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Impact of bile salt adaptation of *Lactobacillus delbrueckii* subsp. *lactis* 200 on its interaction capacity with the gut

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Abstract

In a previous work, bile-salt-resistant derivatives were obtained from non-intestinal lactobacilli. The aim of this work was to investigate the impact of bile adaptation of *Lactobacillus delbrueckii* subsp. *lactis* 200 on morphology, surface properties, in vivo interaction capacity with the gut and ability to activate the gut immune response. Electron microscopy studies, growth kinetics in the presence of bovine and porcine bile, the capacity to deconjugate bile acids, hydrophobicity, autoaggregation and co-aggregation capacities were studied for the parental strain and its bile-resistant derivative in vitro. Additionally, survival in intestinal fluid, the interaction with the gut and the immunomodulating capacities were studied in mice. Bile salt adaptation conferred upon the adapted strain a higher capacity to withstand physiological concentrations of bile salts and greater survival capacity in intestinal fluid. However, bile salt exposure reduced cell hydrophobicity, autoaggregation and adhesion capacities, resulting in reduced persistence in the intestinal lumen and delayed capacity to activate the gut immune response. Insight into the effects of bile salts upon the interaction and immunomodulating capacity of lactobacilli with the gut is provided, relating in vitro and in vivo results.
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Keywords: Bile salts; Lactobacilli; Intestine; Autoaggregation; Adherence; Immune response

1. Introduction

The joint FAO/WHO working group defined probiotics as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). This definition does not distinguish the food or intestinal origin of the probiotic microorganism, but it highlights the fact that health effects must be demonstrated in vivo. In this sense, Haller et al. (2001) showed that functional properties of lactobacilli isolated from the intestine are also present in certain strains isolated from fermented foods. Additionally, similar immunostimulating properties were found in lactobacilli strains independently of their origin (Dogi and Perdigón, 2006; Vinderola et al., 2004). One of the characteristics suggested for the selection of potential probiotic strains is resistance to gastric acidity and bile salts (FAO/WHO, 2002). It is known that oral administration of fermented milks containing traditional starter bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) is able to induce numerous beneficial effects upon consumer health (Guarner et al., 2005; Meydani and Ha, 2000; Perdigón et al., 1988, 2000, 2001; Tejada-Simon et al., 1999). Some of these effects have also been achieved by oral administration of pure cultures of strains of those species. Many studies showed the beneficial effects of yogurt bacteria against certain intestinal disorders (de Moreno de LeBlanc and Perdigón, 2004; Fuller, 1991; Goldin, 1998; Guarner et al., 2005). Specifically, regarding the possible probiotic role of starter microorganisms such as *L. delbrueckii*, Perdigón et al. (1986) reported their ability to activate peritoneal macrophages, suggesting that these bacteria, on passing through the stomach, reach the intestine where they modulate the immune function. These observations were then confirmed by the same group (Perdigón et al., 1999) when it was reported that oral

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administration of a strain of this species induced an increase in the number of IgA-producing cells in the lamina propria of small intestine of mice. In a previous work, we reported on the isolation of bile-adapted strains from non-intestinal lactobacilli (Burns et al., 2008). The process of acquisition of permanent resistance to bile conferred upon strains the capacity to grow under physiological concentrations of bile, which might be a valuable and natural tool for increasing the survival of non-intestinal bacteria in the intestinal environment. Later on, the response to bile stress was characterized by a proteomic approach (Burns et al., 2010). In that study, it was observed that the increase in bile resistance of *L. delbrueckii* subsp. *lactis* 200 was counterbalanced by a decrease in capacity of adhesion to intestinal cell lines. The aim of the present study was to investigate the impact of bile adaptation of *L. delbrueckii* subsp. *lactis* 200 and its bile-adapted strain *L. delbrueckii* subsp. *lactis* 200+ on the morphology, surface properties, in vivo interaction capacity with the gut and the ability to activate the gut immune response.

2. Materials and methods

2.1. Bacterial strains

The microorganisms used in this study were the parental strain *L. delbrueckii* subsp. *lactis* 200 and its bile-resistant derivative *L. delbrueckii* subsp. *lactis* 200+, obtained and characterized in previous studies (Burns et al., 2008, 2010). Depending on the assay, overnight cultures of the parental strain were grown in MRS broth (Biokar, Beauvais, France) at 37 °C for 18 h, whereas *L. delbrueckii* subsp. *lactis* 200+ was grown in MRS broth with or without bovine bile salts (0.50% w/v) (B3883, Sigma Chemical Co., St. Louis, MO, USA).

2.2. Electron microscopy studies

The effect of bile salts on cell morphology was investigated using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). *L. delbrueckii* subsp. *lactis* 200 was grown in MRS broth and *L. delbrueckii* subsp. *lactis* 200+ was grown in MRS containing 0.50% (w/v) bovine bile salts. Overnight (18 h) cultures were harvested by centrifugation (8000 *g*, 10 min, 5 °C), resuspended in phosphate-buffered saline (PBS) solution (pH 7.4) and fixed with glutaraldehyde (according to the instructions of the laboratory incharge of SEM and TEM studies). Samples were sent (at 5 °C) to the Electron Microscopy Service of the Superior Institute for Biological Researches (INSIBIO, UNT-CONICET, Tucumán, Argentina) for SEM and TEM studies. The time between harvesting and fixation of cells and further processing of samples at INSIBIO was less than 18 h.

