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Technological and probiotic characterisation of *Lactobacillus casei/paracasei* strains and their phage-resistant mutants

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ABSTRACT

Technological, biological and functional attributes of two groups of spontaneous bacteriophage-resistant mutants were investigated to verify features similar to those of their respective phage-sensitive parent probiotic strains (*Lactobacillus casei/paracasei*). A derivative from each group was challenged with phages in simulated probiotic product fermentation. Technological performance, compared with the original strains, was demonstrated. Minimal variations were detected in acidifying and enzymatic profiles. No β-glucuronidase activity was observed, but very high α-glucosidase and β-galactosidase activities were recorded. Low proteolytic activities and inability to ferment prebiotics were observed. Within one group, significant differences throughout simulated gastrointestinal-transit were observed. Neither of the strains deconjugated bile salts, but all showed inhibitory activity against four pathogens. Both mutants increased IgA-producing cell numbers in vivo in a manner similar to that of the parent strains. Two derivatives demonstrated improved phage-resistance phenotype compared with the parental strains, resulting in alternatives for industrial rotation schemes.

1. Introduction

According to the definition adopted by the joint FAO/WHO working group (FAO/WHO, 2002), "probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit on the host". The working group agreed that "lactobacilli have a long history of use as probiotics without established risk to humans, and this remains the best proof of their safety". Within the genera *Lactobacillus*, several *Lactobacillus casei* strains are proposed as probiotic. Their health claims are mainly related with immunomodulation of the immune system (Baken et al., 2006; Galdeano & Perdigon, 2006; Peluso et al., 2007; Perdigón, Vintiñi, Alvarez, Medina, & Medici, 1999; Ya et al., 2008), antimicrobial activity against pathogens (Merenstein et al., 2010; Servin, 2004; Verdenelli et al., 2009), and protection against diarrhoea in children (Agarwal & Bhasin, 2002; Guérin-Danan et al., 2001). The first commercially used (1935) and most studied *Lb. casei* strain is *Lb. casei* strain Shirota (LcS), which is used as a starter culture in the manufacture of the dairy drink Yakult. Recently, and after an exhaustive study, an expert panel has documented the intended use of LcS as generally recognised as safe (GRAS) based on scientific procedures (FDA, 2012).

Any biotechnology process that relies on the use of bacteria to produce a molecule or make a product can be disrupted by phage (Garneau & Moineau, 2011). Since the first *Lb. casei* phage isolation in 1965 (Séchaud, Cluzel, Rousseau, Baumgartner, & Accolas, 1988), use of *Lb. casei* as starter cultures for the manufacture of probiotic products has led to the emergence of phage infections severely affecting manufactures of high economic value (Capra, Binetti, Mercanti, Quiberoni, & Reinheimer, 2009; Capra, Quiberoni, Ackermann, Moineau, & Reinheimer, 2006; Séchaud et al., 1988; Watanabe, Takesue, Jin-Nai, & Yoshikawa, 1970). Though different strategies are available to cope with phage infections, the spectrum is reduced for probiotic bacteria (Capra, Mercanti, Rossetti, Reinheimer, & Quiberoni, 2011).

Isolation of spontaneous phage-resistant mutants from sensitive probiotic strains is convenient, simple and natural and it has no regulatory restrictions (Guglielmotti, Briggiler Marcó, Golowczyc, Reinheimer, & Quiberoni, 2007). In our previous work (Capra et al., 2011), ten bacteriophage-resistant mutants derived from two American Type Culture Collection (ATCC) strains (*Lb. casei* ATCC 27139 and *Lactobacillus paracasei* ATCC 27092) were isolated using

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virulent phage MLC-A and their phage-resistant phenotypes were characterised (Capra et al., 2006). Both original collection strains have been used as starters in the manufacture of a fermented milk beverage, and as a consequence their mutants were postulated as their putative substitutes in industrial rotation schemes. However, spontaneous mutations could also alter some other desirable features of the strains (Vinderola et al., 2007). Thus, the aim of this work is to investigate technological, biological and probiotic attributes of the resistant variants to assure they resemble those observed in the strains from which they were derived.

2. Materials and methods

2.1. Bacteria, bacteriophages and culture conditions

Host strains *Lb. paracasei* ATCC 27092 and *Lb. casei* ATCC 27139 (American Type Culture Collection, Manassas, VA, USA) and their mutants (092-b2, 092-b3, 092-sm1, 092-sm2, 092-sm3, 139-b1, 139-b2, 139-b3, 139-sm1, 139-Rsm2) obtained against lytic phage MLC-A (Capra et al., 2011) were used in this study. Strains were grown and routinely reactivated overnight (37 °C) in de Man, Rogosa and Sharpe (MRS) broth (Biokar, Beauvais, France). They were maintained as frozen (−80 °C) stocks in MRS broth in the presence of 15% (v/v) glycerol. Viable cell counts were done in MRS agar incubating 48 h at 37 °C. MLC-A phage particles were replicated in MRS broth with 10 mmol L^{-1} $CaCl_2$ and counted in MRS agar supplemented with 10 mmol L^{-1} $CaCl_2$ and 100 mmol L^{-1} glycine (MRS-Ca-Gly), on commercial *Lb. paracasei* A strain (Capra et al., 2006). Phage stocks were prepared as described by Neviani, Carminati, and Giraffa (1992) and stored at 4 °C (MRS broth) and −80 °C (MRS broth containing 15%, v/v, glycerol). Phage counts, expressed as plaque-forming units per millilitre (pfu mL^{-1}), were carried out by the double-layer plaque titration method (Svensson & Christiansson, 1991), incubating at 34 °C under microaerophilic conditions.

For studies on the inhibition of pathogens, *Salmonella* sp. (strain OMS-Ca), *Staphylococcus aureus* (strain 76) and *Escherichia coli* (strain V517) were grown in Nutrient Broth (Merck Química Argentina, Buenos Aires, Argentina), whilst *Listeria monocytogenes* (strain ATCC 15313) was grown in Brain Heart Infusion (BHI) broth (Britania, Buenos Aires, Argentina). All the strains were grown at 37 °C.

