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Biological and probiotic characterisation of spontaneous phage-resistant mutants of *Lactobacillus plantarum*

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ABSTRACT

Four spontaneous phage-resistant mutants, previously isolated from *Lactobacillus plantarum* ATCC 8014 using a phage cocktail (ATCC 8014-B1 and ATCC 8014-B2), were characterised with regard to their probiotic potential. Phage-resistant mutants exhibited, in general, the same properties as those found for *Lb. plantarum* ATCC 8014 strain. However, mutant M1 evidenced a remarkably high resistance to gastrointestinal passage. Low values of either β-galactosidase activity or hydrophobicity were observed. Antimicrobial activity against Gram-negative and Gram-positive bacteria was evidenced and lactulose was the most fermented carbohydrate. Strains were sensitive to gentamicin, erythromycin, ampicillin and chloramphenicol, whereas production of biogenic amines was not observed. Finally, a selected phage-resistant mutant (M1) produced the same immunological response as the sensitive strain in mice fed for 10 consecutive days. These natural mutants, with similar or improved potentially probiotic characteristics regarding their sensitive strain, could be used during fermented food manufacture to minimise failure due to phage.

1. Introduction

Lactobacillus plantarum has the ability to grow and survive in the wide variety of environmental conditions inherent to meat, vegetables, and dairy products (Corsetti & Valmorri, 2011). Furthermore, interesting features as health-promoting organisms were reported for some *Lb. plantarum* strains, like strain 299v (Molin, 2008; de Vries, Vaughan, Kleerebezem, & de Vos, 2006). Therefore, this species could be used as adjunct culture in cheeses (De Angelis et al., 2008; Mangia, Murgia, Garau, Sanna, & Deiana, 2008; Milesi, McSweeney, & Hynes, 2008) as well as in the development of new functional foods (Corsetti & Valmorri, 2011; Zago et al., 2011).

Any technological factor causing *Lb. plantarum* culture failure or inhibition when it is used as starter/adjunct culture must be considered. As is already known, the ubiquity of bacteriophages in every natural niche makes actively growing bacteria into ideal and easy targets for phage infection, dairy fermentations being a largely recognised scenario suffering the most severe consequences. In the past several years, a lot of strategies have been designed and applied to minimise both phage presence in dairy industries and its economic impact on the processes. Traditional approaches include modified factory design, optimised sanitation and improved processes, enhanced starter media and use of phage-resistant cultures in rotation (Garneau & Moineau, 2011).

Despite these technologies, a high diversity of phages infecting probiotic bacteria is increasingly been documented, as well as a remarkable resistance to harsh heat treatments and traditional sanitisers (Atamer, Neve, Heller, & Hinrichs, 2012; Capra, Binetti, Mercanti, Quiberoni, & Reinheimer, 2009; Suárez & Guglielmotti, 2012). Therefore, facing phage infections of probiotic bacteria has become a new challenge for microbiologists and technologists as a few control strategies might be suitable. In particular, the replacement of a probiotic strain as part of a culture rotation program could be unachievable since health-promoting claims are specifically established for a strain. In this regard, the isolation of spontaneous bacteriophage insensitive mutants (BIMs) from a sensitive strain arises as an alternative for industrial applications. Even if some drawbacks are linked to this approach, their simplicity and no involvement of genetic manipulations turned it into a 'natural' and popular option to be applied in industrial food manufacture (Briggiler Marcó, Quiberoni, & Moineau, 2012). Nevertheless, meticulous examination must confirm that technological and

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potentially probiotic traits from the original sensitive strain are preserved in the phage-resistant derivatives.

In a previous work, the technological performance of the first *Lb. plantarum* BIMs isolated by using a phage cocktail was demonstrated during their use as starter in a fermented milk manufacture (Briggiler Marcó, Mercanti, Reinheimer, & Quiberoni, 2011). In the present study, the resistance to biological barriers and the probiotic potential of four BIMs derived from a sensitive *Lb. plantarum* strain was assessed to verify their suitability to act as replacement strains in industrial rotation starter programs.

2. Materials and methods

2.1. Cultures and culture conditions

In this study, the phage-sensitive strain *Lb. plantarum* ATCC 8014 and its phage-resistant mutants previously isolated (Briggiler Marcó et al., 2011) were used. Strains were routinely grown in MRS broth (Biokar, Beauvais, France) at 37 °C. In addition, for antimicrobial activity assays, *Escherichia coli* V517 (INLAIN Collection), *Salmonella* sp. OMS-Ca (INLAIN Collection) and *Staphylococcus aureus* 76 (INLAIN Collection) were grown in TS (Britania, Buenos Aires, Argentina) broth at 37 °C, whereas *Listeria monocytogenes* ATCC 15313 was grown in BHI (Britania, Buenos Aires, Argentina) broth at 37 °C.

