 20°C might contribute to the greater TBARS content and derive partly from polar lipid degradation. It is possible that the breakdown of polar lipids as a consequence of lipid oxidation with the accumulation of MDA might lead to the loss of membrane integrity. Loss of membrane structure might therefore result in electrolyte leakage and, ultimately, in loss of viability. This hypothesis is in agreement with previous studies, as Castro *et al.* [3] have shown that the decline in viability during the storage of freeze-dried lactobacilli could be attributed to further membrane damage by oxidation, and as proposed by Selmer-Olsen *et al.* [18]. In addition, Borsos-Motovina and Blake [1] showed that electrolyte leakage could be a measure of the loss of membrane integrity from membrane damage. Altogether, the results from the present study and those from our previous work on PUFAs degradation during storage of the freeze-dried *W. paramesenteroides* [5, 25] are in agreement with the notion that membrane lipid degradation and survival of freeze-dried bacteria might be related. A rapid decrease in viability tended to occur during the early storage period, whereas changes in TBARS content and leakage value of the dried strain were found to increase with time. Mechanical damage, linked to intracellular ice formation and recrystallization during freezing, might affect the integrity of the cell structure [9]. Solutes might leak from injured cells more rapidly during

rehydration because the membrane could be mechanically ruptured by the dehydration treatment or because the permeability of an intact membrane could be altered. To make this distinction, changes in the membrane permeability of *W. paramesenteroides* after freeze-drying need to be assessed. In addition, further research is needed to identify the polar lipids that are oxidized or degraded during the storage of freeze-dried strain.

Acknowledgments

We gratefully acknowledge the Federal Research Centre for Nutrition, Institute of Hygiene and Toxicology (Karlsruhe, Germany) for providing *Weissella paramesenteroides* LC11, I. Coulibaly and M. Harden for their technical support on fatty acids analysis, and P. Mottet for his help during preparation of the manuscript.

REFERENCES

1. Borsos-Matovina, V. and T. J. Blake. 2001. Seed treatment with the antioxidant Ambiol enhances membrane protection in seedlings exposed to drought and low temperatures. *Trees* **15**: 163–167.
2. Castro, H. P., P. M. Teixeira, and R. Kirby. 1995. Storage of lyophilized cultures of *Lactobacillus bulgaricus* under different relative humidities and atmospheres. *Appl. Microbiol. Biotechnol.* **44**: 172–176.
3. Castro, H. P., P. M. Teixeira, and R. Kirby R. 1996. Changes in the cell membrane of *Lactobacillus bulgaricus* during storage following freeze-drying. *Biotechnol. Lett.* **18**: 99–104.
4. Champagne, C. P., F. Mondou, Y. Raymond, and D. Roy. 1996. Effect of polymers and storage temperature on the stability of freeze-dried lactic acid bacteria. *Food Res. Int.* **29**: 555–562.
5. Coulibaly, I., A. A. Yao, G. Lognay, J. Destain, M.-L. Fauconnier, and P. Thonart. 2008. Survival of freeze-dried *Leuconostoc mesenteroides* and *Lactobacillus plantarum* related to their cellular fatty acids composition during storage. *Appl. Biochem. Biotechnol.* **157**: 70–84.
6. Halliwell, B. and S. Chirico. 1993. Lipid peroxidation: Its mechanisms, measurement, and significance. *Am. Clin. Nutr.* **57**: 715s–724s.
7. Hamoudi, L., J. Goulet, and C. Ratti. 2007. Effect of protective agents on the viability of *Geotrichum candidum* during freeze-drying and storage. *Food Microbiol. Food Saf.* **72**: M45–M49.
8. Hayashi, T., A. Terao, S. Ueda, and M. Namiki. 1985. Red pigment formation by the reaction of oxidized ascorbic acid and protein in a food model system of low moisture content. *Agric. Biol. Chem.* **49**: 3139–3144.
9. Heckly, R. J. 1985. Principles of preserving bacteria by freeze-drying. *Dev. Ind. Microbiol.* **26**: 379–395.
10. Hurst, A. 1977. Bacterial injury: A review. *Can. J. Microbiol.* **23**: 936–944.
11. Ishibashi, N., I. Tatematsu, S. Shimamura, M. Tomita, and S. Okonogi. 1985. Effect of water activity on the viability of