2.2. Technological characterisation of phage-resistant mutants

2.2.1. Growth in several media

Ability of each mutant and the sensitive strains (control) to grow in MRS broth, sterile reconstituted skim milk (RSM, Sancor C.U.L., Santa Fe, Argentina, 10%, v/v), RSM with added yeast extract (Britania, Buenos Aires, Argentina) and in a commercial enriched medium (BTC12, BIOTEC S.A., Buenos Aires, Argentina) was evaluated. BTC12 is a buffered milk based medium used for propagating both thermophilic and mesophilic lactic acid bacteria in the dairy industry. It was reconstituted (6%, w/v) with added glucose (1%, w/v) and pasteurised at 110 °C for 6 min. For each strain, three subsequent subcultures were carried out at 37 °C in MRS broth and the third subculture was used to inoculate (3%, v/v) every tested media. Incubations were done at 37 °C and, at predetermined times, aliquots were taken for pH measurements (SA 720 pH meter, Orion, Beverly, Massachusetts, USA). In the case of MRS broth, optic density measurements were done (λ = 560 nm, spectrophotometer Metrolab M1700, Buenos Aires, Argentina). Acidification kinetics were plotted as pH values versus time.

2.2.2. Acidifying activity

The best medium for the development of the strains was chosen by considering for selection the one that allowed the cultures to rapidly reach the lowest pH values. The acid-producing ability was studied by inoculating the medium with the third stationary subculture of each strain as described in Section 2.2.1. Incubations were carried out at 37 °C for 24 h. The acidity developed was measured at predetermined times during the experiment by titration with 0.1 mol L^{-1} NaOH to pH 8.4 and the results were expressed as Dornic degrees (°D).

2.2.3. Proteolytic activity

Proteolytic activity was determined by the *o*-phthaldialdehyde spectrophotometric assay (OPA test; Church, Swaisgood, Porter, & Catignain, 1983) and values were expressed as the difference in absorbance at 340 nm (A_{340}) between strain cultures and a control of uninoculated milk.

2.2.4. Culture performance during the simulated manufacture of a probiotic product in presence of phage particles

For each sensitive strain, one phage-resistant mutant was selected to carry out the fermentation of the chosen medium. Both the mutant and its control were grown in presence and absence of phage MLC-A to observe the effect of the infection in the evolution of the culture.

Aliquots of the medium were inoculated in parallel with a final concentration of 10^6 colony-forming units per millilitre (cfu mL^{-1}) of both strains (sensitive and resistant). Afterwards, both cultures were divided into two aliquots and phage MLC-A (final concentration of 10^3 pfu mL^{-1}) was added to one of them. Fermentation was carried out at 37 °C in a temperature controlled bath until pH values for non-infected cultures were stabilised. After finishing the elaboration, samples were taken out from the water bath and preserved at 12 ± 1 °C, a temperature usually found for cold storage in supermarkets in Argentina, to assess the refrigerated shelf-life of the product. Evolution of pH, viable cell counts and infective phage particles (if applicable) were determined during fermentation and at 8–10 day intervals over a one-month period of refrigerated storage. Results were plotted against time.

2.3. In vitro probiotic studies

2.3.1. Determination of enzyme profiles

Enzyme profiles of the two host strains and their resistant mutants were determined with the API ZYM system (bioMerieux, Marcy l'Etoile, France) according to supplier's instructions. All strains were grown in MRS broth to reach an optical density of 1.5 at 560 nm, centrifuged (13,100 × *g*, 10 min, 5 °C) and the pellets used to prepare a suspension with a turbidity of 5–6 McFarland in distilled sterile water. Subsequently, 65 μL of sample were added into each cupule of the API galleries, incubated at 37 °C and read after 4.5 h. The enzymatic activities were graded from 0 to 5 (i.e., 0 to ≥40 nmol of hydrolysed substrate) according to the intensity of the colour by means of the API ZYM colour reaction chart (3, 4 and 5 being considered as positive reactions). All tests were done in duplicate.

2.3.2. Simulated gastrointestinal survival of strains

Gastrointestinal resistance of all strains (sensitive and resistant) was assessed by simulating the conditions in vitro, according to Burns et al. (2011) modified as follows. An aliquot (40 mL) of an overnight culture in MRS broth was centrifuged (5,800 × *g*, 10 min, 5 °C) and washed twice with phosphate buffered saline, pH 7 (PBS) buffer (Sambrook, Fritsch, & Maniatis, 1989). Cells were suspended in 20 mL of RSM and mixed with the same volume of a simulated gastric juice (GJ) comprising $CaCl_2$ (0.22 g L^{-1}), NaCl (16.2 g L^{-1}), KCl (2.2 g L^{-1}), $NaHCO_3$ (1.2 g L^{-1}) and 0.6% (w/v) porcine pepsin (Sigma–Aldrich, St. Louis, MO, USA). A 100 μL sample was removed

for cell counts immediately after admixture and then, pH was quickly brought to pH 2.5 with 1 mol L^{-1} HCl. Samples were incubated 90 min at 37 °C in a water bath and at the end, 100 μL were taken for viable cell counts. Another aliquot (1.4 mL) was centrifuged (2,600 × *g*, 10 min, 5 °C), pellet was suspended in the same volume of simulated duodenal juice (DJ) comprising phosphate buffer (0.1 M, pH 8; Cicarelli, Buenos Aires, Argentina) with added bovine bile (1%, w/v, Sigma–Aldrich) and incubated for 10 min at 37 °C. Cell counts were carried out before centrifuging (4,050 × *g*, 10 min, 5 °C) and pellet was suspended in simulated ileal juice (IJ) comprising 1.3 mL of phosphate buffer with added bile (0.3%, w/v) and porcine pancreatin (0.1%, w/v; Sigma–Aldrich). After a 90 min-incubation at 37 °C, the last viable cell counts were done.

2.3.3. Utilisation of prebiotics

The ability of the sensitive strains and their mutants to ferment prebiotic carbohydrates was explored according to Zago et al. (2011), but with 2% strain inoculums. Seven prebiotics were investigated in this study: kefiran (kindly provided by Dr. Analía Abraham from Centro de Investigacion y Desarrollo en Criotecnologia de Alimentos-CONICET, La Plata, Argentina), xylitol and D + raffinose (Sigma–Aldrich), inulin and oligofructose P95 (Orafti, Tienen, Belgium), Litesse™ polydextrose (Danisco, Denmark), and Promitor™ soluble corn fibre (Tate & Lyle, London, UK).

