BIOTECHNOLOGY LETTERS
Volume 18 No.1 (January 1996) pp.99-104
Received as revised 30th October.

Changes in the cell membrane of *Lactobacillus bulgaricus* during storage following freeze-drying

HELENA P. CASTRO*, PAULA M. TEIXEIRA, ROY KIRBY
Escola Superior de Biotecnologia. Porto - Portugal

SUMMARY
The mechanism of inactivation of freeze-dried *Lactobacillus bulgaricus* during storage in maltodextrin under controlled humidity was investigated. Evidence is presented supporting the hypothesis that membrane damage occurs during storage. A study on the lipid composition of the cells by gas chromatography showed a decrease in the unsaturated and saturated fatty acid content of the cell. Further evidence indicating membrane damage includes a decrease in membrane bound proton-translocating ATPase activity.

INTRODUCTION
Freeze-drying is a method of preservation that depends upon the reduction of water activity by water removal. Microbial cultures can be kept in a "dormant" state, especially if they are kept at low temperatures (Foster, 1962; Clementi and Rossi, 1984). Nevertheless, during storage, bacterial cells are likely to lose their viability and activity (acid production capacity) (Alaeddinoglu *et al.*, 1989). In a previous paper (Castro *et al.*, 1995), a change in the lipid profile during storage of *Lactobacillus bulgaricus* lyophilized in skim milk powder was reported. This change expressed by a decrease in the ratio of the unsaturated to saturated fatty acids (u/s index) was related with lipid peroxidation and was dependent on the water activity at which the lyophilized bacteria were kept. Furthermore the decrease in the u/s index was correlated with a decrease in cell viability. The purpose of this work was to identify the main fatty acids involved in the change of the lipid profile during storage of *Lactobacillus bulgaricus* and to find out reasons for the loss of viability. The effects of phospholipid degradation were further evaluated on the proton-translocating ATPase.
In this article an equilibrium relative humidity of 11% was employed as it was considered to be the best for cell stability (Castro *et al.*, 1995). The use of maltodextrin made possible the post stress recovery of cells not contaminated with insoluble material.

EXPERIMENTAL METHODS

Organism and culture medium

Cultures of *Lactobacillus delbrueckii* ssp. *bulgaricus* NCFB 1489 were grown in Man Rogosa and Sharpe broth (De Man *et al.*, 1964) (MRS) for 16 h, at 37°C and agitated using magnetic stirrer bars to produce early stationary phase cells. Cells were harvested by centrifugation at 16000 g at 4°C and washed twice with cold deionized water. Cells were then suspended in maltodextrin (11%, w/v) at ca. 10^9 cells/mL prior to lyophilization.

Lyophilization and storage

Samples were frozen at -80°C in a deep freezer and then desiccated under vacuum (6.7 Pa) in the freeze-drier (Martin Christ Alpha 1-4) for 24 h. After freeze-drying, the dried bacteria were equilibrated in a sealed flask containing a saturated salt solution of lithium chloride providing an equilibrium relative humidity (RH_{eq}) of 11%, previously reported as optimal for survival (Castro *et al.*, 1995). The flask was held at 20 °C during the study. Samples were removed at intervals for enumeration and analysis.

Enumeration

Prior to plating samples were rehydrated with sterile distilled water at room temperature (20°C) and immediately agitated. Viability was determined on solid media (MRS agar) by the Miles and Misra technique (1938). Plates were incubated at 37°C for 48 h before enumeration.

Lipid analysis

Membrane lipids were analysed by gas chromatography as described by Rozès et al. (1993) using sodium methoxide (1 N) in methanol for methanolysis. For quantitative determination, peak areas of the esterified fatty acids were related to those of the standards. As this methylation procedure does not act on free, but only on esterified fatty acids (Jamieson and Reid, 1965), standard FAME were used to establish calibration curves. Relative response factors were determined for each ester, and these were used in the calculation. The results for fatty acids were computed by using both the proportional values and the absolute amounts obtained in the analysis. The total areas of the main fatty acids present, cyclopropane, palmitoleic, stearic, palmitic and oleic acids were used to determine the relative percent of each fatty acid present, and calculation of the ratio of unsaturated to saturated fatty acids (uns/sat index). For absolute quantifications, concentrations obtained from calibration curves were related to the amount of sample used in the analysis and evaluated by dry weight determination (described in the ATPase assay).

ATPase assay

The proton-stimulated ATPase was assayed as described by Belli and Marquis (1991) using permeabilized cells in the presence of 100 µM N,N'-dicyclohexylcarbodiimide (DCCD). Cells were permeabilized as described previously (Belli and Marquis, 1991) by use of toluene and 2 cycles of freezing and thawing. ATPase specific activities were expressed as micromoles of phosphate released from ATP per g of cell dry weight.

Dry weight determination: Permeabilized cells were harvested by centrifugation and washed with cold deionized water. Samples of permeabilized cells (200 µL) were dried overnight in pre-tared ependorff tubes at 105°C and weighed.