2.2. Resistance to gastrointestinal conditions

The resistance of *Lb. plantarum* ATCC 8014 and its phage-resistant mutants to successive biological barriers (simulated gastric juice, bile and lysozyme) was performed in triplicate and according to Vinderola et al. (2007) slightly modified as follows. Briefly, overnight cultures in MRS broth were centrifuged (10,000 × *g*, 5 min) and washed twice with phosphate buffered saline, pH 7.4 (PBS). Then, pellets were resuspended in a solution (pH 3) containing 0.3% (w/v) pepsin (Sigma–Aldrich, St. Louis, MO, USA) and 0.5% (w/v) sodium chloride, and incubated at 37 °C for 1 h. Cultures were then centrifuged, washed twice with PBS and resuspended in a 0.3% (w/v) bovine bile (Sigma–Aldrich) solution and incubated at 37 °C for 1 h. The cultures were finally centrifuged, washed twice with PBS, resuspended in a solution of lysozyme (Sigma–Aldrich) at 0.1 mg mL^{-1} and held at 37 °C for 1 h. Control suspensions in PBS were also included. At the beginning of the experiment and after each treatment, cell counts on MRS agar were determined.

2.3. Hydrophobicity

The ability of sensitive and phage resistant variant cells to adhere to hydrocarbons was determined according to Vinderola and Reinheimer (2003). The partition of bacterial cells between organic (n-hexadecane) (Cicarelli, San Lorenzo, Santa Fe, Argentina) and aqueous phases was determined by measurement of optical density at 560 nm ($OD_{560\ nm}$). Assays were performed in triplicate and hydrophobicity (%) was calculated as follows:

$$\%\ \text{hydrophobicity} = [(OD_0 - OD)/OD_0] \times 100 \quad (1)$$

where OD_0 and OD are the optical densities before and after extraction with the organic solvent, respectively.

2.4. β-Galactosidase activity

The β-galactosidase activity of *Lb. plantarum* sensitive strain and the phage resistant mutants was determined according to Miller (1972) modified by Vinderola and Reinheimer (2003). *Lb. plantarum* cultures were inoculated (2%, v/v) into lactose-MRS broth and *o*-nitro-β-D-galactopyranoside (ONPG, Sigma–Aldrich) (4 mg mL^{-1}) was used as reaction substrate (Vinderola & Reinheimer, 2003). After the reaction (15 min), absorbance values at both 420 nm and 560 nm were measured and the enzymatic activity (β-gal act; Miller units) was calculated as follows:

$$\beta\text{-gal act} = 1000 \times \left[(OD_{420} - 1.75 \times {OD_{560}}^{a})/(15\ \text{min} \times 0.1\ \text{mL} \times {OD_{560}}^{b})\right] \quad (2)$$

where ${OD_{560}}^{a}$ is the cell density before assay and ${OD_{560}}^{b}$ is the cell density of the reaction mixture. Assays were carried out in triplicate.

2.5. Prebiotic utilisation

The ability of *Lb. plantarum* cultures to ferment several carbohydrates, i.e., raffinose, lactulose, xylitol, inulin (Sigma–Aldrich), oligofructose P95 (Beneo-Orafti, Tienen, Belgium), Litesse™ polydextrose (Danisco, Brabrand, Denmark) and Promitor™ soluble corn fibre (Tate & Lyle, London, UK), was evaluated. Glucose (Sigma–Aldrich) was included as positive control and MRS without carbon source was used as negative control. All carbohydrates and glucose were sterilised by filtration using a 0.22 μm syringe filter unit (Millipore, São Paulo, Brazil). Cultures were inoculated (2%, v/v) into MRS broth added of each carbohydrate or glucose (2%, w/v) and incubated anaerobically at 37 °C for 24 h. Assays were carried out in triplicate and the results were expressed as the percentage of growth (optical density at 560 nm) and calculated with the following formula:

$$\text{Growth}\ (\%) = [(\text{MRSp} - \text{MRSb}) \times 100/(\text{MRSg} - \text{MRSb})] \quad (3)$$

where MRSp is MRS with each prebiotic, MRSb is MRS without carbon source and MRSg is MRS with glucose (Zago et al., 2011).

2.6. Antimicrobial activity

The antimicrobial activity of the sensitive strain and its phage resistant variants on *E. coli* V517 (INLAIN Collection), *Salmonella* sp. OMS-Ca (INLAIN Collection), *L. monocytogenes* ATCC 15313 and *S. aureus* 76 (INLAIN Collection) was determined in triplicate by the well diffusion agar assay (Cardamone et al., 2011). To obtain the cell-free supernatants (CFS), overnight *Lb. plantarum* cultures were centrifuged (12,000 × *g*, 10 min, 5 °C) and then sterilised by filtration (0.22 μm, Millipore, São Paulo, Brazil). To prepare plates containing pathogens, tubes with 20 mL of nutrient agar (Britania) or BHI agar (for *L. monocytogenes*), melted and cooled to 45–50 °C, were inoculated (2%, v/v) with pathogenic cultures (grown at DO_{560} of 0.8 or DO_{560} of 1, for *S. aureus*), vigorously homogenised and poured onto Petri dishes. A volume of 180 μL of CFS of each *Lb. plantarum* strain was placed in a well (10 mm in diameter), previously made in the agar layer. Plates were incubated at 37 °C for 24 h and the diameters of the halos of inhibition were recorded.