2.3.4. Culture growth in presence of bile salts and bile salt deconjugation ability

Bile salts deconjugation was determined according to Taranto, Pesce de Ruiz Holgado, and Font de Valdez (1995). Sodium salts of taurocholic (TC) and taurodeoxycholic (TDC) acids (Sigma–Aldrich) were tested against each strain and streaks on MRS agar were used as growth controls. The presence of an opaque halo of precipitated bile acid around colonies indicated deconjugation of bile salts. A similar assay was carried out in presence of glycocholic acid, though in this case only the growth of the cultures was evaluated.

2.3.5. Hydrophobicity

Partition of bacterial cells between organic (*n*-hexadecane) and aqueous phases was used to estimate the cell surface hydrophobicity of the parent strains and their phage-resistant derivatives, by following the methodology described by Vinderola and Reinheimer (2003).

Cell surface hydrophobicity (*H*%) was calculated with the formulae: $H\% = [(OD_0 - OD)/OD_0]100$, where OD_0 and OD are the absorbance before and after extraction with n-hexadecane, respectively.

2.3.6. Pathogen inhibitory activity and nature of the inhibitory agent

The well-diffusion agar assay (Vinderola, Mocchiutti, & Reinheimer, 2002) was used to investigate the antimicrobial activity of the sensitive strains and their phage-resistant variants against the pathogens previously mentioned, by following the methodology described by Guglielmotti et al. (2007). Also the nature of the pathogen growth inhibition compound present in the supernatants of sensitive- and phage-resistant strains was investigated by carrying out several treatments; heating at 121 °C for 15 min, neutralisation with 1 mol L^{-1} NaOH and incubation with 100 μg mL^{-1} proteinase K (Life Technologies, Carlsbad, CA, USA) or 1 mg mL^{-1} pepsin (Merck) on the cell-free supernatants (CFSs). Subsequently, inhibition halo diameters were measured and recorded.

2.4. In vivo probiotic studies

2.4.1. Animals

Thirty six female BALB/c mice weighing 18–20 g were purchased from the random bred colony of the Centro de Experimentaciones Biológicas y Bioterio, Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (Esperanza, Argentina). Animals were allowed to acclimatise for one week before starting the experiments. Each experimental group consisted of 4 mice housed together in plastic cages and kept in a controlled environment at a temperature of 21 ± 2 °C with humidity at 5 ± 2%, with a 12 h light/dark cycle. Mice were maintained and treated according to the guidelines of the National Institute of Health (NIH, Bethesda, MD, USA).

2.4.2. Feeding procedures

All mice received, simultaneously and ad libitum, a sterile conventional balanced diet (Cooperación, Buenos Aires, Argentina) containing: 230 g kg^{-1} proteins, 60 g kg^{-1} raw fibre, 100 g kg^{-1} total minerals, 13 g kg^{-1} Ca, 8 g kg^{-1} P, 120 g kg^{-1} water, and vitamins. Different treatments were applied in the drinking water (RSM, 1% in non-sterile tap water), renewed daily, which was offered to the animals from 5 p.m. to 8 p.m. Then 5 groups were established: a control group that received drinking water without lactobacilli and 4 lactobacilli-treated groups, which were offered drinking water containing either *Lb. paracasei* ATCC 27092 and *Lb. casei* ATCC 27139 or their phage-resistant mutants for 5 consecutive days. Cell suspensions were prepared daily from overnight cultures of the strains under study at a concentration of approximately 3 × 10^7 cfu mL^{-1}.

2.4.3. Evaluation of safety and functionality

After each feeding period, animals were anesthetised and sacrificed by cervical dislocation as reported by Burns et al. (2012). A liver translocation assay was done to evaluate strain safety (Zacarías, Binetti, Laco, Reinheimer, & Vinderola, 2011). Both the small and the large intestines were removed for histological preparation and paraffin inclusion, according to Vinderola et al. (2005). To assess the number of IgA-producing (IgA+) cells in the intestinal lamina propria, an immunofluorescence test was carried out on histological slices of samples from the ileum, not considering Peyer's patches, and from the large intestine (Zacarías et al., 2011). Results were expressed as the mean number of positive (fluorescent) cells per 10 fields (magnification 100×) for each treatment after counting 60 fields for each animal (4 mice were included in each experimental group).

2.5. Statistics

Data were statistically analysed by means of one-way analysis of variance (ANOVA) tests using the SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). Tests were performed employing the strain as factor, with two categories: parental and derivative. Data were considered significantly different ($P < 0.05$) in variables between groups.

3. Results and discussion

3.1. Technological characterisation of phage-resistant mutants

3.1.1. Growth in several media

Resistant mutants and their parent strains were unable to grow adequately either in RSM or RSM with added yeast extract, as evidenced by pH values of 6.0 or higher after 24 h at 37 °C in the latter case or clot formation just after incubating for 6 d in milk. However, all mutants appropriately developed in MRS broth and

even better in the commercial medium BTC12. At 8 h of incubation, some derivatives seemed to be slightly slower than their corresponding original strains (092-sm1, 092-sm2 and 092-b3 for 27092; 139-sm1 and 139-Rsm2 for 27139); however, they reached similar pH values to those observed for their parental strains at the end of the experiment (24 h) (Fig. 1). Regarding both sensitive strains and their derivatives, pH values at 24 h were between 4.00 and 4.30 for MRS and around 3.75 for BTC12. Culture growth in broth was also monitored by OD measurements (data not shown) obtaining results in accordance with the acidifying kinetics: after 7 h of incubation curve slopes diminished, indicating minor acidification rates.

3.1.2. Acidifying activity

Considering results obtained in the previous assay, BTC12 medium was used for testing acidifying activities. No significant differences ($P = 0.179$) were observed when comparing the acidifying activity (24 h) of each mutant (114 ± 3 °D, $n = 5$) with that of its control original strain *Lb. paracasei* ATCC 27092 (112 ± 1 °D). On the other hand, *Lb. casei* ATCC 27139 exhibited an acidifying ability (124 ± 2 °D) slightly higher ($P < 0.001$) than those showed by its resistant derivatives (111 ± 2 °D, $n = 5$), differing the most with variant 139-b3 (107 ± 1 °C).