Statistical analysis

Data were analysed by standard analysis of variance using the StatView package (Abacus Concepts, Inc., Berkeley, CA). Significance was declared at $P \leq .05$ unless otherwise stated.

RESULTS

The effects of storage of *Lactobacillus bulgaricus* (20°C) on the main unsaturated fatty acids (palmitoleic and oleic acid), the lactobacillic acid (often included with the unsaturated acids) and the saturated fatty acids (palmitic and stearic acid) are shown in Figs. 1 and 2, respectively. Results show a decrease in the unsaturated fatty acid content of membranes. Palmitoleic, oleic and lactobacillic acid behaved similarly. Saturated fatty acids also decreased during all the storage period. Palmitic and stearic acid also showed similar changes.

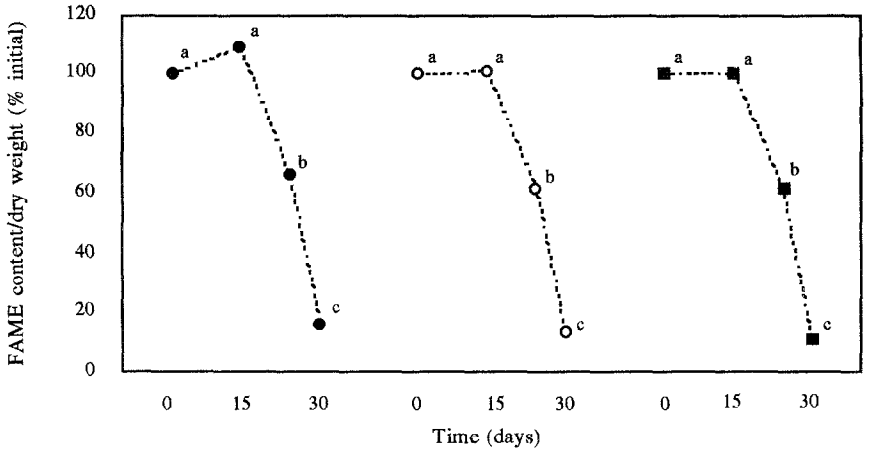

Figure 1. Variation of the percentage of unsaturated fatty acids of Lactobacillus bulgaricus during storage. Palmitoleic acid, ● ; oleic acid, O; lactobacillic acid, ■ (cyclopropane acid was considered an unsaturated fatty acid). Means without a common letter (a,b,c) differ (P≤.05).

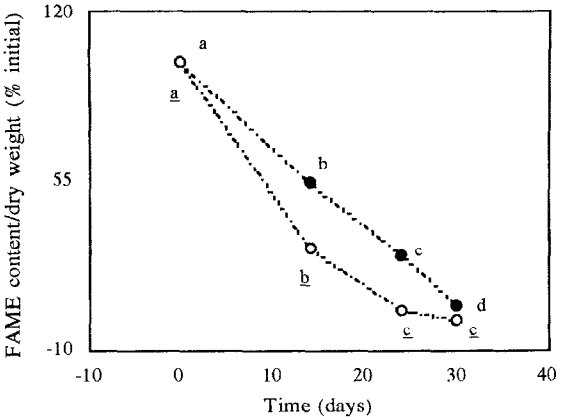

Figure 2. Variation of the percentage of saturated fatty acids of Lactobacillus bulgaricus during storage. Palmitic acid, ● ; stearic acid, O. Means without a common letter (a,b,c,d and a,b,c,d) differ (P≤.05).

Changes in the unsaturated/saturated (u/s) fatty acid index and cell viability are shown in Fig. 3. Results show that cell viability decreased as the u/s index decreased.

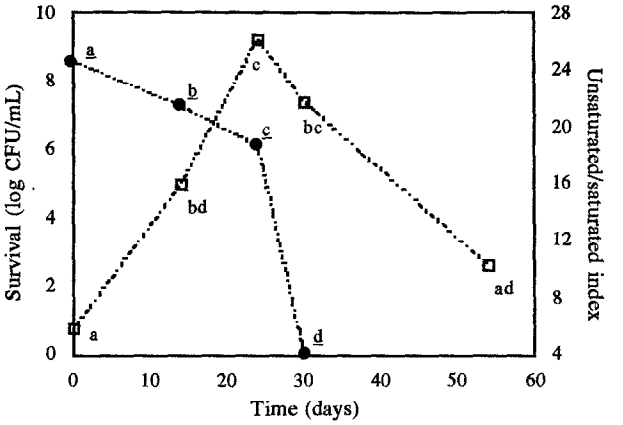

Fig. 3. Comparison of the survival of freeze-dried Lactobacillus bulgaricus during storage with the uns/sat index of the membrane fatty acids. Log CFU/mL, ●; uns/sat index, □. Means without a common letter (a,b,c,d and a,b,c,d) differ (P≤.05).

The proton-translocating ATPase "specific" activity of the citoplasmic membrane is presented in Fig. 4. The ATPase "specific" activity showed a pronounced decrease in the first 14 days of storage, followed by a subsequent slower rate of decline.

