Influence of Bile on β-Galactosidase Activity and Cell Viability of *Lactobacillus reuteri* when Subjected to Freeze-Drying

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ABSTRACT

The effect of bile on β-galactosidase activity and cell viability was investigated using two strains of *Lactobacillus reuteri* that were subjected to freeze-drying. In the presence of 0.15% oxgall, β-galactosidase activity of the whole cells was significantly increased. After lyophilization, the cultures that had been treated with oxgall showed a low survival rate without changes in β-galactosidase activity. The poor resistance of the cells to damage from freeze-drying might be related to the presence of membranous structures containing simple folds and buds of the cell membrane, as was observed by transmission electron microscopy.
(**Key words**: lactic acid bacteria, bile, β-galactosidase, freeze-drying)

Abbreviation key: **β-GAL** = β-galactosidase, **MRSL** = MRS broth containing 2% lactose instead of glucose, **MRSLO** = MRSL plus 0.15% oxgall.

INTRODUCTION

Lactose maldigestion, caused by reduced lactase activity in the intestine, produces abdominal cramping, diarrhea, and flatulence in most populations (14). These symptoms cause many people to avoid the consumption of milk, which serves as a major calcium source in the diet of western nations.

An approach to improve lactose malabsorption relies on the consumption of viable lactic acid bacteria in fermented milks. To remain viable, the potentially probiotic bacteria should be able to resist stress conditions such as bile salts. In fact, the tolerance to bile salts is a very important criterion for selecting probiotic cultures, which have to survive in the intestinal tract. Another criterion might be the ability of the cells to survive freeze-drying, a process commonly used for culture conservation and for the production of concentrated starter cultures. In this regard, Kole and Altossar (9), working with *Leuconostoc oenos*, found a close relationship between the resistance of the cells to damage from freeze-drying and the tolerance to bile salts. Those authors (9) claimed that bile-resistant variants might be suitable for obtaining high yields of viable cells for use as freeze-dried starters.

This work evaluated the effect of bile on the β-galactosidase (**β-GAL**) activity and the survival rate of *Lactobacillus reuteri* that had been subjected to freeze-drying. This microorganism has been proposed as a dietary adjunct because of its beneficial properties on the health of the gastrointestinal tract (16).

MATERIALS AND METHODS

Media and Growth Conditions

The strains of *L. reuteri* (CRL 1098 and 1100) that were used in this study were obtained from the culture collection of Centro de Referencia para Lactobacilos (CERELA, Tucumán, Argentina). The growth medium was MRS (3) broth containing 2% lactose (**MRSL**) instead of glucose. The cultures were subcultured at least three times just prior to experimental use. Bacterial growth in MRSL broth and **MRSLO** broth [MRSL supplemented with 0.15% oxgall (Difco Laboratories, Detroit, MI)] was determined by measuring the absorbance at 560 nm with a Spectronic 20 (Bausch & Lomb, Rochester, NY) spectrophotometer.

Effect of Bile

Freeze-drying. Bacterial cells that had been grown in MRSL were harvested after an 18-h incubation period at 37°C, washed twice with 0.05 *M* phosphate buffer (pH 7.0), and resuspended in buffer to make the total volume equal to 0.2% of the original.

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TABLE 1. Effect of oxgall on β-galactosidase (β-GAL)[1] activity of whole cells of *Lactobacillus reuteri* CRL 1098 and CRL 1100.

Oxgall content	β-GAL Activity	
	CRL 1098	CRL 1100
(%)	(μmol/ml per 10 min)	
0.0	1.5	1.46
0.15	5.12*	5.2*

[1]Values are expressed as micromoles of *o*-nitrophenol released per milliliter of cell suspension per 10 min of incubation at 37°C.

*Significantly different from control ($P < 0.001$).

An aliquot of the cell suspension was mixed in a ratio of 1:1 (vol/vol) with each of the following suspending media: M1 was distilled water (control); M2 was 10% sodium glutamate; M3 was 0.30% oxgall, and M4 was 0.30% oxgall plus 10% sodium glutamate. The final concentration of glutamate and oxgall in the suspension media was 5 and 0.15%, respectively. In a second trial, *L. reuteri* was cultured in MRSLO and then freeze-dried using M1 and M2 as suspension media. Aliquots (0.3 ml) of each bacterial suspension were placed into a series of sterile ampules, frozen overnight at –20°C, and dried under vacuum (6.67 Pa) for 8 h in a chamber-type freeze-drier (Lyovac GT2; Leybold, Köln, Germany) which yielded products with <1% residual moisture. After the freeze-drying cycle had been completed, the ampules were sealed by fusion under vacuum, and the number of viable cells was determined. Each sample of the freeze-dried bacteria was rehydrated to its original fluid volume with distilled water. Appropriate dilutions were pour plated in MRSL agar; the plates were incubated at 37°C for 48 h, and the resulting colonies taken from samples before and after freeze-drying were counted. All samples were processed in duplicate.