In addition, CSF were subjected to several treatments to elucidate the nature of the inhibitor agent: heating at 121 °C for 15 min, neutralisation to pH 7, incubation at 37 °C for 1 h with 200 μg mL^{-1} proteinase K (Invitrogen Life Technologies, Carlsbad, CA, USA) or 1 mg mL^{-1} pepsin (Merck, Darmstadt, Germany). Treated and non-treated (control) CFS were sterilised by filtration (0.22 μm pore diameter) and assayed for remaining activity by the well diffusion agar assay (Coconnier, Liévin, Bernet-Camard, Hudault, & Servin, 1997).

On the other hand, assays in liquid medium were performed according to Lash, Mysliwiec, and Gourama (2005) and modified as follows: 20 mL of TS or BHI (for *Listeria*) broth, inoculated with 2 μL of each pathogen, were added of 3 mL of each CFS or MRS broth (control). Mixtures were then divided in aliquots, incubated at 37 °C and at intervals of 90 min, the OD_{560} was determined.

2.7. Antibiotic susceptibility testing

The minimum inhibitory concentration (MIC) of vancomycin, gentamicin, tetracycline, erythromycin, ampicillin, streptomycin and chloramphenicol (Sigma–Aldrich) for *Lb. plantarum* strains was evaluated in triplicate using the microdilution test. Antibiotics were dissolved in distilled water or water:ethanol (6:4 for erythromycin and ampicillin; 5:5 for chloramphenicol) and sterilised by filtration (0.22 μm syringe filter units; Millipore). Overnight cultures of assayed strains were diluted in LSM broth (90%, w/v, Iso-Sensitest, Oxoid Ltd, Basingstoke, UK; 10%, w/v, MRS, Biokar, Beauvais, France) adjusted at pH 6.7 to reach a cell concentration of 10^5 cfu mL^{-1} (Rosetti, Carminati, Zago, & Giraffa, 2009). Cell suspensions and antibiotics (test ranges: 0.125–512 μg mL^{-1}) were placed in microplates and incubated at 37 °C for 48 h. After incubation, the MIC was determined as the lowest antibiotic concentration at which no growth was observed. Interpretation of results was based on the breakpoints (BP) adopted by technical guidance of the European Food Safety Authority (2008).

2.8. Biogenic amine production

Biogenic amine production by sensitive and phage resistant strains was evaluated according to Bover-Cid and Holzapfel (1999). Bacterial cultures were streaked in triplicate on the medium plates with each amino acid (histidine, tyrosine, lysine and ornithine) (Sigma–Aldrich) and without them (control). Petri dishes were incubated at 37 °C for 3 days under microaerophilic conditions. Biogenic amine production was confirmed by indicator colour changes in response to the amine formation.

To confirm the results obtained in agar, the same assay was also performed in liquid medium. For this purpose, overnight cultures were inoculated (2%, v/v) in the medium described by Bover-Cid and Holzapfel (1999) with and without amino acids. After incubation at 37 °C for 30 h, the result was considered as positive if a colour change in the medium was observed. In addition, pH values were determined.

2.9. In vivo trials

2.9.1. Animals and feeding procedures

The phage-sensitive strain *Lb. plantarum* ATCC 8014 and one phage-resistant mutant (M1), selected according to its behaviour with respect to resistance to gastrointestinal assays, were used for the in vivo trials.

Thirty female BALB/c mice (16–19 g) were obtained from the random-bred colony of the Centro de Experimentaciones Biológicas y Bioterio, Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (Esperanza, Santa Fe, Argentina). Animals were allowed to stand at the INLAIN animal facility for a week before the experiments. Each experimental group consisted of four mice housed in plastic cages kept under controlled conditions of temperature (21 ± 2 °C) and relative humidity (55 ± 2%), and with a 12 h light/dark cycle. Mice were maintained and treated according to the guidelines of the National Institute of Health (USA). The control group received (intragastric intubation) 150 μL day^{-1} $mouse^{-1}$ of 10% (w/v) skim milk for 6 consecutive days whereas three groups received a cell suspension of each strain (2×10^8 cfu mL^{-1} $mouse^{-1}$) in 10% (w/v) skim milk for 3, 6 or 10 consecutive days. The cell suspension was prepared daily from an overnight culture washed twice with PBS buffer.

