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Application of fluorescent techniques to evaluate the survival of probiotic lactobacilli to bile acid

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Abstract

Purpose of work To apply a fluorescent dye as an alternative technique to evaluate the survival of potentially probiotic lactobacilli to bile acids (BA) as first step in the design of probiotic functional foods.

The use of lactic acid bacteria (LAB) in the functional food design depends on their ability to survive in the gastrointestinal tract where bile is an important natural barrier. Bile is mainly constituted by conjugated BA, which can be hydrolyzed to free BA and taurine or glycine. Changes in the transmembrane electrical potential ($\Delta\Psi$) of probiotic LAB strains due to the effect of conjugated and free BA were measured and showed that the majority of the tested LAB strains had greater sensibility to free BA than to their respective conjugated acids. Variations in the $\Delta\Psi$ of the microorganism correlated well with bacterial viability determined by standard plate count method. We therefore propose the DiSC₃-based

fluorescent technique as a rapid and effective method to evaluate the resistance of probiotic lactobacilli to bile as first step for strain selection to be included in functional foods.

Keywords Bile acid · *Lactobacillus* · Probiotic · Transmembrane electrical potential

Introduction

The development of functional foods containing lactic acid bacteria (LAB) with beneficial effect on human health requires the rational selection of LAB strains able to survive during the gastrointestinal passage. In the duodenal loop, probiotic LAB are challenged by toxic detergent-like compounds such as bile salts. Thus, the bile tolerance is an important criterion in the selection of LAB strains to be used as dietary supplements.

Bile is mainly constituted by conjugated bile acids (BA) whose hydrolysis releases free BA plus taurine or glycine. This phenomenon takes place due to the bile salt hydrolase (BSH) activity, an enzyme present exclusively in certain species of the intestinal microbiota (Begley et al. 2006; Jones et al. 2008). BA affect the membrane integrity of most Gram-positive and Gram-negative bacteria causing growth inhibition and even cell death (Kurdi et al. 2006) due to their deteritive character, which present a toxicity pattern similar to SDS and other similar detergents

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(Fernandez Murga et al. 2001; Begley et al. 2002; Noriega et al. 2004). The response to this effect depends on the strain as well as on the concentration and type of BA used.

Assessment of active or viable microorganisms under stress conditions is often difficult. Plate count technique is the standard method to measure the cellular viability based on the cell ability to reproduce and form colonies; however, long incubation times (2–14 days) are required to visualize results. Furthermore, the traditional plate count method cannot be applied for many species for which an adequate growth medium has not yet been defined (Kell et al. 1998) or for many bacteria that remain viable but non-cultivable as consequence of exposition to an environmental stress like, for instance, the presence of bile (Ben Amor et al. 2002; Mary et al. 2002). Alternative fluorescent methods are currently employed to determine bacterial viability but they often work for a limited subset of bacterial groups. In this context, the fluorescent nucleic acid binding dyes with two or more positive charges, such as propidium iodide (PI) TO-PRO-3 and Sytox Green, are among the compounds used as indicators of cell death (Joux and Lebaron 2000). These probes are currently used successfully; however, they could stain the cell if the membrane is completely damaged. The DiSC₃(5) probe could accumulate into the lipid bilayer of energized cells (whose membranes are still intact) resulting in the auto-quenching of the fluorescence. If the membrane is disrupted, the dye will be released into the medium, increasing the fluorescence. This allows monitor the kinetics of alteration, i.e. the process can be followed in real time.

Previous studies have evidenced significant differences in bile tolerance among strains of different *Lactobacillus* species (Mishra and Prasad 2005; Noriega et al. 2004). In this study, the cytoplasmic membrane depolarization effect of BA on seven probiotic LAB strains was determined using the membrane potential-sensitive dye DiSC₃(5) probe (Breewer and Abbe 2004). Our results show that the assessment of depolarization of the cytoplasmic membrane by BA correlates well with bacterial cell viability, measured assessed by plate count. For the first time, it is demonstrated the application of the DiSC₃-based fluorescent technique as a rapid and effective method to evaluate the resistance of probiotic lactobacilli to BA as first step for strain selection.