2.3. Growth kinetics in the presence of bovine and porcine bile salts

The ability of strains to grow in the presence of bovine (0.50% w/v) or porcine bile salts (0.15%, 0.30% and 0.50% (w/v), B8631, Sigma) was determined. MRS broth was inoculated (ca. 10^6 CFU/ml) with an overnight culture (washed twice with PBS) of each strain and incubated in a water bath at 37 °C. Cell growth was monitored periodically by measures of absorbance (Metrolab 1700 UV–Vis spectrophotometer, Buenos Aires, Argentina) at 560 nm. Cell viability was assessed by the colony count technique on MRS agar (37 °C, 72 h, aerobiosis).

2.4. Bile acid deconjugation

The capacity of the strains to deconjugate specific bile acids was determined according to the method of Taranto et al. (1995). The following bile acids/salts were added (0.50% w/v) to MRS agar before autoclaving: Sigma T4009: taurocholic acid; Sigma T0875: taurodeoxycholic acid; Sigma T6260: sodium taurochenodeoxycholate; Sigma G0759: sodium glycochenodeoxycholate; Sigma G2878: glycocholic acid hydrate and Sigma G7132: sodium glycocholate hydrate. Plates were prepared on the day of use and kept in the dark at 5 °C. Overnight cultures of *L. delbrueckii* subsp. *lactis* 200 grown in MRS broth and *L. delbrueckii* subsp. *lactis* 200+ grown in MRS-bovine bile (0.50% w/v) were streaked on the surface of the culture media containing individual bile salts. Plates were incubated anaerobically (anaerBox, bioMérieux) at 37 °C for 72 h in the dark. The commercial probiotic strains *Lactobacillus acidophilus* A9 and *L. acidophilus* CSL, kept in the INLAIN culture collection, were used as positive controls of deconjugating strains.

2.5. Hydrophobicity of cells grown in the presence of bile salts or temporarily exposed to bile salts (bile shock)

The bile-resistant derivative was grown overnight in MRS + 0.50% (w/v) bovine bile or in MRS + 0.15% (w/v) porcine bile, whereas the parental strain was grown in MRS broth. Cell hydrophobicity was assessed as described by Burns et al. (2008). To assess the effects of a temporal bile salt exposure (bile shock) on cell hydrophobicity, overnight cultures of both the strains were obtained in MRS broth, centrifuged and washed twice as described above. Cells were resuspended in MRS containing (or not) 0.50% (w/v) bovine bile or 0.15% (w/v) porcine bile. Cell suspensions were incubated at 37 °C for 30 min. Cells were recovered by centrifugation, washed twice with PBS and cell hydrophobicity was assessed as described by Burns et al. (2008).

2.6. Autoaggregation assay

Autoaggregation capacity was studied by direct observation of cell suspensions. The quick formation of cell aggregates (floccules), with a random movement, induced error signals in the spectrophotometer and then it was not possible to use spectrophotometric techniques. Overnight cultures were obtained in MRS broth for the parental strain and in MRS + 0.50% (w/v) bovine bile for the bile-resistant derivative. Cells were centrifuged (8000 *g*, 10 min, 5 °C), washed twice with PBS and resuspended in the same buffer to a concentration of ca. 10^8 CFU/ml. Cell suspensions were

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vigorously vortexed and allowed to stand at room temperature. Pictures were taken periodically over a 30 min period to monitor the degree of autoaggregation.

2.7. Co-aggregation with Salmonella enterica serovar Typhimurium

S. enterica serovar Typhimurium OMS-Ca, kept in the INLAIN culture collection, was used as model pathogen for the co-aggregation assay. *Salmonella* Typhimurium was grown and counted in tryptone soy (Britania, Buenos Aires, Argentina) broth and agar, respectively (37 °C, 24 h, aerobiosis). Cell suspensions from overnight cultures were prepared as described above in PBS (pH 7.4). *L. delbrueckii* subsp. *lactis* 200 and *Salmonella* Typhimurium were mixed in a CFU:CFU relation of 1:1 or 10:1 (lactobacilli/*Salmonella*) in a test tube (10 ml final volume). The cell suspension was vigorously vortexed and allowed to stand at 37 °C. Aliquots of the supernatant were obtained every 1 h over a 3 h period and cell counts of *Salmonella* Typhimurium were performed. Cell suspensions of pure cultures were used as controls.