In all assays, treated and control animals received ad libitum tap water and a sterile conventional balanced diet (Cooperación, Buenos Aires, Argentina) containing: protein, 230 g kg^{-1}; raw fibre, 60 g kg^{-1}; total minerals, 100 g kg^{-1}; Ca, 13 g kg^{-1}; P, 8 g kg^{-1}; water, 120 g kg^{-1}; and vitamins.

After each feeding period, mice were anesthetised (100–200 μL $mouse^{-1}$) intraperitoneally with a rodent cocktail (9 parts ketamine (100 mg mL^{-1}) + 9 parts xylazine (20 mg mL^{-1}) + 3 parts acepromazine (10 mg mL^{-1}) + 79 parts sterile saline). Animals were sacrificed by cervical dislocation and the small intestine was removed and washed with cold PBS. Translocation assays were carried out according to Burns et al. (2012).

2.9.2. Determination of secretory IgA in the intestinal fluid

Small intestine contents were recovered in a falcon tube by pressing the small intestine from the duodenum to the distal ileum, weighed and diluted in PBS containing protease inhibitors (1%, v/v) (P8340, Sigma–Aldrich) at a ratio of 1:4. After centrifugation (5000 × *g*, 30 min, 5 °C), the supernatant was carefully collected and stored at −80 °C. The supernatant was used for secretory IgA (S-IgA) quantification using an ELISA kit (BD Biosciences Pharmingen, San Diego, CA, USA) following the manufacturer's protocol (Rodrigues et al., 2009). The absorbance was read at 492 nm with a microplate reader (Thermo Scientific™ Multiskan FC). The results were calculated against standard curves generated using known amounts of a purified IgA standard included in the kit. They were expressed as micrograms per gram of intestinal content (μg g^{-1}).

2.9.3. Determination of cytokines in intestinal homogenates

Cytokines were quantified in homogenates prepared from small intestine samples (160–180 mg). The intestinal samples were added of PBS containing protease inhibitors (1%, v/v, Sigma–Aldrich), 10 mmol L^{-1} EDTA (Sigma–Aldrich) and 0.05% (v/v) Tween 20 (Sigma–Aldrich) in a relation of 1 mL every 100 mg of intestinal tissue and were placed in an ice bath and homogenised using an Ultra Turrax T8 Homogenizer (Ika Labortechnik, Staufen, Germany). After centrifugation (10,000 × *g*, 10 min, 5 °C), the supernatants were carefully collected and stored at −80 °C. Concentrations of interleukin (IL)-10 and gamma interferon (IFN γ) in supernatants were determined by ELISA using commercially available antibodies (BD Biosciences Pharmingen) according to the instructions supplied by the manufacturer. Absorbance values were read at $\lambda = 492$ nm using a microplate reader (Thermo Scientific™ Multiskan FC). Cytokine levels were expressed as nanograms per gram of intestinal tissue (ng g^{-1}) (Negrao-Corrêa et al., 2004).

3. Results and discussion

3.1. Selection of spontaneous phage-resistant mutants

Phage-resistant mutants studied in this work were selected according to the results obtained during the characterisation of their phage-resistance phenotype as well as their technological properties (Briggiler Marcó et al., 2011). This characterisation, carried out in a previous work, included the phage-resistance stability, level of resistance (i.e., efficiency of plaquing, EOP), adsorption rates and the spontaneous release of phage particles from the resistant variants. The technological characterisation involved acidification kinetics in a reconstituted commercial milk-medium (EM-glucose medium) similar to those used to propagate the probiotic cultures in the dairy industry (Briggiler Marcó et al., 2011).

Based on the results obtained, four phage-resistant mutants (M1, M3, MC4 and MC6) were chosen. M1 and M3 were isolated by using phage ATCC 8014-B1 whereas MC4 and MC6 were obtained using a phage cocktail (ATCC 8014-B1 and ATCC 8014-B2). The four mutants exhibited high levels of resistance (EOP of 10^{-8}–10^{-10}) and stability (up to the seventh culture). Undetectable counts of phage plaques (<10 pfu mL^{-1}) in the supernatants of phage-resistant mutants were obtained suggesting that virions are not released spontaneously. Variants were not able to bind phage particles since adsorption rates below 16.7% were found (Briggiler Marcó et al., 2011). Regarding technological characterisation, phage-resistant mutants evidenced a behaviour similar to that of the sensitive strain, reaching pH values <4.6 within 4 (M1 and M3) and 6 h (MC4 and MC6) in EM-glucose medium at 37 °C. At 24 h, *Lb. plantarum* ATCC 8014 reached a pH value of 3.54 and 1.31% of acidity in EM-glucose medium. Under these conditions, the four mutants developed acidity percentages ranging between 0.97 and 1.21 and pH values from 3.64 to 3.84 (Briggiler Marcó et al., 2011).