Materials and methods

Microorganisms and culture conditions

Lactobacillus (L.) reuteri CRL 1098, selected for its probiotic properties such as hypocholesterolemic effect (Taranto et al. 2000) and cobalamin production (Taranto et al. 2003), and the strains *L. reuteri* CRL 1100 and CRL 1101, *L. acidophilus* CRL 44 and CRL 1072, *L. casei* CRL 203, and *L. delbrueckii* subsp. *bulgaricus* CRL 494, able to stimulate the immune system (unpublished data), were obtained from the CERELA Culture Collection (San Miguel de Tucumán, Argentina). Cultures were grown in MRS broth at 37°C for 16 h before use.

Preparation of cell suspensions

Bacterial cells were inoculated in fresh MRS medium (1%, v/v) and incubated until mid-growth phase at 37°C. Then, cells were harvested at 14,600×g for 10 min, washed twice with 50 mM HEPES buffer (pH 7.2), resuspended in the same buffer to an OD₅₆₀ value of 0.5, and kept on ice until use.

Transmembrane electrical potential ($\Delta\Psi$) assays

Changes in the membrane potential of energized lactobacilli strains were measured with the fluorescent probe 3,3'-dipropylthiadicarbocyanine iodide [DiSC₃(5); Molecular Probes Products]. Stock solution was prepared in ethanol at 2 mM.

Each cell suspension (2 ml) was energized with the addition of glucose to 5 mM. Immediately, the fluorescent probe DiSC₃(5) was added at 2 μM. All assays were conducted with agitation at 37°C. The fluorescence intensity was monitored with a fluorimeter (Cary Eclypse Varian) with excitation and emission of 640 and 670 nm, respectively (slit widths of 5 nm). The conjugated [glycocholic acid (GCA), taurocholic acid (TCA), glycodeoxycholic acid (GDCA), and taurodeoxycholic acid (TDCA)], and free BA [cholic acid (CA) and deoxycholic acid (DCA)] (Sigma-Aldrich Inc.) were added at 0.5, 1, 1.5, 2, and 2.5 nM. At the end of each assay, Triton X-100 was added (2%, v/v) to completely dissolve the cell membrane. Data of dissipation percentage were calculated considering Triton dissipation as 100%.

Viability assays

An overnight culture of each strain was plated on MRS agar and MRS agar supplemented with 0, 0.5, 1, 1.5, 2 or 2.5 mM conjugated or deconjugated BA. The plates were incubated at 37°C for 48 h. The results were expressed as cell death percentage, which was calculated by using the following equation: $100 - (\log \text{c.f.u. A}/\log \text{c.f.u. B} \times 100)$, where A = treatment with BA and B = control.

Statistical analysis

All the assays were carried out in triplicate. Statistical analyses were performed by using Minitab 14 software (Pennsylvania, USA). Comparisons were accomplished by ANOVA general linear model followed by Tukey's post-hoc test and $P < 0.05$ was considered as differences statistically significant and interaction effects. Cellular viability data were assessed using an $8 \times 6 \times 4$ factorial design (microorganisms \times BA type \times BA concentration). Variance analysis of the data was performed.

Results

The effect of different concentrations (0.5–2.5 mM) of conjugated (GCA, TCA, GDCA and TDCA) and free (CA and DCA) BA on the viability and the transmembrane electrical potential ($\Delta\Psi$) of seven lactobacilli strains was evaluated by the traditional plate counting method and by a fluorescent technique based on the membrane potential-sensitive dye DiSC₃(5). In this latter method, the probe accumulates inside the intact lipid bilayer of energized cells, which after disruption of the cell membrane by using BA the dye is released into the medium increasing the fluorescence. According to this, a fluorescence increase indicates the dissipation of the $\Delta\Psi$. Thus, the maximum fluorescence corresponding to the total $\Delta\Psi$ dissipation (100%) was obtained by using Triton X-100.

The addition of cholic-conjugated acid to the *L. delbrueckii* subsp. *bulgaricus* CRL 494, *L. casei* CRL 203 and *L. acidophilus* CRL 1072 cells disrupted the cell membrane reaching a $\Delta\Psi$ dissipation percentage value of 31–34% in the presence of 2.5 mM of this compound (Table 2). The sensitivity to high GCA concentrations of the CRL 494, 203 and

1072 strains was also evidenced with the plate count method although the cell death percentage values obtained were between 14 and 16% (Table 1). *L. reuteri* CRL 1100 and CRL 1101 showed scarce cellular viability loss (cell death percentage $\leq 2\%$) with all the GCA concentrations assayed while an increment in the $\Delta\Psi$ dissipation percentage ($\geq 13\%$) was observed with BA concentration higher or similar to 2 mM (Tables 1, 2).