2.8. Survival in mouse intestinal fluid

The intestinal fluid of conventional mice was obtained from control mice of the animal trial described below. Briefly, the small intestine was obtained and quickly placed on a cold plate containing PBS (5 °C). The intestine was flushed with 2 ml of sterile distilled water. The intestinal content was recovered, centrifuged (8000 *g*, 15 min, 4 °C) and filter-sterilized (0.45 and 0.22 μm, Millipore, San Pablo, Brazil). The intestinal fluid was fractioned and kept at −76 °C until used. Overnight cultures of *L. delbrueckii* subsp. *lactis* 200 in MRS broth and *L. delbrueckii* subsp. *lactis* 200+ in MRS broth containing 0.50% (w/v) of bovine bile salts were obtained and washed as described above. Cell suspensions were centrifuged (8000 *g*, 15 min, 4 °C) and resuspended in the intestinal fluid to a final cell concentration of 10^8 CFU/ml. Cell suspensions were kept at 37 °C in a water bath. Cell viability was monitored periodically by cell counts on MRS agar. The sterility of the intestinal fluid was also checked on routine culture media.

2.9. Animal trial

2.9.1. Animals

A total of 18 (used in Section 2.9.2) + 35 (used in Section 2.9.3) female BALB/c mice (19–21 g) were obtained from the vrandom bred (in-bred) colony of the Centro de Experimentaciones Biológicas y Bioterio, Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (Esperanza, Santa Fe). Animals were allowed to stand at the INLAIN animal facility for a week before starting the assay. Each experimental group (sampling point) consisted of 5 mice housed together in plastic cages and kept in a controlled environment at 21 ± 2 °C with humidity at 55 ± 2%, with a 12 h light/dark cycle. Mice were maintained and treated according to the guidelines of the National Institute of Health (NIH, USA). The experiments with animals were approved by the Ethical Committee for Animal Experimentation of the Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (Esperanza, Santa Fe).

2.9.2. Study of the interaction of lactobacilli with the gut

Fluorescein-isothiocyanate-labeled *L. delbrueckii* subsp. *lactis* 200 (grown in MRS broth) and *L. delbrueckii* subsp. *lactis* 200+ (grown in MRS-bovine bile) were orally administered to mice (a single dose of 10^8 cells/mouse) according to Vinderola et al. (2004) by gavage. Samples of the small intestine (the last four 3-cm sections of the distal small intestine) were obtained after 10, 30 and 60 min for tissue fixation (3 animals by sampling point), paraffin inclusion and observation of fluorescent cells on histological slices of the intestine. It was previously verified that fluorescent labeling did not change cell viability or hydrophobicity of the strains used. Images from all the tissues collected were observed by two independent operators in a blinded way.

2.9.3. Study of the immunomodulating capacity of the strains

Overnight cultures of *L. delbrueckii* subsp. *lactis* 200 grown in MRS broth and *L. delbrueckii* subsp. *lactis* 200+ grown in MRS-bovine bile (0.50% w/v) were obtained daily, washed twice with PBS and administered to mice by gavage (ca. 5×10^7 CFU/mouse/day) for 3, 6 or 10 consecutive days. The safety (translocation assay and examination of hematoxylin–eosin stained histological slices of the small intestine), the percentage of phagocytosis and the number of IgA+ cells in the small intestine lamina propria were studied according to Vinderola et al. (2005). All small intestine sections (three) obtained from all mice were examined.

2.10. Statistical analysis

Except for the animal trials (conducted once), all in vitro experiments were replicated at least twice. Data were analyzed using the one-way ANOVA procedure of SPSS software (SPSS Inc., Chicago, IL, USA). The differences between means were detected by Duncan's Multiple Range test. Data were considered significantly different when $P < 0.05$.

3. Results

3.1. In vitro characterization of bile salt adaptation

Scanning and transmission electron microscopy studies were carried out in order to study the effects of bile salt adaptation on cell morphology. No changes were observed between *L. delbrueckii* subsp. *lactis* 200 and its bile-resistant derivative when both strains were grown in MRS broth. However, the growth of *L. delbrueckii* subsp. *lactis* 200+ in the presence of 0.50% (w/v) of bovine bile salts induced elongation of bacterial cells and an irregular display of the cell wall and membrane, observed by TEM, when compared to the parental strain grown in MRS broth (pictures not shown).

Fig. 1 shows the growth kinetics of the parental strain and the bile-resistant derivative in the presence of bovine and porcine

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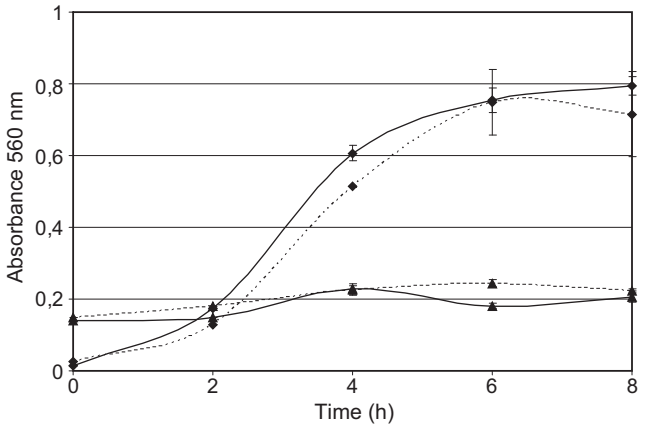