3.2. Resistance to gastrointestinal conditions

Gastrointestinal conditions (low pH in the stomach, pepsin, bile salts and lysozyme) result in the destruction of most microorganisms ingested. However, in some cases, similar biological effects were observed working with either non-viable bacteria or viable microorganisms (Vinderola et al., 2007). In this work, phage-sensitive strain *Lb. plantarum* ATCC 8014 showed reductions of 4.8 log orders when it was subjected to simulated digestion (Fig. 1). A similar behaviour was observed for *Lb. plantarum* strains isolated from sauerkraut and exposed to simulated gastric juice (pH 3) for 180 min (Yu et al., 2013). Vizoso Pinto, Franz, Schillinger, and Holzapfel (2006) studied the resistance of *Lb. plantarum* strains suspended in milk (including *Lb. plantarum* ATCC 8014) to simulated digestion conditions and found a good survival of *Lb. plantarum* strains after passage for simulated gastric juice (pH 2.5) and simulated duodenum juice (pH 7.2). *Lb. plantarum* ATCC 8014, in particular, showed high viability (reductions of 0.5 log orders) under these simulated gastrointestinal conditions. Possibly, milk exerts a protective role of cultures during the gastrointestinal passage. Thus, it is important to consider the food vehicle in which the probiotic culture would be included.

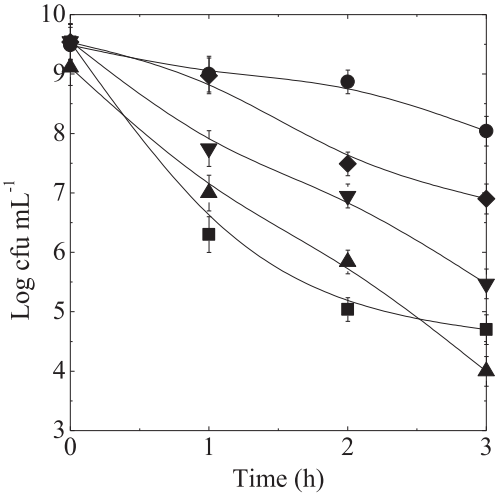

Fig. 1. Resistance of *Lb. plantarum* phage-sensitive strain ATCC 8014 (■) and its phage-resistant mutants M1 (●), M3 (◆), MC4 (▲) and MC6 (▼) to acidic conditions (pH 3, 1 h), bile salts (0.3%, 1 h), and lysozyme (100 ppm, 1 h). Each value is the mean ± standard deviation of three independent trials.

In addition to their improved phage-resistance, the variants studied in the present work exhibited, on advantage, a better performance under simulated gastrointestinal conditions in comparison with those obtained for their parent strain, mutant M1 being the best one. For this mutant, a cell viability loss of only 1.4 log orders was found. In general, exposure of the mutants to acidic conditions produced the highest viability losses (0.5–3.24 log units). In the presence of bile salts or lysozyme, cell suspensions evidenced more resistance since the cell counts decreased from 0.13 to 1.26 and from 0.34 to 1.84 log units, respectively (Fig. 1). For all strains assessed, the viability of cells suspended in PBS remained unchanged throughout the assay (data not shown).

3.3. β-Galactosidase activity

It is well documented that cultures with high β-galactosidase activity might be used as dietary adjunct to moderate lactose maldigestion in the gut (Belicová, Mikulásová, & Dusinky, 2013). In this work, phage-resistant mutants evidenced higher β-galactosidase activity than the sensitive strain *Lb. plantarum* ATCC 8014. However, it was low in comparison with those reported for other LAB (Guglielmotti, Briggiler Marcó, Golowczyc, Reinheimer, & Quiberoni, 2007; Vinderola & Reinheimer, 2003). Mutants M1 and MC6 showed the highest enzymatic activity (230 and 250 UM, respectively) whereas for *Lb. plantarum* ATCC 8014 the β-galactosidase activity was 60 UM. Several authors reported a variable β-galactosidase activity of *Lb. plantarum* strains (Belicová et al., 2013; Cebeci & Gürakan, 2003; Vizoso Pinto et al., 2006; Yu et al., 2013; Zago et al., 2011).

Similar to the behaviour observed in this work, Vallejo, Marguet, and Etchechoury (2008) reported that *Lactobacillus* strains isolated from Argentinian cheeses with a low β-galactosidase activity evidenced ability to grow in a medium containing lactose. In these cases, lactose would possibly be hydrolysed by other enzyme. Besides permease pathway in which lactose is hydrolysed by β-galactosidase, bacteria can use the phosphoenol pyruvate–phosphotransferase (PEP/PTS) system in which phospho-β-galactosidase (p-β-galactosidase) takes part (Mäyra-Mäkinen, 2004). In the present work, phage-resistant mutants evidenced a low β-galactosidase activity, but they would be able to hydrolyse lactose through p-β-galactosidase and could reduce the lactose intolerance symptoms. In this sense, Honda et al. (2007) specifically suggest the use of *Lactobacillus gasseri* strains with high p-β-galactosidase activity to diminish the problems associated to lactose maldigestion.