The presence of cholic-conjugated acid did not affect the cell viability of *L. reuteri* CRL 1098 and *L. acidophilus* CRL 44. Moreover, changes in the $\Delta\Psi$ of these strains were not observed indicating the absence of the cell membrane damage by the effect of GCA.

GDCA affected both the cell viability and $\Delta\Psi$ for the majority of the strains, greater effects being observed at high concentrations. *L. delbrueckii* subsp. *bulgaricus* CRL 494 was the most sensitive strain showing 67 and 74% of cell death and $\Delta\Psi$ dissipation, respectively, with the addition of 2.5 mM GDCA. The CRL 203, CRL 1072 and CRL 1101 strains were also affected by this BA showing $\Delta\Psi$ dissipation percentage values between 40 and 54% at the higher concentrations assayed. Even though the deleterious effect of the GDCA was also evidenced in the cellular viability loss of these strains, the obtained cell death percentage values were lower (38.4–46.1%) than those detected for the $\Delta\Psi$ dissipation (Table 1).

The cell viability of the CRL 1098 and CRL 44 strains was only affected with high GDCA concentrations (≥ 2 mM) displaying cell death percentage values lower than 13%. Similarly, the presence of 2.5 mM GDCA induced changes in the $\Delta\Psi$ with dissipation percentage values near to 20%.

Regarding the effect of free BA, CA affected the cell membrane integrity of the CRL 494, CRL 1100, CRL 1101, CRL 203 and CRL 1072 strains showing values of $\Delta\Psi$ dissipation percentages between 34 and 54%. The adverse effect of the CA on these strains was also evidenced by applying the standard plate count technique (cell death percentage $\geq 48\%$). *L. reuteri* CRL 1098 was not affected by this free BA showing no $\Delta\Psi$ dissipation and cell death percentages of 9% but only at the highest CA concentration. On the other hand, *L. acidophilus* CRL 44 was slightly affected by CA; the effect on this strain was only evidenced by a moderate increase in the $\Delta\Psi$ dissipation (15%).

Table 1 Effect of conjugated and free bile acids on the survival of probiotic strains