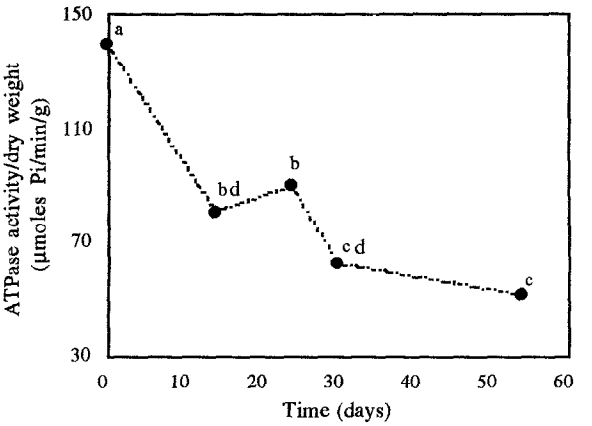

Fig. 4. Evolution of the ATPase "specific activity" of freeze-dried Lactobacillus bulgaricus during storage. Means without a common letter (a,b,c,d) differ (P≤.05).

DISCUSSION

Results presented here were considered, for practical purposes, as values for membrane composition, as most of the lipids of Gram-positive microorganisms are found predominantly in the cell membrane (Kates, 1964). The method of analysis allowed conclusions about the integrity of the phospholipids, because the process of methylation did not acted on free, but only

on esterified fatty acids (Jamieson and Reid, 1965). Results presented here showed alterations of the lipid composition of the cell membrane during storage. It is possible that two different mechanisms of phospholipid degradation were involved: oxidation and lipolysis. Following freeze-drying, saturated fatty acids immediately started to decrease. Autoxidation of saturated fatty acids is extremely slow at room temperature (Nawar, 1976); the observed decrease in saturated fatty acids is probably a consequence of lipolysis, caused by the bacteria themself, in spite of the low water activity (Roissart, 1986). As a matter of fact, lactic acid bacteria may present a certain (although weak) lipolytic power (Stadhouders and Veringa, 1973). Unsaturated fatty acids also decreased during the storage time. Contrarily to their saturated analogs, unsaturated fatty acids are much more susceptible to oxidation (Nawar, 1976). The mechanism of induced degradation could be explained by the fact of a certain period being required before free radicals are formed (Nawar, 1976). Lipolysis of unsaturated phospholipids might not be important in membrane degradation, because of inaccessibility to the lipolytic enzyme (Lehninger *et al.*, 1993). The calculation of the unsaturated/saturated index (u/s index) allowed to define in time the importance of each mechanism in phospholipid degradation. The u/s index showed an initial increase in the first 30 days, probably associated essentially with lypolysis of saturated fatty acids. After an "induction" period, oxidation of unsaturated fatty acids also became important, leading to a decrease in the u/s index.

The consequences of the change of the lipid profile can be very important to the cell. An increase in the u/s index does not seem to be closely related with cell survival, but the increase of the proportion of esterified saturated fatty acids strongly correlated with lost of cell viability (Castro *et al.*, 1995). Saturated fatty acids pack well into a paracrystalline array, unlike unsaturated and branched side chains that do not stack as tightly. The consequence of an increase in the proportion of the saturated fatty acids, is an increase in the phase transition temperature of the membrane, presumably due to a change of the membrane lipids from a liquid to a more crystalline phase (Watson *et al.*, 1973), consequently decreasing membrane fluidity (Bruch and Thayer, 1983).

A decrease in the ATPase activity was also observed during the study probably as a consequence of alterations in lipid composition as has previously been found by incubation of inner mitochondrial membranes with ascorbate or cysteine (Santiago *et al.*, 1973). The attack of free radicals on the fatty acid moiety of phospholipids causes a lowering of the hydrophobicity owing to the introduction of hydrophilic groups, weakening the hydrophobic interactions with catalytic proteins, which are essential for their activity (Wiesner *et al.*, 1981). Aditionally the direct interaction of lipid peroxides and their degradation products with the enzyme, could also be responsible for its degradation (Cheftel *et al.*, 1976).

Biological membranes are predominantly held together by noncovalent bonds, which make them highly impermeable to small ions (In't Veld *et al.*, 1992). A lowering of the hydrophobicity of membrane phospholipids owing to the introduction of hydrophilic groups and a decrease in

ATPase activity might partially explain why upon storage a progressive reduction in the activity of freeze-dried lactic acid bacteria is observed (El- Sadek *et al.*, 1975, Alaeddinoglu *et al.*, 1989). According with Malis and Bonventre (1986), oxidation in mitochondria is responsible for an increase in the permeability of the cell membrane to protons. It is possible that in *L. bulgaricus* a collapse of the pH gradient across the cell membrane occurs, as a consequence of the increase in the net conductance to H^+ (or OH^-) aggravated by a defficient mechanism of proton extrusion (Maloney, 1990). This process may lead to the death of the cell by energy failure.

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