3.4. Hydrophobicity

Cell wall hydrophobicity facilitates the first interaction between bacteria and the intestinal epithelial cells (Schillinger, Guigas, & Holzapfel, 2005; Vinderola & Reinheimer, 2003).

In the present study, *Lb. plantarum* ATCC 8014 and its phage-resistant mutants evidenced low hydrophobicity percentages (<24%), which were similar to those reported for *Lb. plantarum* strains isolated from dairy fermented products (Mathara et al., 2008; Zago et al., 2011) and a fermented seaweed (Kantachote et al., 2010). Although hydrophobicity may be helpful for adhesion process, it is not a prerequisite for a strong adherence capacity (Guglielmotti et al., 2007). While some authors reported a correlation between hydrophobicity and adhesion to the mucosal surface (Ehrmann, Kurzak, Bauer, & Vogel, 2002; Van Tassell & Miller, 2011), other results indicate hydrophobicity values do not correlate with adhesion properties (Mathara et al., 2008). In this sense, *Lb. plantarum* strains with low hydrophobicity values (24–44%) evidenced high percentage adhesion to HT 29 MTX cells (71–76%)

(Mathara et al., 2008). Therefore, specific adhesion assays may be useful for *Lb. plantarum* mutants characterised in this study.

3.5. Prebiotic utilisation

Prebiotics are defined as non-digestible carbohydrates that selectively stimulate the growth and/or activity of beneficial bacteria in the colon, thus improving the host health (Zago et al., 2011). Thus, they are used as functional ingredients in food industry (e.g., dairy products, meat, cereals, and beverages) (Caselato de Sousa, Freitas dos Santos, & Sgarbieri, 2011). Among carbohydrates assayed in this work, lactulose produced the highest growth rates (72.4–88.9%) in comparison with glucose. When corn fibre was evaluated, lower fermentation rates were observed (33.4–36.1%). Raffinose, xylitol, inulin, polydextrose and oligofructose were poorly fermented by *Lb. plantarum* strains studied in this work (4.3–29.2%) (Table 1). Similar behaviour was observed for *Lb. plantarum* strains isolated from cheeses (Zago et al., 2011).

3.6. Antimicrobial activity

In this work, when the well diffusion agar assay was performed, CFSs of *Lb. plantarum* ATCC 8014 and its phage-resistant mutants were able to produce total inhibition halos on *Salmonella* sp. OMS-Ca, *E. coli* V517 and *S. aureus* 76. However, partial inhibition halos on *L. monocytogenes* ATCC 15313 were obtained, suggesting a lower inhibitory effect on this pathogen. In all cases, antibacterial activity disappeared when CFSs were neutralised, indicating that the inhibitory compounds would be acidic. A total inhibitory effect produced by acidic compounds was observed only when MRS broth acidified with lactic acid was assayed whereas in the presence of MRS broth acidified with HCl, a partial inhibition was evidenced. These results may indicate the antibacterial activity is due to the lactic acid produced by lactic acid bacteria and not only to the low pH values reached by the strains in the medium (data not shown).

Besides the inhibitory effect of organic acids, several authors reported the production of bacteriocins by *Lb. plantarum* strains (Atrih, Rekhif, Michel, & Lefebvre, 1993; Lash et al., 2005; Messi, Bondi, Sabia, Battini, & Manicardi, 2001; Mills et al., 2011; van Reenen, Dicks, & Chikindas, 1998; Todorov, 2009). However, in this work, the well diffusion agar assay was not useful to evidence an inhibitory effect produced by a proteinaceous component. Possibly, and according to Lash et al. (2005), the diffusion of high size-proteinaceous compounds would be difficult through the agar medium and consequently the inhibitory effect may not be observed. For this reason, a methodology in liquid medium was carried out. In the presence of CFS, *E. coli* V517 showed a growth percentage of 16.8 ± 2.8% whereas for *Salmonella* sp. OMS-Ca a higher inactivation was obtained (3.2 ± 0.1%). Similar to results obtained with well diffusion agar assay, neutralised CFS were not able to inhibit pathogenic bacteria. Additionally, a reduced growth rate of both pathogens was observed in the presence of either thermally treated CFS or CFS treated with pepsin (Table 2). Similarly, antimicrobial activity was observed against *S. aureus* 76 (15.3 ± 0.7%) and *L. monocytogenes* ATCC 15353 (25.0 ± 1.1%). Similar results were observed for *Lb. plantarum* strains isolated from fermented products (Belicová et al., 2013; Kantachote et al., 2010; Vizoso Pinto et al., 2006; Yu et al., 2013). However, Zago et al. (2011) reported *Lb. plantarum* strains isolated from cheeses were not able to inhibit pathogens.