β-***GAL Activity.*** Harvested cells from cultures in MRSL were washed twice with 0.05 *M* phosphate buffer (pH 7.0) and resuspended in the same buffer to yield an absorbance of 0.80. To investigate the effect of bile, washed cells were resuspended in buffer containing oxgall at the final concentration of 0.15% for 20 min. The β-GAL activity was determined according to the method of Noh and Gilliland (11) by using *o*-nitrophenyl-β-D-galactopyranoside (Sigma Chemical Co., St. Louis, MO) as the substrate. Activity was expressed as micromoles of *o*-nitrophenol released per milliliter of cell suspension per 10 min of incubation. To determine the β-GAL activity in freeze-dried cells, samples were rehydrated to their original volume with phosphate buffer and then permeabilized by the addition of 100 μl of toluene and acetone (1:9, vol/vol) per milliliter of cell suspension under agitation for 5 min before the assays.

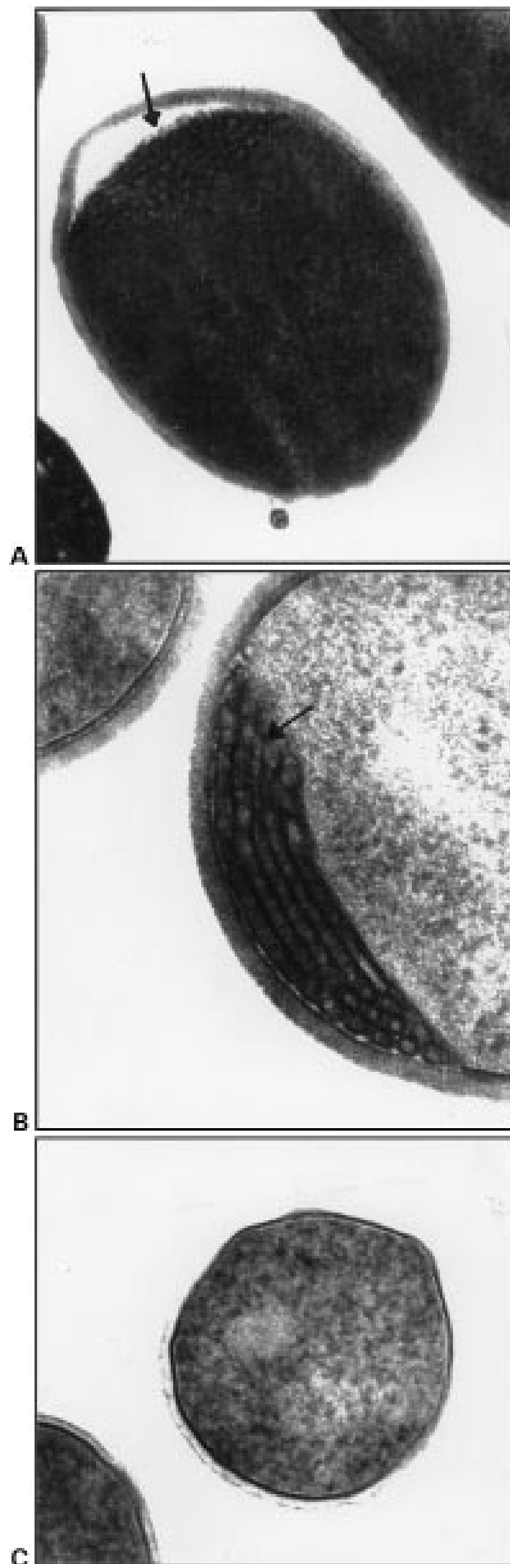

Figure 1. Transmission electron microscopy examination of cells of *Lactobacillus reuteri* CRL 1098 after 20 min of oxgall treatment (A, B) in phosphate buffer, pH 7.0, and untreated (C) in phosphate buffer, pH 7.0. Magnification: A) ×82,640, B) ×140,600, and C) ×82,640.

Transmission Electron Microscopy

Cells from cultures grown in MRSL and MRSLO at the early stationary phase were harvested by centrifugation at 9000 × *g* for 15 min, washed once with 0.1 *M* sodium phosphate buffer, pH 7.4, and resuspended (1:1, vol/vol) in the same buffer containing 2.5% (vol/vol) glutaraldehyde and 1% (wt/vol) $CaCl_2$. Samples were kept overnight at 4°C and were embedded in agar. A second fixation was in 1% OsO_4 and uranile acetate (1:1, vol/vol) for 2 h at room temperature (20°C), followed by dehydration in ethanol series and embedding in durcupan. Staining was performed according to methods described by Reynolds (12). Thin sections were examined in an electron microscope (EM 109 Zeiss; Zeiss, Köln, Germany).

