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Functional properties of *Lactobacillus plantarum* strains: A study *in vitro* of heat stress influence

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ABSTRACT

Functional properties of two *Lactobacillus plantarum* strains (Lp 813 and Lp 998) were evaluated (*in vitro*) before and after heat adaptation and shock. The stress conditions were selected considering a previous work: 55 °C – 15 min (heat shock) and 45 °C – 30 min (thermal adaptation), both performed in MRS broth. The functional properties evaluated were: a-survival to simulated gastrointestinal tract (GIT: saliva + bovine pepsina solution brought to pH 2.3 in 90 min, bile solution 1% w/v pH 8–10 min and bile + pancreatin solution pH 8–30 min), b-co-aggregation with *Saccharomyces cerevisiae* to simulate intestinal epithelia adhesion, c- auto- and co-aggregation with enteric pathogens (*Escherichia coli* and *Salmonella enteritidis*) and d-use of prebiotic compounds (inulin, soluble corn fiber, lactulose and raffinose) evaluated for kinetics of growth. Heat treatments (generally) affected the studied functional properties as follows: a-diminished the resistance to acidic step and improved survival undergoing bile-pancreatin step in GIT simulation, b-mean values of auto-aggregation and co-aggregation with yeast or pathogens were not significantly modified, despite an increase in variability and c-prebiotic use diminished in the case of strains subjected tothermal treatments except for raffinose, which showed to have been better employed in this last case. These results could be very useful when it comes to the selection of microorganisms to be used and conserved in processes and methodologies that involve high temperatures as a stress factor (spray drying or cheese elaboration).

1. Introduction

Lactobacillus plantarum is a versatile species of lactic acid bacteria (LAB), capable of fermenting milk, vegetables, coffee, meat and silage, that is also widely used as a starter or adjuvant culture for the production of fermented food. It can be found in numerous ecological niches, including the gastrointestinal tract of humans and animals (Chibanni-Chennoufi et al., 2004; de Vries et al., 2006). In addition, *L. plantarum* shows a wide phenotypic diversity, which allows to find strains with different metabolic capacities to be used in many industrial processes (Siezen and van Hylckama Vlieg, 2011). Some *L. plantarum* strains are considered "potential probiotics". According to FAO/WHO (2002) probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host". Recently, some authors (Hill et al., 2014; Sanders et al., 2014) suggested that under the "probiotic" concept should be included all "live microorganisms potentially beneficial to host's health".

Microorganisms used in food manufacture, including probiotics, are subjected to adverse conditions (e.g. changes in temperature, pH, electrolytes and oxidative agents) during their conservation and/or production process (Zotta et al., 2008). These factors could affect the viability and/or functional properties of microorganisms. Sophisticated defense mechanisms against stress conditions were developed by lactic acid bacteria (LAB) in order to survive when exposed to sudden environmental changes (Van de Guchte et al., 2002; Serrazanetti et al., 2009). Probiotic selection criteria should include not only the evaluation *in vitro* and *in vivo* of functional properties in optimal conditions but also after stress, with the aim of determining the maintenance of such properties (Makinen et al., 2012). The successful application of probiotic strains in food industry will undoubtedly depend on their intrinsic or acquired ability to maintain their viability and interesting functional properties, assuring the quality and potential probiotic-health claims of the final product. In this regard, available information about the effect of diverse stress factors on microbial functional properties is really limited (Gardiner et al., 2000).

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The aim of our work was to investigate the heat stress effect (adaptation and/or shock) on functional properties (*in vitro*) of strains with demonstrated probiotic potential.

2. Materials and methods

2.1. Strains and culture conditions

L. plantarum strains used (Lp 813 and Lp 998) were isolated from Italian and Argentinean cheeses and were proposed as potential probiotic microorganisms in a previous work (Zago et al., 2011). The strains were stored frozen at −20 °C and −80 °C, in MRS broth (Biokar, Beauvois, France), added with glycerol (15% v/v) as cryoprotective agent. They were routinely reactivated in MRS (Biokar, Beauvois, France) broth (24 h−34 °C) and stocked in fridge. *Saccharomyces cerevisiae* strain (INLAIN Collection) was used for co-aggregation assays (Adlerberth et al., 1996; Zago et al., 2011), to simulate intestinal adhesion mediated by mannose-like receptors. This strain was replicated periodically in ME broth (Malt Extract), incubating at 25 °C during 24 h without shaking. Two microorganisms, *Escherichia coli* V517 and *Salmonella enteritidis* OMS-Ca (INLAIN Collection), were used for co-aggregation with enteric pathogens. These strains were replicated periodically in TS broth (Tryptone Soy, Britania, Buenos Aires, Argentina), incubating at 37 °C during 24 h. All strains were stored by freezing at −20 °C and −80 °C, in proper culture medium (ME and TS broth), added with glycerol (15% v/v) as cryoprotective agent.