Fig. 1. Growth kinetics of *L. delbrueckii* subsp. *lactis* 200 (▲) and *L. delbrueckii* subsp. *lactis* 200+ (◆) in MRS broth containing 0.50% (w/v) bovine bile (straight line) and MRS broth containing 0.15% (w/v) porcine bile (dashed line).

bile salts. No growth was observed in the presence of 0.30% or 0.50% (w/v) of porcine bile salts, even for the bile-resistant derivative. The capacity of *L. delbrueckii* subsp. *lactis* 200+ to grow in the presence of 0.50% (w/v) bovine bile salts matched approximately with the growth in broth containing 0.15% (w/v) of porcine bile salts. Cell counts of *L. delbrueckii* subsp. *lactis* 200+ after 8 h of growth in the presence of 0.50% (w/v) bovine bile were 7.42 ± 0.15 log CFU/ml, whereas in the presence of 0.15% (w/v) porcine bile, cell counts were 7.33 ± 0.18 log CFU/ml.

Table 1 shows the effects of bile salt adaptation of *L. delbrueckii* subsp. *lactis* 200 on the capacity of growing and deconjugating individual bile acids/salts. Sodium glycochenodeoxycholate inhibited the growth of all strains assessed, even the two probiotic reference strains. Bile salt adaptation conferred upon *L. delbrueckii* subsp. *lactis* 200+ the capacity to grow in the presence of glycocholic acid, whereas previous growth in 0.50% (w/v) bovine bile additionally conferred upon the strain the capacity to grow in the presence of taurodeoxycholic acid and sodium taurochenodeoxycholate. However, bile salt adaptation did not confer upon the strain the capacity to deconjugate any of the individual bile acids/salts assessed. Only two individual bile acids (taurocholic and taurodeoxycholic acids) were deconjugated by the probiotic reference strains.

Not every orally given probiotic bacteria can multiply in the gut in the presence of bile salts along its gastrointestinal transit. Then, the effects of bovine and porcine bile on cell hydrophobicity (in cells grown in or temporarily exposed to

Table 1
Effect of bile salt adaptation on the deconjugation capacity of individual bile acids/salts.

Strain	Bile acid/salt					
	Taurocholic acid	Taurodeoxycholic acid	Na taurochenodeoxycholate	Na glycochenodeoxycholate	Glycocholic acid hydrate	Na glycocholate hydrate
L. lactis 200 grown in MRS	g-nd	ng	ng	ng	ng	g-nd
L. lactis 200+ grown in MRS	g-nd	ng	ng	ng	g-nd	g-nd
L. lactis 200+ grown in MRS + 0.50% bovine bile	g-nd	g-nd	g-nd	ng	g-nd	g-nd
L. acidophilus A9 grown in MRS	g-d	g-d	g-nd	ng	g-nd	g-nd
L. acidophilus CSL grown in MRS	g-d	g-d	g-nd	ng	g-nd	g-nd

ng: no bacterial growth (ng) on agar surface; g-nd: bacterial growth (g) but no bile salt deconjugation (nd); g-d: bacterial growth (g) and bile salt deconjugation (d).

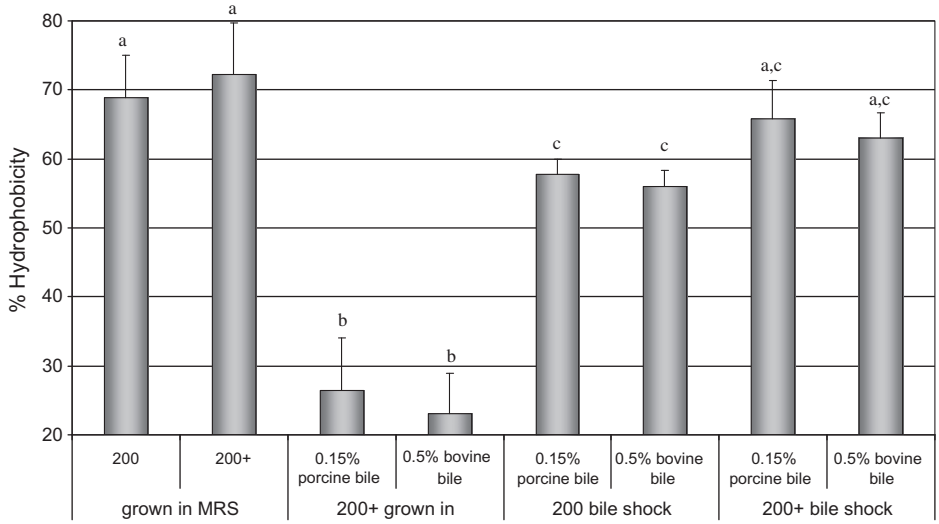

Fig. 2. Effects of growth in and exposure to (bile shock) porcine and bovine bile salts on the cell hydrophobicity of *L. delbrueckii* subsp. *lactis* 200 and its bile-resistant derivative *L. delbrueckii* subsp. *lactis* 200+. Columns with different superscript letters are significantly different ($P < 0.05$).