3.1.3. Proteolytic activity

Proteolytic activity was measured in BTC12 medium. Phage-resistant derivatives showed values comparable with those of the corresponding parent strains. All obtained values were very low (<0.070) evidencing a deficient proteolytic capacity. Only variant 139-Rsm2 gave a slightly higher value (0.142 ± 0.020), but still reflects poor proteolytic performance. Floros, Hatzikamari, Litopoulou-Tzanetaki, and Tzanetakis (2012) also observed low extracellular proteolytic activity on *Lb. paracasei* strains isolated from Greek cheeses.

3.1.4. Culture performance during the manufacture simulation of a probiotic product in presence of phage particles

Based on their similar ability to grow in BTC12 medium as their parental strains, on their survival through the gastrointestinal tract (GIT) and on their suitable phage-resistant phenotype, previously studied (Capra et al., 2011), one phage-resistant mutant for each sensitive strain was selected for the challenge of developing in presence of active phage particles. Culture evolution was monitored in BTC12 medium in presence and absence (control) of active phage MLC-A particles.

Variants 092-sm1 and 139-b2 were used for simulating the dairy fermentation process. Variant 092-sm1, infected or not, and its parent (*Lb. paracasei* ATCC 27092, used as a sensitive control) reached a pH value < 4 at 22 h-incubation. Unsurprisingly, the infected sensitive strain was only able to partially reduce the pH (almost at 5.5) at the same incubation time (Fig. 2A). After 7.5 h, cell counts were between 1.1×10^8 and 2.8×10^8 cfu mL^{-1} for the mutant and the uninfected sensitive strain. In phage presence, the latter replicated to about 5.0×10^7 cfu mL^{-1} after 3.5 h of incubation and the number of cfu was dramatically reduced to 2.0×10^5 cfu mL^{-1} after 7.5 h of incubation. In that case and in parallel, phage titre rose to 3.6×10^8 pfu mL^{-1} whereas a lesser amount (1.0×10^2 pfu mL^{-1}) than the initial inoculum was recovered from the infected mutant culture after 7.5 h of incubation. Thereafter, titres remained almost constant until the end of the experience (Fig. 2B).

Phage-resistant strain 139-b2 was not disturbed by phage MLC-A presence (Fig. 3A). While pH for the sensitive strain stopped at 5.7 (22.5 h), all the other cultures (the same strain without phage or resistant variant with or without phage) reached lower pH values (3.8, 4.4 and 4.5, respectively). At 7 h of incubation, all cell counts were higher than 10^8 cfu mL^{-1}, except for the sensitive strain (9.7×10^5 cfu mL^{-1}). At the same time, phage titres rose up to 2.7×10^7 pfu mL^{-1} when the phage successfully propagated on *Lb. casei* ATCC 27139, but only 10^2 pfu mL^{-1} were counted from the challenged resistant variant (139-b2) after 7 h of incubation (Fig. 3B).

Figs. 2A and 3A showed that until 5.5 h in the fermentation time, both resistant and sensitive strains shared identical behaviour. After that time, the sensitive cell counts dropped dramatically and the slopes of acidification curves tended to diminish, coinciding with phage titres (Figs. 2B and 3B) that exceeded 10^6 pfu mL^{-1} (1 $\log_{10}$ over the critical threshold estimated for phage concentrations; Emond & Moineau, 2007).

For both groups, pH values and cell viability of sensitive strains (uninfected) and resistant mutants (infected or not) remained unaffected after 21 d at refrigerated storage and cell counts were around 1×10^9 cfu mL^{-1} (Figs. 2C and 3C). Although in Argentina there is still no legislation about the minimal probiotic bacterial counts that should be present in fermented milks, there is an international trend considering that an adequate amount to provide health benefits should be 10^7–10^8 cfu mL^{-1} standing until the end

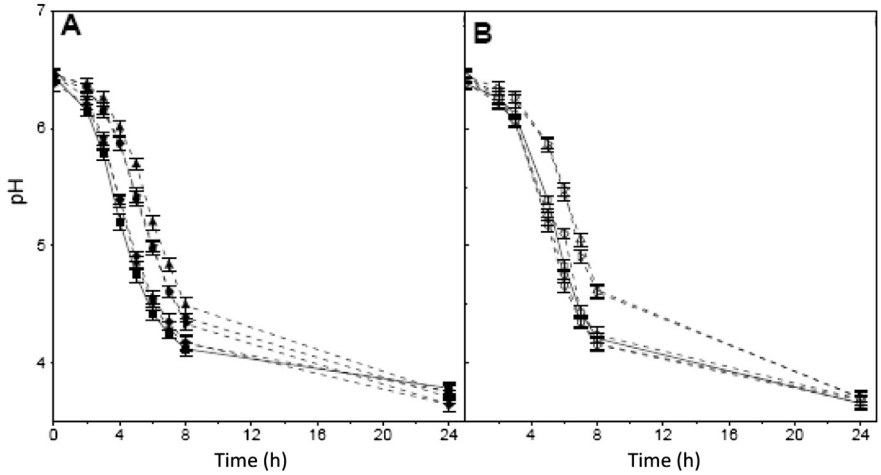

Fig. 1. Acidification kinetics in BTC12 medium of (A) *Lactobacillus paracasei* ATCC 27092 (■) and (B) *Lactobacillus casei* ATCC 27139 (□) and their respective phage-resistant mutants: ▼, 092-b2; ●, 092-b3; ◆, 092-sm1; ▲, 092-sm2; ▶, 092-sm3; ▽, 139-b1; ○, 139-b2; ◇, 139-b3; △, 139-sm1; ▷, 139-Rsm2. Error bars represent the standard deviation of three determinations.