- freeze-dried bifidobacteria and lactic acid bacteria, pp. 227–232. I. I. R.-I. I. F. Commission C1, Tokyo, Japan.
12. Kostinek, M., I. Specht, V. A. Edward, C. Pinto, M. Egounlety, C. Sossa, *et al.* 2007. Characterisation and biochemical properties of predominant lactic acid bacteria from fermenting cassava for selection as starter cultures. *Int. J. Food Microbiol.* **114**: 342–351.
 13. Kostinek, M., I. Specht, V. A. Edward, U. Schillinger, C. Hertel, W. H. Holzapfel, and C. M. A. P. Franz. 2005. Diversity and technological properties of predominant lactic acid bacteria from fermented cassava used for the preparation of Gari, a traditional African food. *Syst. Appl. Microbiol.* **28**: 527–540.
 14. Lefèvre, G., M. Beljean-Leymarie, F. Beyerle, D. Bonnefont-Rousselot, J. P. Cristol, P. Théron, and J. Torreilles. 1998. Evaluation de la peroxydation lipidique par le dosage des substances réagissant avec l'acide thiobarbiturique. *Ann. Biol. Clin.* **56**: 305–319.
 15. Martos, G. I., A. P. Ruiz Holgado, G. Oliver, and G. F. De Valdez. 1999. Use of conductimetry to evaluate *Lactobacillus delbrueckii* ssp. *bulgaricus* subjected to freeze-drying. *Milchwissenschaft* **54**: 128–130.
 16. Raharjo, S. and J. N. Sofos. 1993. Methodology for measuring malondialdehyde as a product of lipid peroxidation in muscle tissues: A review. *Meat Sci.* **35**: 145–169.
 17. Raharjo, S., J. N. Sofos, and G. R. Schmidt. 1992. Improved speed, specificity, and limit determination of an aqueous acid extraction thiobarbituric acid-C18 method for measuring lipid peroxidation in beef. *J. Agric. Food Chem.* **40**: 2182–2185.
 18. Selmer-Olsen, E., S. E. Birkeland, and T. Sørhaug. 1999. Effect of protective solutes on leakage from and survival of immobilized *Lactobacillus* subjected to drying, storage and rehydration. *J. Appl. Microbiol.* **87**: 429–437.
 19. St. Angelo, A. J. 1996. Lipid oxidation in foods. *Crit. Rev. Food Sci. Nutr.* **36**: 175–224.
 20. Tarladgis, B. O., A. M. Pearson, and L. R. Jr Dugan. 1964. Chemistry of the 2-thiobarbituric acid test for determination of oxidative rancidity in foods: (II) Formation of the TBA-malonaldehyde complex without acid-heat treatment. *J. Sci. Food Agric.* **15**: 602–607.
 21. Teixeira, P. C., M. H. Castro, F. X. Malcata, and R. M. Kirby. 1995. Survival of *Lactobacillus delbrueckii* ssp. *bulgaricus* following spray drying. *J. Dairy Sci.* **78**: 1025–1031.
 22. van Ginkel, G. and A. Sevanian. 1994. Lipid peroxidation-induced membrane structural alterations. *Methods Enzymol.* **233**: 273–288.
 23. Vandana, V., M. S. L. Karuna, P. Vijayalakshmi, and R. B. N. Prasad. 2001. A simple method to enrich phospholipid content in commercial soybean lecithin. *J. Am. Oil Chem. Soc.* **78**: 555–556.
 24. Wang, Y.-C., R.-C. Yu, and C.-C. Chou. 2004. Viability of lactic acid bacteria and bifidobacteria in fermented soymilk after drying, subsequent rehydration and storage. *Int. J. Food Microbiol.* **93**: 209–217.
 25. Yao, A. A., I. Coulibaly, G. Lognay, M.-L. Fauconnier, and P. Thonart. 2008. Impact of polyunsaturated fatty acid degradation on survival and acidification activity of freeze-dried *Weissella paramesenteroides* LC11 during storage. *Appl. Microbiol. Biotechnol.* **79**: 1045–1052.
 26. Yao, A. A., F. Bera, C. M. A. P. Franz, W. H. Holzapfel, and P. Thonart. 2008. Survival rate analysis of freeze-dried lactic acid bacteria using the Arrhenius and z-value models. *J. Food Prot.* **71**: 431–434.
 27. Zhao, G. and G. Zhang. 2005. Effect of protective agents, freezing temperature, rehydration media on viability of malolactic bacteria subjected to freeze-drying. *J. Appl. Microbiol.* **99**: 333–338.