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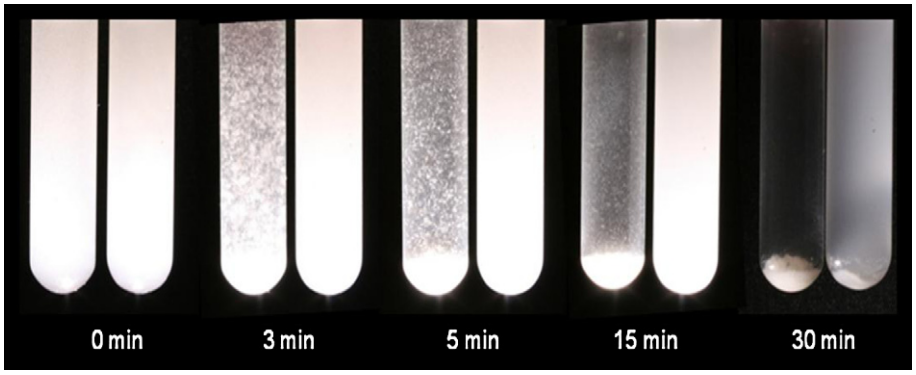

Fig. 3. Effects of bovine bile on the autoaggregation capacity of the bile-resistant derivative *L. delbrueckii* subsp. *lactis* 200+ (right tube of the pair at each time), compared to the parental strain *L. delbrueckii* subsp. *lactis* 200 (left tube of the pair at each time).

Table 2
Co-aggregation of *L. delbrueckii* subsp. *lactis* 200 and *Salmonella enteritidis*.

Salmonella/lactobacilli ratio	Cell count (log CFU/ml) of *Salmonella enterica* serovar Typhimurium in the supernatant at different times (h) of autoaggregation			
	0	1	2	3
1:0 (control)	8.15 ± 0.23[a]	7.97 ± 0.16[a]	7.79 ± 0.07[a]	7.85 ± 0.10[a]
1:1	8.08 ± 0.16[a]	7.82 ± 0.15[a]	7.91 ± 0.10[a]	8.04 ± 0.12[a]
1:10	8.22 ± 0.09[a]	8.01 ± 0.14[a]	7.89 ± 0.15[a]	8.11 ± 0.11[a]

Values in rows with the same superscript letters are not significantly different ($P > 0.05$).

bile salts, called bile shock in this study) were studied and are reported in Fig. 2. Both strains displayed high cell hydrophobicity (above 70%) when grown in the absence of bile salts, whereas growth of *L. delbrueckii* subsp. *lactis* 200+ in both porcine and bovine bile reduced the mean value of this parameter below 30%. The temporal exposure to bile salts (bile shock) significantly reduced the hydrophobicity of the parental strain, but had no effect on the hydrophobicity of the bile-resistant derivative.

The effects of bile salts upon the autoaggregation capacity of the strains are shown in Fig. 3. The autoaggregation capacity of *L. delbrueckii* subsp. *lactis* 200 did not differ from that of *L. delbrueckii* subsp. *lactis* 200+ when both strains were grown in MRS broth in the absence of bile salts (pictures not shown). The bile-resistant derivative grown in the presence of bile salts was evidently less autoaggregative, compared to the parental strain. In the co-aggregation assay with the enteropathogen *Salmonella* Typhimurium (Table 2), the pathogen and the parental strain *L. delbrueckii* subsp. *lactis* 200 were suspended at ratios of 1:0 (control), 1:1 and 1:10. Cell counts of the pathogen did not differ from that of the control (suspension of the pathogen alone) for the different co-aggregation times assessed, indicating that *L. delbrueckii* subsp. *lactis* 200 was not effective in co-aggregating with the pathogen for its elimination from the supernatant.

The survival of *L. delbrueckii* subsp. *lactis* 200 and *L. delbrueckii* subsp. *lactis* 200+ in mouse intestinal fluid was studied. Cell death up to 3 log cycles was observed for the parental strain after 24 h of incubation. A significantly higher survival capacity (0.5 log cycle) was observed for the bile-resistant derivative compared to the parental strain (Fig. 4).

3.2. In vivo characterization of the effects of bile salt adaptation on the interaction capacity with the gut

FITC-labeled bacteria were used to study the impact of bile salt adaptation on the interaction capacity of *L. delbrueckii* subsp. *lactis* 200 and *L. delbrueckii* subsp. *lactis* 200+ with the small intestine (Fig. 5). This technique enabled us to confirm that the higher in vitro autoaggregation capacity of *L. delbrueckii* subsp. *lactis* 200, compared to the bile-resistant derivative, was verified during the in vivo transit of the strain