Bile acid	Strain						
	1,098	1,101	1,100	44	203	1,072	494
CA (mM)							
0.5	3.7 ± 0.1*	3.2 ± 0.7	1.3 ± 0.3	9.6 ± 0.4	2.2 ± 0.4	0.9 ± 0.7	10.5 ± 0.4
1	7.3 ± 1.1	33.5 ± 1.1	11.3 ± 0.5	8.9 ± 1.3	13.5 ± 1.1	3.5 ± 0.6	23.5 ± 1.1
1.5	8.3 ± 1.2	49.7 ± 0.8	19.1 ± 1.5	10.5 ± 0.8	12.4 ± 0.7	6.0 ± 0.8	43.5 ± 2.8
2	8.6 ± 0.6	52.2 ± 2.2	37.5 ± 1.6	14.3 ± 3.2	41.9 ± 1.5	17.2 ± 2.2	57.2 ± 2.2
2.5	8.9 ± 0.2	45.9 ± 3.2	39.0 ± 1.8	15.3 ± 1.9	49.2 ± 2.2	38.1 ± 1.8	58.1 ± 1.8
DCA							
0.5	4.7 ± 0.7	28.3 ± 1.9	18.3 ± 0.9	20.9 ± 2.4	13.4 ± 1.9	9.8 ± 1.2	17.8.5 ± 1.9
1	12.3 ± 1.3	33.6 ± 1.9	23.5 ± 1.6	29.9 ± 2.7	33.1 ± 1.8	17.3 ± 1.6	23.5 ± 1.1
1.5	28.8 ± 1.6	36.5 ± 1.7	30.1 ± 1.3	41.5 ± 1.6	43.5 ± 1.4	17.6 ± 1.9	66.8 ± 3.2
2	31.6 ± 2.6	46.2 ± 2.2	36.6 ± 242	44.7 ± 2.2	52.5 ± 3.2	37.6 ± 2.9	99.9 ± 0.1
2.5	41.9 ± 3.2	53.5 ± 2.4	39.8 ± 2.3	50.4 ± 2.3	77.8 ± 1.7	69.0 ± 2.8	99.9 ± 0.1
GCA							
0.5	0.9 ± 0.1	1.6 ± 0.6	1.2 ± 0.2	1.4 ± 0.3	7.2 ± 0.3	7.9 ± 0.7	4.1 ± 0.3
1	1.0 ± 0.8	1.5 ± 0.3	1.4 ± 0.3	1.3 ± 0.2	8.2 ± 0.6	8.5 ± 0.6	6.1 ± 1.1
1.5	1.1 ± 0.2	1.8 ± 0.8	1.3 ± 1.1	1.7 ± 0.2	8.1 ± 0.7	13.8 ± 1.1	6.6 ± 0.8
2	0.8 ± 0.1	1.6 ± 0.5	1.5 ± 0.2	1.5 ± 0.5	12.5 ± 1.2	13.2 ± 0.9	15.7 ± 1.6
2.5	0.9 ± 0.2	1.8 ± 0.	1.4 ± 0.5	3.4 ± 0.6	14.0 ± 1.1	14.3 ± 1.2	16.1 ± 0.8
GDCA							
0.5	9.6 ± 0.9	2.3 ± 0.5	1.4 ± 0.3	12.4 ± 0.8	13.7 ± 0.8	23.1 ± 1.1	37.1 ± 1.4
1	9.3 ± 2.1	3.5 ± 0.6	7.7 ± 0.9	9.9 ± 1.2	13.5 ± 1.1	22.6 ± 1.4	43.6 ± 1.9
1.5	10.9 ± 2.2	5.2 ± 0.6	9.6 ± 1.1	12.8 ± 0.9	22.4 ± 1.2	28.1 ± 1.2	57.3 ± 2.2
2	9.8 ± 1.6	25.3 ± 1.5	15.7 ± 1.2	12.3 ± 1.2	33.1 ± 1.3	31.2 ± 0.8	65.2 ± 2.3
2.5	12.6 ± 2.1	38.4 ± 1.2	18.2 ± 1.2	13.5 ± 1.1	46.1 ± 1.3	34.2 ± 1.5	67.1 ± 2.8

To determine the cell viability, a 16 h-old culture of each strain was plated on MRS agar and MRS agar supplemented with 0, 0.5, 1, 1.5, 2 or 2.5 mM conjugated and free bile acids. After incubation (37°C for 48 h) colonies were counted and results were expressed as cell death percentage (%) by using the following equation: 100 – (log c.f.u. A / log c.f.u. B × 100), where A = cell treatment with BA and B = Control

Strains: *L. reuteri* CRL 1098, 1100 and CRL 1101, *L. acidophilus* CRL 44 and CRL 1072, *L. casei* CRL 203, and *L. delbrueckii* subsp. *bulgaricus* CRL 494. Bile acids used: glycocholic acid (GCA), glycodeoxycholic acid (GDCA), cholic acid (CA) and deoxycholic acid (DCA)

* Values are means ± SD

The most harmful BA compound was clearly the DCA, which affected in the same extent both the cell survival and the $\Delta\Psi$ of the all the strains studied. However, the extent of the effect depended on the strain and on the DCA concentration. An imbalance in the cell membrane, reflected as an increase in the $\Delta\Psi$ dissipation (values $\geq 80\%$), was observed by the addition of DCA to the sensitive strains CRL 494, CRL 203 and CRL 1072 (Table 2). These results were comparable to those obtained with the standard plate count technique (Table 1). The remaining strains (CRL 1098, CRL

1101, CRL 1100 and CRL 44) were also affected by this compound reaching $\Delta\Psi$ dissipation percentage values between 59 and 65% with high (≥ 2 mM) free BA concentrations.