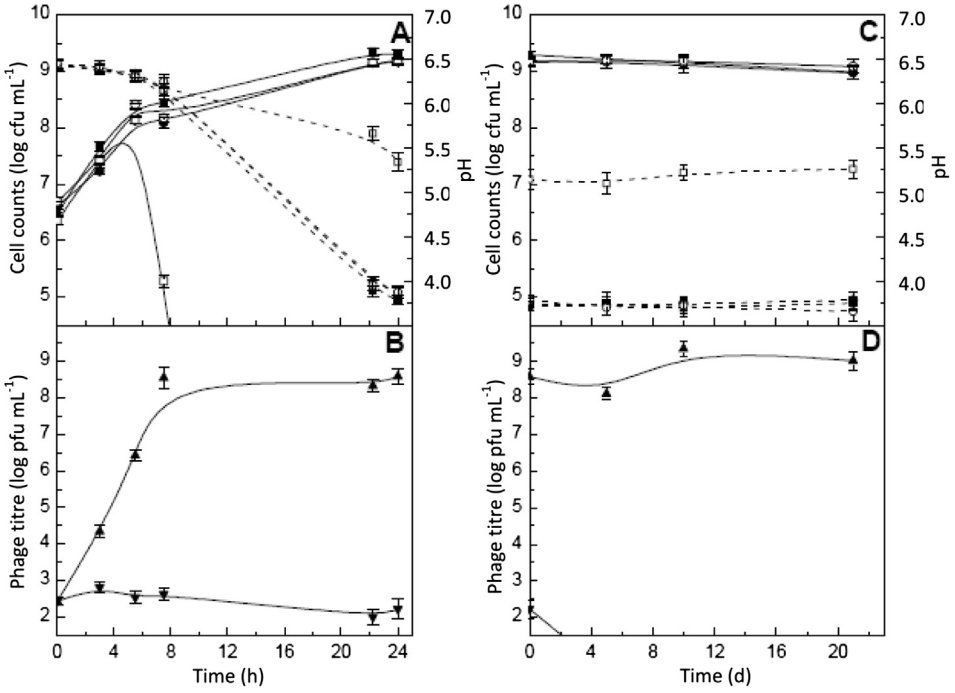

Fig. 2. Cell counts (solid lines) and pH (dashed lines) values during fermentation (A) and subsequent refrigerated storage (C) in BTC12 medium by using *Lactobacillus paracasei* ATCC 27092 (□,■) or its mutant 092-sm1 (○,●), infected (□,○) or not (■,●) with phage MLC-A, and titres of phage MLC-A during fermentation (B) and the subsequent refrigerated storage (D) of BTC12 medium by using *Lb. paracasei* ATCC 27092 (▲) or its mutant 092-sm1 (▼). Error bars represent the standard deviation of three determinations.

of the specified shelf-life (Vinderola et al., 2011). The ability of both selected mutants to carry out fermentation even in presence of phages evidenced their effective performance over passing the technological capacity of their parental strains. Similar results were obtained by Briggiler Marcó, Mercanti, Reinheimer, and Quiberoni (2011) for *Lactobacillus plantarum* spontaneous phage-resistant mutant obtained against a lytic phage cocktail. Although spontaneous phage-resistance mutants have been obtained for almost all species in the dairy industry, their use in rotational schemes is still under study in the case of probiotic strains (Briggiler Marcó,

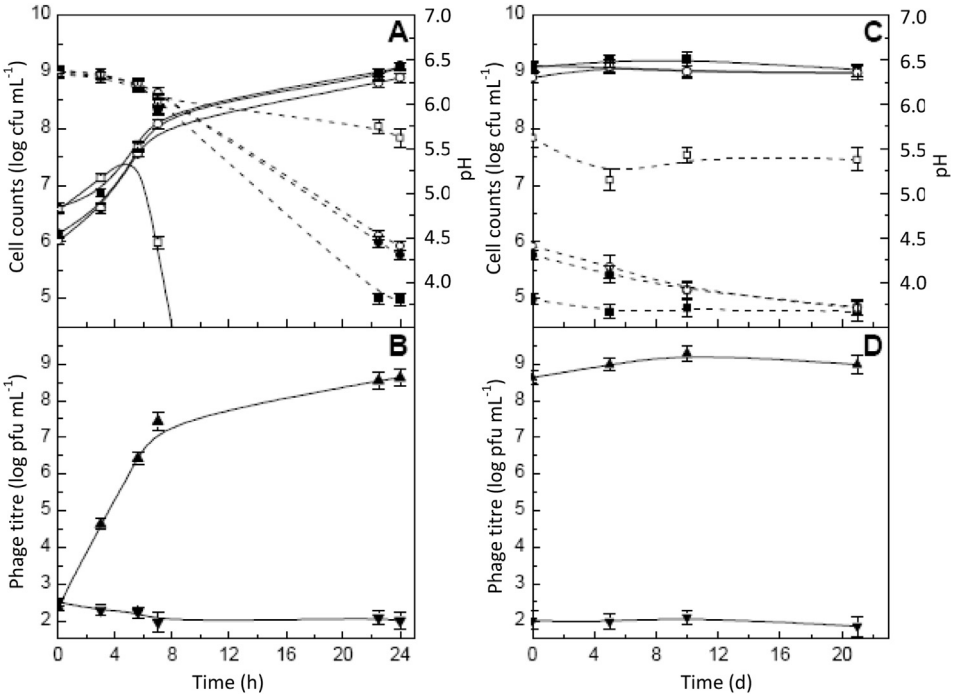

Fig. 3. Cell counts (solid lines) and pH (dashed lines) during fermentation (A) and subsequent refrigerated storage (C) in BTC12 medium by using *Lactobacillus casei* ATCC 27139 (□,■) or its mutant 139-b2 (○,●), infected (□,○) or not (■,●) with phage MLC-A, and titres of phage MLC-A during fermentation (B) and the subsequent refrigerated storage (D) of BTC12 medium by using *Lb. casei* ATCC 27139 (▲) or its mutant 139-b2 (▼) are also shown. Error bars represent the standard deviation of three determinations.

Moineau, & Quiberoni, 2012; Capra et al., 2009). It could be a valid alternative when proper functional studies guaranteed analogous features for parental and derivative strains.

3.2. In vitro probiotic studies

3.2.1. Determination of enzyme profiles

If the strains consumed with the functional food are able to survive the imposed biological hurdles, a large number of microorganisms will transit along the GIT, delivering their enzymes and other substances into the bowel (Djouzi, Andrieux, Degivry, Bouley, & Szylit, 1997).

Several enzyme activities (alkaline phosphatase, lipase (C14), trypsin, α-chymotrypsin, β-glucuronidase, α-mannosidase, α-fucosidase, α-galactosidase and N-acetyl-β-gucosaminidase) were not detected in any of the strains tested. Absence of β-glucuronidase and β-glucosidase activities deserves special note in view of the carcinogenic potential of these enzymes (De Preter et al., 2008; Nguyen, Kang, & Lee, 2007). Intestinal lactobacilli may manifest those enzymatic activities (Mroczyńska & Libudzisz, 2010); therefore it is worth studying their existence since a positive result is a negative feature from a probiotic point of view.