In short, results obtained suggest that, besides lactic acid, thermolabile proteinaceous compounds would also produce antibacterial activity against Gram-negative bacteria (*E. coli* V157 and *Salmonella* sp. OMS-Ca). On the other hand, the inhibition of Gram-positive bacteria (*S. aureus* 76 and *L. monocytogenes* ATCC 15353) would be produced by compounds other than organic acids/proteins or by a mixture of them. Taking into account the results obtained in the present work, the phage-resistant mutants of *Lb. plantarum* could help control the growth of pathogens during food manufacture and subsequent storage. In this sense, it will be mandatory to test the potential protective cultures on a food matrix.

3.7. Determination of antibiotic susceptibility and biogenic amine production

One of the required properties of probiotic strains is their safety for human consumption (Vizoso Pinto et al., 2006). Thus, the antibiotic resistance and biogenic amine production for the *Lb. plantarum* strains were assayed. Regarding assays with antibiotics and according to Break Point defined by the FEEDAP Panel (European Food Safety Authority, 2008), the five *Lb. plantarum* strains were sensitive to gentamicin, erythromycin, ampicillin and chloramphenicol (Table 3). For tetracycline, a MIC identical to the BP value was demonstrated by the variants. The same behaviour was observed for mutant M3 with ampicillin (Table 3). However, this is not considered to be cause for concern according to what was reported by EFSA (2012) for *Lb. plantarum* strains and it could be related to normal variations of the assayed technique.

As reported by several authors, *Lb. plantarum* strains evidenced intrinsic resistance to vancomycin and tetracycline (EFSA, 2008, 2012; Mathara et al., 2008). On the contrary, several authors found *Lb. plantarum* strains to be resistant to various antibiotics (Belicová et al., 2013; Kantachote et al., 2010; Mathara et al., 2008; Vizoso Pinto et al., 2006; Yu et al., 2013; Zago et al., 2011). Antibiotic resistance can be considered as an advantage or a disadvantage. Antibiotic-resistant strains could be given during an antibiotic treatment. However, if antibiotic resistance genes are present on plasmids, they could be transferred by conjugation to other bacteria including pathogens (Cebeci & Gürakan, 2003). Consequently, studies about the presence of antibiotic resistance genes on chromosomes or on plasmids are mandatory.

Biogenic amines are organic basic compounds generated mainly by decarboxylation of amino acids. In foods, they are produced

Table 1
In vitro probiotic characteristics of phage-sensitive strain *Lb. plantarum* ATCC 8014 and its phage-resistant mutants (M1, M3, MC4, MC6).[a]

Strain	β-gal	H (%)	Growth in presence of						
			Lactulose	Raffinose	Xylitol	Inulin	Polydextrose	Corn fibre	Oligofructose
ATCC 8014	60 ± 4.3	14.1 ± 2.1	88.9 ± 2.5	7.7 ± 1.2	4.9 ± 0.8	20.6 ± 0.9	18.2 ± 1.1	35.3 ± 1.8	18.7 ± 1.2
M1	230 ± 10.1	16.5 ± 3.5	75.6 ± 2.7	29.2 ± 2.3	4.7 ± 0.7	18.4 ± 0.8	19.5 ± 0.9	36.1 ± 1.9	17.8 ± 0.9
M3	150 ± 9.6	24.5 ± 4.1	72.4 ± 3.0	15.2 ± 2.0	4.8 ± 0.7	21.8 ± 1.1	20.7 ± 0.9	35.2 ± 2.0	19.6 ± 1.0
MC4	125 ± 8.5	23.1 ± 6.2	87.6 ± 2.6	8.2 ± 1.1	4.3 ± 0.5	20.6 ± 1.2	18.3 ± 1.0	33.4 ± 1.5	19.2 ± 1.3
MC6	250 ± 7.6	15.0 ± 2.1	77.3 ± 2.4	19.6 ± 1.8	4.7 ± 0.6	18.9 ± 1.1	17.7 ± 1.2	34.6 ± 1.3	18.7 ± 1.2

[a] Values are the mean of three determinations ± standard deviation, values for growth are expressed as growth cell percentage in presence of each carbohydrate with respect to glucose (100% utilisation), used as control: β-gal, β-galactosidase activity in Miller units; H, hydrophobicity percentage.