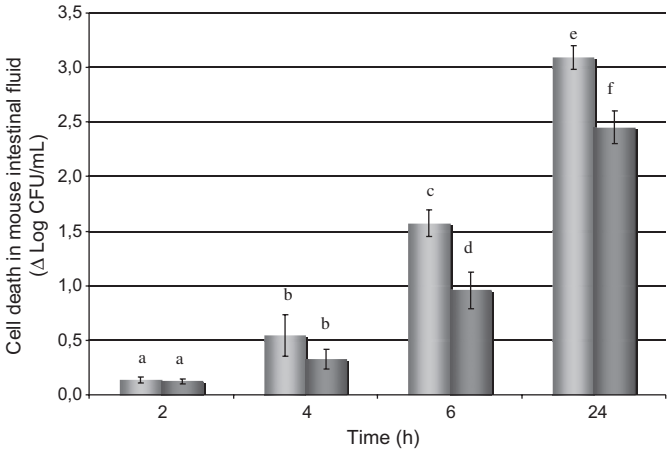

Fig. 4. Effects of bile salt adaptation on survival of cells suspended in mouse intestinal fluid. *L. delbrueckii* subsp. *lactis* 200 grown in MRS broth (▒), *L. delbrueckii* subsp. *lactis* 200+ grown in MRS broth containing 0.50% (w/v) of bovine bile salts (■). Columns with different superscript letters are significantly different ($P < 0.05$).

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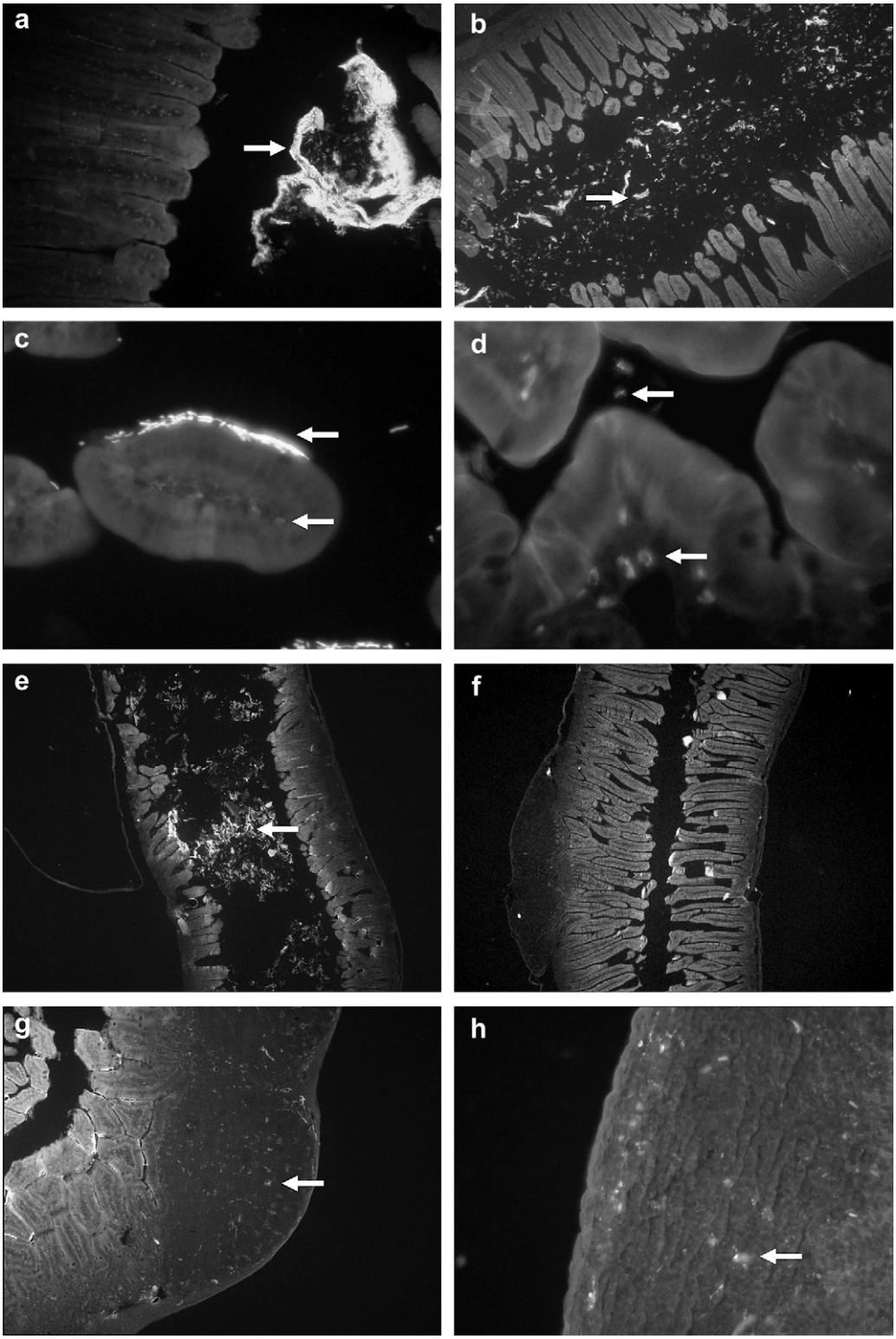

Fig. 5. Interaction with the small intestine of FITC-labeled *L. delbrueckii* subsp. *lactis* 200 (pictures on the left column) and its bile-resistant derivative *L. delbrueckii* subsp. *lactis* 200+ (pictures on the right column) after 10 min (a,b), 30 min (c,d) or 60 min (e–h) of their oral administration to mice. Magnification: 4× (b,e–g), 10× (a,h), 40× (c,d). Arrows indicate cell aggregates in the intestinal lumen (a,b,e), individual lactobacilli cells in the lumen (d), adhering to villi (c) or internalized into villi (c,d) or cell debris internalized in Peyer's patches (g,h).