Table 1 lists only the enzymatic activities found in the parental strains and compares them with activities from their respective derivatives. No differences were recorded among enzymatic profiles for ATCC 27092 and their derivatives and only some minor differences were found for ATCC 27139 group.

Regarding aminopeptidases, all strains in both groups exhibited very high leu-arylamidase and val-arylamidase activities (>40 nmol hydrolysed substrate), while they showed a weak cys-arylamidase activity (<10 nmol, data not shown). Aminopeptidases can release amino acids that may contribute positively to the cheese flavour or, in some cases, can cause bitterness defects that should be avoided (Floros et al., 2012). Similar enzyme activities were observed by Floros et al. (2012) for *Lb. paracasei* (12) and *Lb. plantarum* (7) strains isolated from different types of Greek traditional cheeses.

Esterase activities of parental and derivative strains tested were weak in all cases. Only for esterase/lipase C8, a strong activity (20 nmol hydrolysed substrate) was found for the majority of the strains, except for a weak activity registered in 139-sm1 and 139-Rsm2 (ca. 10 nmol). Acid phosphatase activity was stronger for ATCC 27092 group than for ATCC 27139 group. For the latter, only 139-b1 (c.a. 33 nmol) differed from the rest of the group (23–28 nmol hydrolysed substrate). Phosphohydrolase was present in all strains tested and no differences were recorded among them. In relation to glycosylases, very strong activities were registered for α-glucosidase (both groups, without differences) and for β-galactosidase (139-b variants showing higher activities than its parental strain). The activity of α-glucosidase is important because the enzyme may improve fermentation of resistant starch, leading to butyrate production, improved bowel habits and increased stool output. The importance of β-galactosidase is well known and refers to an improvement in lactose digestion (Djouzi et al., 1997).

Table 1
Results of API Zym for *Lactobacillus paracasei* ATCC 27092 and *Lactobacillus casei* ATCC 27139 and their phage-resistant mutants.

Enzyme activity	Reaction level (VS/S/W)[a]			
	27092	092-Mutants[b]	27139	139-Mutants[b]
Esterase lipase (C8)	0/1/0	0/5/0	0/1/0	0/3/2
Leucine arylamidase	1/0/0	5/0/0	1/0/0	5/0/0
Valine arylamidase	1/0/0	5/0/0	1/0/0	5/0/0
Acid phosphatase	1/0/0	5/0/0	0/1/0	1/4/0
Phosphohydrolase	0/1/0	0/5/0	0/1/0	0/5/0
β-Galactosidase	1/0/0	5/0/0	0/1/0	3/2/0
α-Glucosidase	1/0/0	5/0/0	1/0/0	5/0/0

[a] VS, number of strains with very strong reaction (grades 4 and 5 according to colour chart: ≥30 nmol); *S*, number of strains with strong reaction (grades 3 to <4: 20 to <30 nmol); *W*, number of strains with weak reaction (grades 2 to <3: 10 to <20 nmol).
[b] Five isolates were tested.

3.2.2. Study of simulated gastric acid resistance

To be effective as functional strains, probiotics must overcome physical and chemical barriers imposed by the GIT (Del Piano et al., 2006) and reach the bowel in viable, active and abundant form. *Lb. casei* is both acid and bile resistant and remains viable at a pH range of 3.0–7.0. This is in agreement with its survival along the GIT shown by large number of viable cells found in ileum (to exert a physiologic effect) and in faeces (Djouzi et al., 1997; Fujimoto, Matsuki, Sasamoto, Tomii, & Watanabe, 2008; Goldin et al., 1992). Resistance in the GIT was assessed by simulating the biological barriers, with the aim of comparing derivative strains resistance against the ones of their parents.

Significant differences were found between some derivatives in comparison with *Lb. casei* ATCC 27139, while the other group behaved practically identically all through the transit simulation (Fig. 4) except for variant 092-sm3, which differed from the *Lb. paracasei* ATCC 27092 after the incubation in simulated ileum juice ($P = 0.006$). For the *Lb. casei* ATCC 27139 group only 139-b2 did not show any significant difference ($P > 0.05$) all through the experiment. All the other variants had lesser resistance to transit through the GIT than their parental strain. Therefore, 139-b2 was selected among 139-derivatives for the simulated probiotic manufacture as reported above. In all cases, the highest cell viability falls (around 4 log orders) were registered after the incubation with simulated gastric juice, with cell counts at the end of the simulated GIT conditions between 10^3 and 10^5 cfu mL^{-1} for both groups.

Similar results were obtained by Burns et al. (2011), though they considerably improved cell survival of a strain of *Lactobacillus delbrueckii* in gastric juice by using more concentrated RSM (20%, w/v). On the other hand, Vinderola et al. (2011) reported complete (8 log reduction) cell death after 90 min-incubation with GJ at pH 2.50 for a *Lb. casei* in a commercial fermented milk sample. Paéz et al. (2012) testing *Lb. casei*/*paracasei* strains in skim milk also observed the greatest reductions (6 log) after incubation in GJ. Corsetti et al. (2008) reported a remarkable recovery of viability even for *Lb. casei* strains that were very sensitive to acid stress (GJ) after the incubation in simulated intestinal fluid.

3.2.3. Utilisation of prebiotics

Neither the mutants nor the parental strains were able to ferment prebiotics efficiently (Table 2). The growth of both groups was slightly stimulated with polydextrose (<10% for ATCC 27092- and <7% for ATCC 27139 groups) and with soluble corn fibre (between 10–15% for ATCC 27092 and 12–20% for ATCC 27139 group), finding no significant differences ($P > 0.05$) among each parental strain and their derivatives. All other prebiotic tested were poorly fermented (≤3.1%) by all strains. A considerable growth was found in basic MRS broth (without glucose) consistent with the ability of *Lb. casei* to metabolise citrate (Weinrichter, Luginbühl, Rohm, & Jimeno, 2001).