2.2. Heat pre-treatment (adaptation) and heat shock

Functional properties were assessed in the strains Lp 813 and Lp 998 in the following conditions: i) control (without heat treatments), ii) after heat shock (HS) and iii) after heat adaptation and later heat shock (HA + HS). Cultures in stationary growth phase (16 h, approx. 10^9 UFC/ml) were used for the assays. They were centrifuged (6000 *g*−10 min, 15 °C), washed twice with 10 mM phosphate buffer pH 7 (PB7) and suspended in MRS broth to initial volume. Heat treatments were applied as follows: heat adaptation (45 °C − 30 min) and/or heat shock (55 °C − 15 min). The heat stress conditions were determined in a previous study of our work group (Ferrando et al., 2015). After heat treatments the cultures were centrifuged (6000 *g* − 10 min, 15 °C), washed with PB7 buffer and subjected to the functional properties evaluation.

2.3. In vitro functional properties

2.3.1. Survival to simulated gastrointestinal tract (GIT)

The pellets obtained as described in 2.2 were suspended in saliva solution (Binetti et al., 2013) addition of bovine pepsin (3 g/l) to initial volume, according to Zago et al. (2011) with modifications. The suspensions were acidified with HCl (3 N) from pH 4.0 until 2.3, incubating at 37 °C between each pH modification as indicated in Table 1. After acid stress the suspensions were centrifuged (6000 *g* − 10 min, 15 °C), suspended in bovine bile 1% (w/v), prepared in phosphate buffer (0.1 M pH 8), to initial volume and incubated at 37 °C for 10 min. Then, suspensions were centrifuged, suspended in bile-pancreatin solution (0.3% and 0.1% w/v, respectively), prepared in phosphate buffer (0.1 M pH 8), and incubated at 37 °C during 1 h. Samples were taken at different times (Table 1) and cell counts were performed. Resistance index (RI), defined as $RI = \log N_0/N_f$ (N_0 = initial cell count; Nf = final cell count) was calculated in each case.

Table 1
Methodological conditions to simulate the gastro intestinal tract (GIT) passage of *L. plantarum* strains and sampling points.

GIT step[a]	Cellular suspension	Incubation time (37 °C)
1	Saliva + pepsine solution[b]	
	pH 4.0	30 min
	pH 3.0	30 min
	pH 2.5	15 min
	pH 2.3	15 min
2	Bile (1% w/v), pH 8[c]	10 min
3	Bile + pancreatin (0.3 + 0.1%, w/v), pH 8[d]	60 min

[a] Cell count points (MRS agar, 48 h−34 °C), before and after each step.
[b] Saliva solution (0.125 M NaCl, 0.007 M KCl, 0.045 M $NaHCO_3$) added of bovine pepsin (3 g/l).
[c] Bovine bile solution prepared in phosphate buffer (0.1 M pH 8).
[d] Bile-pancreatin solution prepared in phosphate buffer (0.1 M pH 8).

2.3.2. Co-aggregation with Saccharomyces cerevisiae as simulation of intestinal adhesion

The methodology proposed by Zago et al. (2011) was applied with modifications: *L. plantarum* cultures obtained as described in 2.2 were suspended in PBS buffer (0.1 M pH 7.2), concentrating 5 times in relation to the initial volume of culture. *S. cerevisiae* was inoculated in ME broth (4% v/v), incubating at 25 °C for 24 h, without shaking. The cultures were centrifuged (6000 *g* − 10 min, 15 °C), washed twice with PBS and suspended in the same buffer to final concentration of 1% (w/v). The co-aggregation mix consisted of 2 ml of yeast suspension, 1 ml of *L. plantarum* suspension and 1 ml of PBS buffer. The yeast auto-aggregation (control) was performed with 2 ml of yeast suspension and 2 ml of PBS buffer. The mixes were gently shaken for 20 min and left to rest for 1 h at room temperature. 10 μl of supernatant were carefully taken and the yeast count was performed in Neubauer chamber.

Co-aggregation percentages were calculated using the following formula: % Co-aggregation (% Co) = $[1 - (N_f/N_c)] \times 100$, where N_f = yeast final count in the mix supernatant and N_c = yeast final count in yeast auto-aggregation (control) supernatant.