through the intestinal lumen. After 10 min of oral administration, cell clusters or autoaggregates of the parental strain were observed in the gut lumen and also adhered to the gut epithelium (small intestine villi). At the same time, fluorescent cells of the bile-resistant derivative were observed in the intestinal lumen in cell autoaggregates of smaller size and lower numbers of cells were observed attached to villi compared to the parental strain. The lower autoaggregation

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capacity of the bile-resistant derivative observed in vitro was confirmed in vivo. After 30 min of intragastric administration of *L. delbrueckii* subsp. *lactis* 200, a higher quantity of cells was observed adhered to the intestinal epithelium compared to the bile-resistant derivative. Cell debris were also observed internalized in villi and in Peyer's patches, probably in contact with immune cells of the gut. Finally, after 60 min of gavage, fluorescent cells of *L. delbrueckii* subsp. *lactis* 200 were still observed, but were more dispersed along the lumen than at previous times. For the bile-resistant derivative, the lumen was observed to be almost devoid of fluorescent cells. For both strains, cell debris were observed inside Peyer's patches.

For the study of the capacity of strains to activate the gut immune response, mice received overnight cultures of the strains for 3, 6 or 10 consecutive days by gavage. The study of translocation of intestinal microbiota to liver was negative (no colonies developed on agar plates where liver homogenates had been plated), suggesting that the dose of bacteria used was safe. The study of the intestinal architecture by hematoxylin–eosin staining showed no lymphocyte infiltrates, edema or mucosal atrophy. No significant morphological changes in the overall architecture of the small intestine were observed in comparison with control mice (pictures not shown). As regards functional parameters studied (phagocytosis of peritoneal macrophages and number of IgA-producing cells in the small intestine lamina propria), it was observed that oral administration of the two strains did not modify the phagocytic activity of peritoneal macrophages (data not shown) when compared to the control value of the percentage of phagocytosis (10.5 ± 2.4%). The study of proliferation of IgA-producing cells in the small intestine lamina propria showed that the oral administration of the parental strain was more effective in enhancing the gut natural defenses; the number of IgA-producing cells augmented for all feeding periods assessed, reaching a maximum by the 10th day of feeding. The bile-resistant derivative was not effective in stimulating the gut response for the 3-day feeding period and the maximum reached in the response by day 10 was significantly smaller compared to that induced by the parental strain for the same feeding period (Fig. 6).

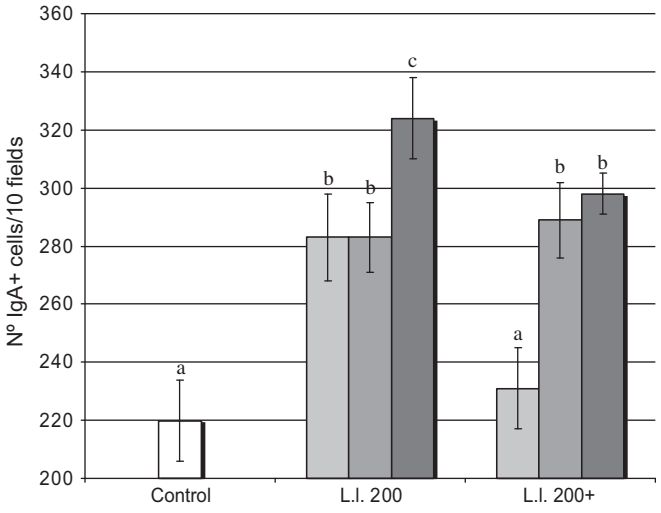

Fig. 6. Effects of oral administration of *L. delbrueckii* subsp. *lactis* 200 (L.l.200) and its bile-resistant derivative *L. delbrueckii* subsp. *lactis* 200+ (L.l.200+), for 3 (▒), 6 (▒) or 10 (■) consecutive days, on the number of IgA-producing cells of the small intestine lamina propria of mice. Columns with different superscript letters are significantly different ($P < 0.05$).

4. Discussion

Effects of bile salt adaptation of non-intestinal strain *L. delbrueckii* subsp. *lactis* 200 on cell morphology, surface properties, capacity of interaction with the small intestine and immune response were studied. SEM and TEM studies showed changes in cell size (elongation) and in the membrane and cell wall structure (shrunken, irregular display of the cell membrane); these morphological changes might anticipate differences in the comparative behavior of the two strains observed later on, since many cell properties, such as hydrophobicity, adhesion and antigenicity rely on structures found on the bacterial surface (Skurnik et al., 2010). Our results are in line with those of Taranto et al. (2006), who showed, using TEM, detachment of certain regions of the cell membrane from the cell wall in *Lactobacillus reuteri* CRL 1098 after exposure to bile acids. Moreover, it was reported that cells become shrunken after exposure to bile (Leverrier et al., 2003; Valdez et al., 1996), as observed in this work for the bile-resistant derivative. Growth kinetics in the presence of bovine or porcine bile salts confirmed that porcine bile salts possess greater inhibitory activity than bovine bile salts (Begley et al., 2005). Although porcine bile salts have a chemical composition closer to human bile salts than bovine bile salts (Begley et al., 2005), most studies of characterization of tolerance to bile are performed using bovine bile salts (Collado and Sanz, 2007; Lee et al., 2008; Oh et al., 2000). This should be taken into account for better in vitro characterization of tolerance to bile of potential probiotic strains. *L. delbrueckii* subsp. *lactis* 200+ was able to grow in the presence of certain individual bile acids, although the strain was not able to deconjugate any of them. Deconjugation of bile acids is a known detoxification mechanism of bile resistance in strains of intestinal origin (Begley et al., 2005), but had not yet been reported for food-derived probiotic strains.