3.2.4. Culture growth in presence of bile salts and bile salt deconjugation ability

The release of free bile salts through the deconjugation of conjugated bile salts in the small intestine results in the excretion of more bile salts in the faeces, being the major means by which cholesterol is removed from the body (Brashears, Gilliland, & Buck, 1998). Moreover, bile salt deconjugation may also be linked to bile

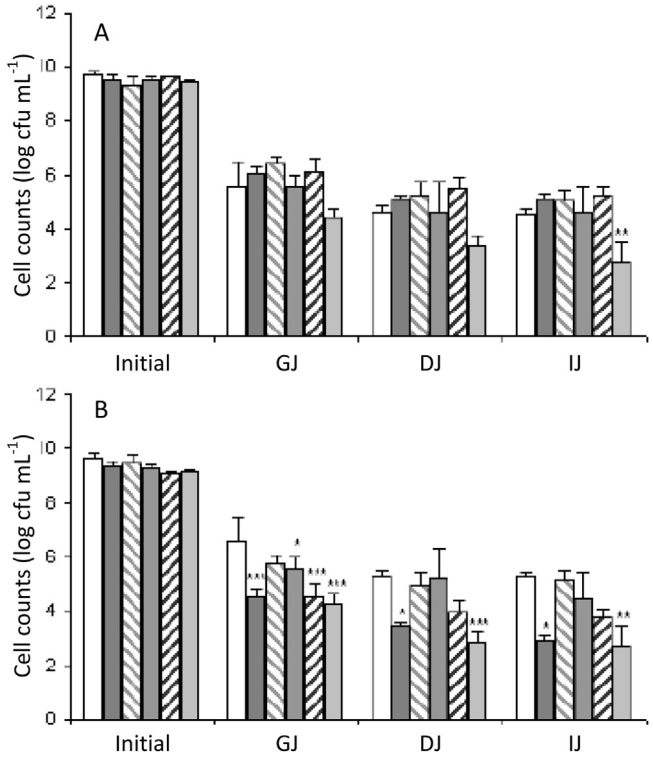

Fig. 4. Counts (log cfu mL^{-1}) of (A) *Lactobacillus paracasei* ATCC 27092 (□) and (B) *Lactobacillus casei* ATCC 27139 (□) and their phage-resistant derivative strains after chemical simulation of gastrointestinal conditions. (A): ■, 092-b2; ▧, 093-b3; ▩, 092-sm1; ▨, 092-sm2; ▫, 092-sm3. (B): ■, 139-b1; ▧, 139-b2; ▩, 139-b3; ▨, 139-sm1; ▫, 139-Rsm2. Gastrointestinal conditions are: GJ, simulated gastric juice; DJ, simulated duodenal juice; IJ, simulated ileal juice. $^{*}P \leq 0.02$, $^{**}P \leq 0.009$, $^{***}P \leq 0.002$. Error bars represent the standard deviation of three determinations.

tolerance and strain survival of the strains in the gut (Ramasamy, Abdullah, Wong, Karuthan, & Ho, 2010). In our work no bile salt deconjugation was detected for any of the strains tested. Regarding growth patterns, derivatives behaved almost identically to their parental strains. The *Lb. paracasei* ATCC 27092 group showed moderate or severe inhibition in the presence of glycocholic acid and bile salts, respectively, and the parental strain displayed slightly less inhibition than its derivatives (results are not shown). The other group showed no inhibition in the presence of glycocholic acid and minor inhibition in bile salts. Besides, practically no differences were found among *Lb. casei* ATCC 27139 and its derivatives. Only 139-b variants in the presence of TDC were demonstrated to be more resistant than the original strain, showing the same growth as in the MRS agar, while the sensitive strain suffered a slight inhibition. Similarly, Vinderola and Reinheimer (2003) did not observe deconjugation for a *Lb. casei* strain, though it showed tolerance to the sodium salts of taurocholic acid, taurodeoxycholic acid and glycocholic acid. Some other authors (Brashears et al., 1998; Ramasamy et al., 2010) demonstrated the ability of 12 *Lactobacillus* strains isolated from GIT to deconjugate sodium glycocholate and sodium taurocholate at varying degrees. Bile salt hydrolase activity in *Lb. casei* has higher substrate specificity towards glycine-conjugated bile (not tested here) compared with taurine-conjugated bile (Liong & Shah, 2005).

3.2.5. Hydrophobicity

Hydrophobicity values (H%) determined for *Lb. paracasei* ATCC 27092 and its phage-resistant derivatives were low (1–11%) and no significant differences ($P > 0.1$) were found between them (Table 2). Implementation of this methodology was not appropriate for the other group since 3 out of 5 derivatives showed autoaggregation capacity (data not shown).

3.2.6. Pathogen inhibitory activity

Lactobacilli have been shown to possess inhibitory activity towards enteropathogens due to their production of several antimicrobial compounds (Verdenelli et al., 2009). In this work, all tested strains showed some inhibitory activity against the four assayed pathogens, though variable antibacterial effects were observed. While total inhibition halos (diameters ranging from 1.35 to 1.85 cm) were obtained against *Salmonella* and *Listeria*, weaker inhibitions (partial halos, diameters ranging from 1.50 to 2.00 cm) were produced against *E. coli* and *S. aureus*.

Regarding the nature of the agent, no inhibition was recorded for any of the pathogens with all the strains tested when the supernatants of the latter were neutralised to pH 7. Therefore, the inhibition could be attributable to an acidic antimicrobial compound itself, to a compound that requires an acidic environment to exert its power or to the low pH value itself. It has been reported

Table 2
Hydrophobicity values and growth in presence of prebiotics for *Lactobacillus paracasei* ATCC 27092 and *Lactobacillus casei* ATCC 27139 and their phage-resistant mutants.