Discussion

Lactic acid bacteria strains that are candidates to be used as probiotics as well as those that are members of the human indigenous intestinal microbiota must

Table 2 Effect of conjugated on the transmembrane electrical potential ($\Delta\Psi$) of probiotic strains

Bile acid	$\Delta\Psi$ dissipation percentage (%)						
	Strain						
	1,098	1,101	1,100	44	203	1,072	494
CA (mM)							
0.5	0	4	0	0	10	9	12
1	0	17	13	0	22	15	25
1.5	0	30	22	0	35	22	31
2	0	40	31	12	43	30	39
2.5	0	45	35	17	51	34	54
DCA (mM)							
0.5	10	12	19	15	20	10	21
1	14	28	27	21	32	21	31
1.5	21	45	38	29	51	41	43
2	48	60	54	52	69	65	65
2.5	60	65	59	63	78	70	81
GCA (mM)							
0.5	0	0	0	0	10	8	12
1	0	4	0	0	14	11	13
1.5	0	9	0	0	18	15	17
2	0	13	10	3	22	19	21
2.5	0	17	15	7	35	31	36
GDCA (mM)							
0.5	0	4	0	0	15	10	18
1	0	15	5	0	21	16	25
1.5	4	23	12	12	28	22	34
2	11	35	19	17	35	48	61
2.5	19	42	35	23	40	54	74

To determine the $\Delta\Psi$ dissipation, washed cells (OD₅₆₀ of 0.5) were mixed with DiSC₃(5) probe (2 μ M) in Hepes buffer. Cells of different strains were energized with glucose (5 mM), and the respective BA were added separately at different concentrations (0.5, 1, 1.5, 2 or 2.5 mM). Triton X-100 was added at 2% (v/v) to completely dissolve the cell membrane to reach 100% dissipation (control). Strains: *L. reuteri* CRL 1098, 1100 and CRL 1101, *L. acidophilus* CRL 44 and CRL 1072, *L. casei* CRL 203, and *L. delbrueckii* subsp. *bulgaricus* CRL 494. Bile acids used: glycocholic acid (GCA), glycodeoxycholic acid (GDCA), cholic acid (CA) and deoxycholic acid (DCA).

The values shown are averages of the data from three independent experiments, carried out with different set of bacterial suspensions; standard deviation was $\leq 3\%$

cope with the deteritive effect of BA. These compounds exert an inhibitory effect on most of Gram-positive and Gram-negative strains (Succi et al. 2005; Šušković et al. 2000). As reported by other authors (Mishra and Prasad 2005; Noriega et al. 2004), we

found that the response to BA depends of the strain as well as on the concentration and type of acid used.

L. delbrueckii subsp. *bulgaricus* CRL 494 was the most sensitive strain whereas *L. reuteri* CRL 1098 and, to a lesser extent *L. acidophilus* CRL 44, showed a clear resistance to BA, being these two strains were sensitive to high DCA concentrations (≥ 2 mM). The inhibitory effect of conjugated and free BA would be due to their deteritive character, which causes changes in the $\Delta\Psi$ that affect the microorganism survival.

Guohong et al. (2010) suggested a relationship between capacities of lactobacilli to resist BA toxicity and their capacities to express BSH activity. Our results show that the resistant CRL 1098 (Taranto et al. 2000) and CRL 44 strains (unpublished data) display high levels of BSH activity (100% hydrolysis) but the moderately sensitive strains, CRL 1101 and 1100, shows important levels of activity too (85 and 95% hydrolysis, respectively). These observations suggest that the BSH would be an enzyme involved in the bile tolerance of the strains but another factor would be implicated in this aspect.

In conclusion, a rapid and sensitive method based on fluorescent techniques that evaluates the survival of probiotic lactobacilli to conjugated and free BA is proposed. This method is based on the assessment changes in the $\Delta\Psi$ during stress towards BA, using the membrane potential-sensitive dye DiSC₃(5). The efficacy and potentiality of this fluorescent dye to assess cell viability was demonstrated by the correlation of direct viable counts. The evaluation of $\Delta\Psi$ changes would allow distinguishing the effect of low concentrations of conjugated BA on the survival of moderately sensible strains to bile since these changes would not be detectable by plate counting. The proposed procedure allows to assess in a quickly, sensitive and single test a wide range of concentrations using small sample volumes, thereby reducing environmental contamination and reagent cost. In addition, it can be used as the main indicator of the physiologic state of the cytoplasmic membrane of the microorganisms (main target of BA), thereby giving an additional parameter in measuring cellular viability.

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