Reproducibility

All results presented in this paper are expressed as the mean of three assays.

RESULTS AND DISCUSSION

The presence of oxgall in the culture medium had no effect on the specific growth rate (determined by the slope of a semilogarithmic plot of absorbance at 560 nm vs. time) of *L. reuteri* CRL 1098 and CRL 1100 ($\mu = 0.8\ h^{-1}$). However, differences in the β-GAL activity, as evaluated in a buffer system, were observed. As shown in Table 1, enzyme activity was over 3-fold higher with 0.15% oxgall for *L. reuteri* CRL 1098 and CRL 110 than was the enzyme activity of the untreated cultures.

The cells that were treated with oxgall maintained their viability, and the colony counts of the cell suspensions in buffer remained essentially the same (data not shown). Therefore, the increase in enzyme activity that we found could be attributed to a detergent effect of the bile salts upon the cell membrane, thus resulting in enhanced cell permeability and more substrate entering the cell. These increased levels of β-GAL activity might explain the beneficial effect of fermented milks in cases of lactose intolerance (8, 10). Similar effects were reported for fermented products containing species that were not bile resistant, such as *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*.

To determine whether the increase in permeability was related to changes in the cellular ultrastructure, *L. reuteri* CRL 1098 and CRL 1100 were treated with 0.15% oxgall or were left untreated (control) and examined by transmission electron microscopy. Figure 1A shows the partial detachment of the membrane from the cell wall and the expulsion of mesosome-like elements into the space between the wall and the contracted cytoplasm. These membranous structures presented a flat and disorganized distribution. They contained relatively simple folds and buds of the membrane and were surrounded by a single dense layer with a faint layer on the cytoplasmic side, filling a narrow space between the cytoplasmic membrane and the cell wall (Figure 1B). In some cases, the wall layer was loosely associated with the cell. These mesosome-like structures were not visible in untreated cells of *L. reuteri* (Figure 1C). The presence of such folds and evaginations of the cell membrane were associated with secretory functions in *Bacillus subtilis* (4, 15). In *Lactobacillus planta-*

TABLE 2. Effect of bile on the β-galactosidase (β-GAL) activity[1] and the viability of freeze-dried *Lctobacillus reuteri* CRL 1098 and CRL 1100.

Suspension medium[2]	CRL 1098		CRL 1100	
	Viability	β-GAL	Viability	β-GAL
	(cfu/ml)	(μmol/ml per 10 min)	(cfu/ml)	(μmol/ml per 10 min)
M1	$1.2 \times 10^{9\,b}$	5.1	$5.0 \times 10^{8\,c}$	5.0
M2	$2.4 \times 10^{10\,a}$	5.1*	$2.3 \times 10^{10\,a}$	5.2*
M3	$1.0 \times 10^{8\,c}$	5.0*	$2.5 \times 10^{8\,c}$	5.2*
M4	$1.4 \times 10^{8\,c}$	4.9*	$1.3 \times 10^{10\,b}$	5.2*
M†	ND[3]	4.9*	ND	5.0*

[a,b,c]Means within a row with no common superscripts differ ($P < 0.01$).

[1]Values are expressed as the micromoles of *o*-nitrophenol released per milliliter of cell suspension per 10 min of incubation at 37°C. The viable count before freeze-drying was standardized to 3.3×10^{10} cfu/ml; the β-GAL activity was 5 μmol/ml per 10 min.

[2]Media: M1, distilled water (control); M2, 10% sodium glutamate; M3, 0.15% oxgall; M4, 0.15% oxgall plus 10% sodium glutamate; and M†, cells grown in MRS broth containing 2% lactose instead of glucose plus 0.15% oxgall and freeze-dried in M1 and M2.

[3]Not detected.

*Not significantly different from control ($P > 0.05$).

rum (7), these structures would be related with biosynthetic and biochemical functions.

Some authors (1, 2) indicate that the primary site of damage during freezing or freeze-drying would be the lipid fraction of the cell membrane. The detergent properties of the bile acids might result in the selection of cells with greater resistance to damage from freeze-drying (9). The effect of oxgall on *L. reuteri* CRL 1098 and CRL 1100 during freeze-drying is shown in Table 2. The CRL 1098 and CRL 1100 cultures showed a reduction of 1 to 2 log units in viable counts after lyophilization in M1, respectively, compared with the viable counts (3.3×10^{10} cfu/ml) before the process. The damage that was inflicted to the cells was partially overcome by the addition of the cryoprotector (glutamate) to the suspending medium M2.