Microorganism and strain	Hydrophobicity values (*H%*)	Growth (%) in the presence of 2% (w/v) prebiotic[a]						
		Oligofructose	Polydextrose	Soluble corn fibre	Kefiran	Raffinose	Xylitol	Inulin
Lactobacillus paracasei								
ATCC 27092[b]	1.5	3.1 ± 2.1	8.5 ± 2.6	12.4 ± 3.0	0.3 ± 0.2	0.2 ± 0.1	0.3 ± 0.2	0.4 ± 0.2
092-sm1[c]	6.0	1.9 ± 0.9	9.7 ± 0.1	14.4 ± 1.3	0.2 ± 0.1	0.2 ± 0.2	0.1 ± 0.1	0.2 ± 0.1
092-sm2[c]	6.8	0.5 ± 0.3	6.1 ± 1.2	10.2 ± 0.3	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
092-sm3[c]	4.8	0.2 ± 0.1	6.4 ± 1.3	12.7 ± 1.2	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
092-b2[c]	8.9	2.2 ± 1.2	9.1 ± 0.9	13.3 ± 0.7	0.1 ± 0.1	0.1 ± 0.2	0.1 ± 0.0	0.2 ± 0.1
092-b3[c]	11.0	0.6 ± 0.1	7.4 ± 0.5	12.7 ± 1.7	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
Lactobacillus casei								
ATCC 27139[c]	nd[d]	0.5 ± 0.3	6.2 ± 0.2	13.0 ± 1.9	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.2
139-b1[c]	nd	2.4 ± 0.7	5.0 ± 0.3	13.7 ± 0.4	0.3 ± 0.2	0.3 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
139-b2[c]	nd	0.5 ± 0.4	5.1 ± 0.7	12.5 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.0 ± 0.1	0.1 ± 0.1
139-b3[c]	nd	0.1 ± 0.1	4.0 ± 0.9	12.4 ± 1.4	0.1 ± 0.0	0.2 ± 0.1	0.0 ± 0.1	0.1 ± 0.1
139-sm1[c]	nd	0.8 ± 0.5	3.2 ± 2.9	17.7 ± 3.8	0.1 ± 0.1	0.2 ± 0.1	0.0 ± 0.2	0.1 ± 0.1
139-Rsm2[c]	nd	2.3 ± 1.3	6.5 ± 0.1	19.0 ± 3.7	0.3 ± 0.2	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.0

[a] Values are the mean of two determinations ± standard deviation; compared with a control in basic MRS broth (without glucose) supplemented with 2% (w/v) of glucose.
[b] Phage-sensitive strain.
[c] Phage-resistance derivatives.
[d] nd, Not determined.

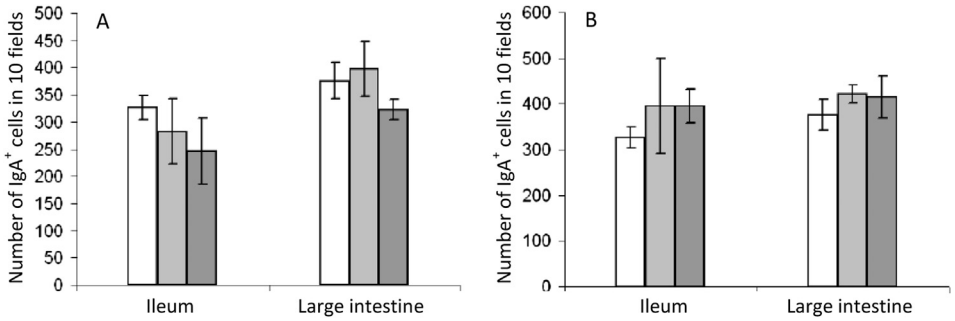

Fig. 5. Effect of the oral administration (approximately 3×10^7 cfu day^{-1} mouse^{-1}) of parental strains (■) *Lactobacillus paracasei* 27092 (A) and *Lactobacillus casei* 27139 (B) and their respective resistance derivatives (■) 092-sm1 (A) and 139-b2 (B) for 5 consecutive days on the number of IgA + cells on histological slices of the ileum and large intestine, compared with control animals that received 1% skim milk (□). Error bars represent the standard deviation of three determinations.

that the bactericidal effect of *Lactobacillus* on *E. coli* depends both on its lactic acid production (undissociated) and the pH reduction, being the cytotoxic properties bacteriostatic (3.2–62 mmol L^{-1}) or bactericidal (over 62 mmol L^{-1}) depending on the concentration of undissociated lactic acid (Ogawa et al., 2001).

The inhibition observed for *Salmonella* was altered (total inhibition halos became partial halos) when CFSs were treated with porcine pepsin, probably indicating a proteinaceous nature of the inhibitor/s. In contrast, no clear differences were observed with the other treatments in comparison with non-treated CFS. Finally, almost all derivatives behaved similarly to their respective parents concerning the inhibition of the tested pathogens. The only exceptions were 139-Rsm2 (against *E. coli*) and 092-sm2 and 092-b2 (against *Listeria*), which were significantly less inhibitory than their original sensitive strains.

3.3. *In vivo probiotic studies*

Many *Lactobacillus* species regulate the innate and adaptive immune responses, because of a dose- and strain dependent effect (Baken et al., 2006; Gregoret, Perezlindo, Vinderola, Reinheimer, & Binetti, 2013; Tsai, Cheng, Liao, & Pan, 2010; Ya et al., 2008). Derivatives 092-sm1 and 139-b2 were selected based on their technological and in vitro probiotic performances and were used in parallel with their original strains (controls) for in vivo assays. Bacterial translocation is a phenomenon caused by a diminished intestinal barrier, resulting in the passage of bacteria through the gut to extra intestinal organs probably causing bacteraemia. Many factors may promote it, including intestinal mucosal injury, immunodeficiency in the host, and an abnormal intestinal bacterial flora (Ishibashi & Yamazaki, 2001). It is considered a putative adverse effects caused by regular probiotic feeding, also being the probiotics itself capable to translocate and cause damage in immunocompromised patients (Liong, 2008). Strains (derivatives and parents) at the conditions used did not induce translocation of enterobacteria to the liver (translocation assay was negative). In relation with the functional attribute evaluated, no significant differences were found in the number of IgA-producing cells induced by the derivatives compared with their corresponding parent strain neither at ileum nor at large intestine (Fig. 5).

4. Conclusions

The performance of 10 phage-resistant mutants spontaneously derived from 2 sensitive probiotic strains was evaluated. Technological, biological and functional attributes obtained for derivatives 092-sm1 and 139-b2 demonstrated that, in addition to their improved phage-resistance phenotype, they resemble their parental probiotic strains.

Several strategies to curtail phage attacks are restricted in the case of probiotic strains. Industrial rotation scheme is one of the restricted measures due to the following facts: probiotic attributes are strain-specific, studies to guarantee those attributes must be made on each particular strain and they cannot be extended to other strains. The evidence that derivatives of a proven probiotic strain only differ in the improved phage-resistance really deserves a special mention.

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