Growth of *L. delbrueckii* subsp. *lactis* 200+ in the presence of porcine or bovine bile salts significantly reduced its cell hydrophobicity and autoaggregation capacity, possibly due to the detergent action of bile acids on cell membrane fatty acids (Begley et al., 2005). Cell hydrophobicity and autoaggregation capacity have been related to the adhesion capacity of the strain (Basson et al., 2007). They have also been related to the capacity of the strain to prevent the host from enteric infections by co-aggregating with intestinal pathogens for its removal from the lumen (Golowczyc et al., 2007) or by direct adhesion to the gut epithelium interfering with attachment of pathogens (Gopal et al., 2001). In this study, the parental strain presented a high autoaggregation capacity but was not able to coaggregate to *Salmonella* Typhimurium. Taking into account the adhesive capacity of the strain, the lack of co-aggregation capacity could be interpreted as a positive feature, since

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co-aggregation to a pathogen and simultaneous adhesion to mucosal surfaces could prolong the retention time of the pathogen close to sites of infection at the mucosal surfaces.

In a previous study (Burns et al., 2010), it was observed that bile salts reduced the adhesion capacity of *L. delbrueckii* subsp. *lactis* 200+ to the intestinal cell line HT29-MTX. Gueimonde et al. (2005) reported that bile salts reduced adhesion of bifidobacteria to intestinal mucus. It was hypothesized that production of one or several adhesion proteins might be downregulated under bile stress. In this sense, the proteomic approach previously used to study the impact of bile adaptation on protein expression (Burns et al., 2010) showed that expression of the protein elongation factor Tu was downregulated under bile stress. This protein was reported to be involved in cell-to-cell adhesion (Siciliano et al., 2008). The loss of adhesion capacity of *L. delbrueckii* subsp. *lactis* 200+ was verified in vivo in this study when fluorescent cells were used to study the interaction of the strain with the small intestine. The lower adhesion capacity of the bile-resistant derivative resulted in reduced persistence in the gut, since, after 60 min of oral administration, no fluorescent cells were observed in the lumen. However, this strain acquired a greater capacity to survive in the intestinal fluid. An autoaggregative strain of *Lactobacillus crispatus* showed higher persistence in the human intestine when compared to oral administration of its non-autoaggregating mutant (Cesena et al., 2001). The same pair of strains was used later in mice. In that study, greater capacity of the autoaggregating phenotype to increase expression of Toll-like receptor 2 and regulatory cytokines IL-10 and IL-6 was observed (Voltan et al., 2007). A proteomic approach applied to the same strains showed downregulation of the molecule elongation factor Tu in the mutant phenotype (Siciliano et al., 2008), in accordance with results of our proteomic approach (Burns et al., 2010). In relation to the capacity of the strains to promote health benefits for the host, positive effects were observed on the gut mucosa (increase in the number of IgA-producing cells), but not at distant sites (lack of capacity to enhance peritoneal macrophage phagocytic capacity). The capacity of strains of the species *L. delbrueckii* to promote innate gut defenses mediated by IgA was previously reported (Perdigón et al., 1999; Vinderola et al., 2007). The immunomodulating capacity of the strains was significantly modified by bile salt adaptation. The bile-resistant derivative was less effective for inducing an immune response, in relation to the feeding periods and the magnitude of the response achieved. Bile salt adaptation delayed induction of the immune response (from day 3 to day 6), probably due to the lower adhesion capacity and persistence of the bile-resistant derivative in the intestinal lumen. The higher survival capacity in the intestinal fluid might have partially restored the undesirable effects of bile salt adaptation, such as loss of hydrophobicity and autoaggregating and adhesion capacity. Adhesion to mucosal surfaces has been pointed out as a necessary but insufficient condition for achieving a probiotic effect (Bibiloni et al., 1999). A recent study showed that there is no direct relationship between adhesion and inmmunomodulating capacity, due to the many and sophisticated mechanisms that allow a microorganism to interact with the host and modulate the gut immune response (Kotzamanidis et al., 2010). Finally, this study underlines the importance of in vivo studies to support predictions made from in vitro results. Bile salt adaptation might have appeared to be an achievement without side-effects. However, in vivo studies showed undesirable modifications of the capacity of the bile-resistant derivative to interact with the gut (reduced persistence) and to induce an immune response (delay).

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