When oxgall was present in the freezing medium (M3), cell viability of *L. reuteri* CRL 1098 decreased sharply (12-fold) after freeze-drying, and *L. reuteri* CRL 1100 showed only a 2-fold reduction in viable counts, compared with the results obtained in M1. The presence of glutamate in M4 was ineffective in overcoming the deleterious effect of the bile salts upon the viability of freeze-dried *L. reuteri* CRL 1098, but glutamate had a strong protective effect upon strain CRL 1100 (Table 2). Despite the loss in viability, the dried cells showed no changes in β-GAL activity.

In a second trial, cultures were grown in MRSLO broth and then freeze-dried in M1 and M2 suspension media. No viable cells were obtained after the process even in the presence of glutamate (Table 2). Interestingly, the β-GAL activity remained fairly unchanged in those freeze-dried cells that were not culturable. This phenomenon appears to reflect a mechanism for bacterial survival; that is, metabolic growth processes would continue in the absence of cell division under stressful environmental conditions (13). The nonculturable status of those cells indicates the necessity of using suitable plating media for recovering sublethally injured cells (17).

A lack of a relationship between cell viability and enzyme activity was also reported for some strains of *Lactobacillus acidophilus* during refrigerated storage (5) and for *Lactobacillus helveticus* that had been subjected to spray-drying at low outlet air temperatures (6).

The low survival rate after freeze-drying of the cells that had been treated with oxgall might be related to the structural changes (Figure 1) that destabilized the cells, rendering them more sensitive to injury from freeze-drying. From these results, bile resistance clearly was not always accompanied by enhanced viability, as has been reported previously by Kole and Altossar (9).

In conclusion, despite the positive effect of bile on β-GAL activity of whole cells, bile produced structural changes in the cell membrane that rendered the cells more sensitive to damage from freeze-drying. This phenomenon raises the question of whether those structural changes also take place in vivo and whether they play a role in the sensitivity of probiotic cells to the stress conditions prevailing in the gastrointestinal tract.

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REFERENCES

1 Breunan, M., B. Warusinail, M. Johnson, and B. Ray. 1986. Cellular damage in dried *Lactobacillus acidophilus*. J. Food Prot. 49:47.

2 Crowe, J. H., and L. M. Crowe. 1982. Induction of anhydrobiosis: membrane changes during drying. Cryobiology 19:317.

3 De Man, J. C., M. Rogosa, and M. E. Sharpe. 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 42:123.

4 Ghosh, B. K., M. G. Sargent, and J. O. Lampen. 1968. Morphological phenomena associated with penicillinase induction and secretion in *Bacillus licheniformis*. J. Bacteriol. 96:1314.

5 Gilliland, S. E., T. E. Staley, and L. J. Bush. 1984. Importance of bile tolerance of *Lactobacillus acidophilus* used as dietary adjunct. J. Dairy Sci. 67:3045.

6 Johnson, J.A.C., and M. R. Etzel. 1994. Properties of *Lactobacillus helveticus* CNRZ-32 attenuated by spray-drying, freeze-drying and freezing. J. Dairy Sci. 78:761.

7 Kakefuda, T., J. T. Holden, and N. M. Utech. 1967. Ultrastructure of the membrane system in *Lactobacillus plantarum*. J. Bacteriol. 93:472.

8 Kim, H. S., and S. E. Gilliland. 1983. *Lactobacillus acidophilus* as a dietary adjunct for milk to aid lactose digestion in humans. J. Dairy Sci. 66:959.

9 Kole, M., and I. Altossar. 1984. Bovine bile resistance increases *Leuconostoc oenos* 44.40 viability upon lyophilization. Appl. Environ. Microbiol. 47:1150.

10 Lin, M. Y., D. Savaiano, and S. Harlander. 1991. Influence of nonfermented dairy products containing bacterial starter cultures on lactose maldigestion in humans. J. Dairy Sci. 74:87.

11 Noh, D. O., and S. E. Gilliland. 1993. Influence of bile on cellular integrity and β-galactosidase activity of *Lactobacillus acidophilus*. J. Dairy Sci. 76:1253.

12 Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17: 208.

13 Roszak, D. B., and R. R. Colwell. 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. 51:365.

14 Sandine, W. E., and C. M. Daly. 1979. Milk intolerance. J. Food Prot. 42:435.

15 Sargent, M. G., B. K. Ghost, and J. O. Lampen. 1968. Localization of cell-bound penicillinase in *Bacillus licheniformis*. J. Bacteriol. 96:1329.

16 Speck, M. L., W. J. Dobrogosz, and I. Casas. 1993. *Lactobacillus reuteri* in food supplementation. Food Technol. 47(7):90, 92.

17 Valdez, G. F., G. S. Giori, A. P. Ruiz Holgado, and G. Oliver. 1986. Composition of the recovery medium and its influence on the survival rate of freeze-dried lactic acid bacteria. Milchwissenschaft 41:286.