Table 2
Cell growth (%) of pathogenic bacteria in the presence of cell-free supernatants of *Lb. plantarum* ATCC 8014 and its phage-resistant mutants.[a]

Pathogen	Cell growth (%)			
	CFS	CFSnt	CFStt	CFSps
E. coli V517	16.8 ± 1.8	99.8 ± 0.2	25.0 ± 2.1	43.4 ± 3.5
Salmonella sp. OMS-Ca	3.2 ± 0.1	97.5 ± 1.2	12.7 ± 0.8	8.6 ± 0.6
S. aureus 76	15.3 ± 0.7	72.1 ± 1.3	14.1 ± 0.4	30.0 ± 2.3
L. monocytogenes ATCC 15353	25.0 ± 1.1	39.8 ± 3.1	29.4 ± 2.3	27.6 ± 3.5

[a] Values are the mean of the results obtained for the five strains. Abbreviations are: CFS, cell-free supernatants; CFSnt, neutralised cell-free supernatants; CFStt, cell-free supernatants with thermal treatment; CFSps, cell-free supernatants treated with pepsin.

through the microflora decarboxylase activity. Several toxicological problems resulting from the consumption of food containing high levels of biogenic amines have been reported (Bover-Cid & Holzapfel, 1999; Karovičová & Kohajdová, 2005). Thus, the inability to form biogenic amines could be a desirable selection criterion for strains to be included in foods. Belicová et al. (2013) studied the ability of 11 *Lb. plantarum* strains isolated from cheeses to form biogenic amines from tyrosine, histidine and ornithine. Three out of 11 strains were able to decarboxylate tyrosine into tyramine whereas none of them produced histamine and putrescine.

In our study, decarboxylase activity was evidenced neither by *Lb. plantarum* ATCC 8014 nor by its four phage-resistant mutants, since a colour change in the medium was not observed, suggesting biogenic amines were not produced (data not shown).

3.8. Animal trials

To study the capacity of *Lb. plantarum* strains to promote gut defences, the quantification of both IgA in intestinal fluid and the cytokines released in the small intestine were determined following their administration to conventional mice. At the mucosal surfaces, especially the gut, IgA is the main immunoglobulin where its main function is to exert the immune exclusion of invading pathogens (Brandtzaeg et al., 1987). As a functional trait, the capacity of enhancing mucosal IgA and cytokines such as IL-10 in the gut after oral administration is a desirable attribute for probiotic bacteria (Galdeano, de Moreno de Leblanc, Carmuega, Weill, & Perdigón, 2009; Zacarías, Binetti, Laco, Reinheimer, & Vinderola, 2011).

The phage-resistant mutant M1 showed the same behaviour as its sensitive parent strain *Lb. plantarum* ATCC 8014. They were able to induce the production of 190 ± 10 μg IgA per g of intestinal fluid after 10 days of treatment. As regards the levels of cytokines, the release of IL-10 was 2.9 ± 0.1 ng g^{-1} whereas for INFγ a concentration of 0.190 ± 0.1 ng g^{-1} was obtained. Zago et al. (2011) reported *Lb. plantarum* strains isolated from cheeses enhanced the number of Ig-A producing cells in mice fed for 2, 5 and 7 consecutive days (by immunofluorescence test). The translocation assay was negative. Regarding the ability to promote gut defences, a similar performance of phage-resistant mutants and their respective sensitive strains was reported for *Lactobacillus casei/paracasei* and *Lactobacillus delbrueckii* (Capra, Tibaldo, Vinderola, Reinheimer, & Quiberoni, 2014; Vinderola et al., 2007).

Table 3
Minimum inhibitory concentration values of diverse antibiotics for *Lb. plantarum* strains.[a]

Strain	Antibiotic						
	Van	Gen	Tet	Ery	Amp	Str	Cmp
ATCC 8014	>512	1	32	<0.125	1	8	4
M1	>512	<0.25	32	<0.125	0.5	8	2
M3	>512	<0.25	32	<0.125	2	8	4
MC4	>512	<0.25	32	<0.125	0.5	8	4
MC6	>512	<0.25	32	<0.125	0.5	2	4
BP	n.r.	16	32	1	2	n.r.	8

[a] Values are in mg mL^{-1}. Abbreviations are: Van, vancomycin; Gen, gentamycin; Tet, tetracycline; Ery, erythromycin; Amp, ampicillin; Str, streptomycin; CMP, chloramphenicol; BP, breakpoint suggested by FEEDAP panel for *Lb. plantarum*; n.r., not required.

4. Conclusions

Spontaneous phage-resistant mutants studied in this work exhibited potentially biological/probiotic characteristics similar to or enhanced than the phage-sensitive strain *Lb. plantarum* ATCC 8014. Some of these properties (resistance to gastrointestinal conditions and β-galactosidase activity, in particular) were improved for some mutants. Specifically, M1 and MC6 mutants showed the best adaptation to gastrointestinal passage and the highest β-galactosidase activity. In addition, the four phage-resistant mutants as well as the sensitive strain showed safety characteristics since they were sensitive to antibiotics and were not able to generate biogenic amines. These natural mutants, with a good technological performance previously demonstrated (Briggiler Marcó et al., 2011) and improved potentially health promoting properties, could be included in culture rotation programs, in replacement of their sensitive strain, as a tool to minimise the phage infection during fermented food manufacture.

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