2.3.3. Lactobacillus plantarum auto-aggregation and co-aggregation with enteric pathogens

The protocol proposed by Kos et al. (2003) was modified as follows: *L. plantarum* cultures obtained as described in 2.2 were suspended in PBS buffer to final optical density ($O.D._{560nm}$) of 0.5. Pathogen cultures were centrifuged, washed twice with PBS buffer and suspended in the same buffer to final $O.D._{560nm}$−0.5.

Identical volumes of *L. plantarum* and pathogen suspensions were mixed to evaluate the co-aggregation. To determine pathogen auto-aggregation, individual suspensions were used. Optical density (560 nm) was determined at 0 and 5 h, ensuring that the samples were left to rest in between readings. Aggregation percentages were calculated using the following formula: % Aggregation (% Au) = $[1 - (OD_f/OD_i) \times 100$, where OD_f = optical density (560 nm) at 5 h and OD_i = optical density (560 nm) at 0 h.

2.3.4. Utilization of prebiotics

Growth kinetics of strains were performed using modified MRS broth, replacing glucose with different prebiotics as a carbon source. Glucose was tested as control, while inulin, soluble corn fiber, lactulose or raffinose at 20 g/l of final concentration were the prebiotics assayed. Different media were inoculated at 2% (v/v) with *L. plantarum* cultures obtained as described in 2.2 and incubated 20 h at 34 °C. Optical density (570 nm) values were determined every 30 min and growth kinetics plotted. The μ_{max} (maximum specific speed, $\mu_{max} = \ln OD_f - \ln OD_0/\theta_f - \theta_0$; OD_f = final optical density; OD_0 = initial optical density; θ_f = final time; θ_0 = initial

time) were calculated for each condition.

2.4. Statistical treatment

All experiences were performed by triplicate and in independent assays. Data processing was made applying one-way ANOVA and Student's test (t), using the 170 IBM SPSS® Statistics Version 2.0.

3. Results

3.1. Survival to simulated gastrointestinal tract (GIT)

Survival of both strains was similar, showing a reduction of 4 log orders in total cell count after the complete treatment. Analyzing in particular the impact of each step, the last one (bile-pancreatin) was the more lethal, reducing approx. 2.2 log orders of cell count (Fig. 1A and B). Moreover, Lp 813 was slightly more resistant than Lp 998 in the first two steps.

When the strains were subjected to heat shock, the remaining cells were significantly more sensitive to the first step (acification), showing 2 to 3 log orders of cell death more than in the controls. Bile shock (step 2) resistance was not affected by heat shock, whereas survival to bile-pancreatin (step 3) significantly increased for both strains in comparison with the controls, showing 2 log orders more of viable cell counts (Fig. 1A and B).

Heat adaptation prior to heat shock caused different behavior in both strains undergoing the first step. While Lp 998 showed a significant increase of 2 log orders in survival compared with cells only exposed to heat shock (without adaptation), Lp 813 did not show significant differences in either stress condition. On the other hand, the response of both strains undergoing steps 2 and 3 was not different, with or without heat treatments (Fig 1A and B).

3.2. Co-aggregation with Saccharomyces cerevisiae as simulation of intestinal adhesion

Under control conditions, both *L. plantarum* strains showed a similar interaction with the *S. cerevisiae* strain, with % Co mean values of 77% and 68% for Lp 813 and Lp 998, respectively. When heat treatments were performed, a reduction in the mean percentages was obtained for both strains in relation to the controls (Fig. 2). After heat shock, mean values were 54.4% and 56.8% for Lp 813 and Lp 998, respectively. When heat adaptation was applied prior to shock, Lp 813 showed higher co-aggregation (70.7%) whereas Lp 998 showed lower interaction (38.2%). Besides, a high variability of RI was observed, what would justify the absence of significant differences ($\alpha = 0.05$, one-way ANOVA) among mean values for both strains in each condition or for each strain in different conditions.

3.3. Lactobacillus plantarum auto-aggregation and co-aggregation with enteric pathogens

Both strains showed similar mean values of auto-aggregation, namely 5%–10% in control conditions. Mean values slightly increased after heat treatments, showing higher response variability (Fig. 3). No significant differences (one-way ANOVA, $\alpha = 0.05$) were found between strains in the same condition or among three conditions for the same strain.

Co-aggregation percentages (% Co) of the *L. plantarum* strains with enteric pathogens were similar for both strains against both

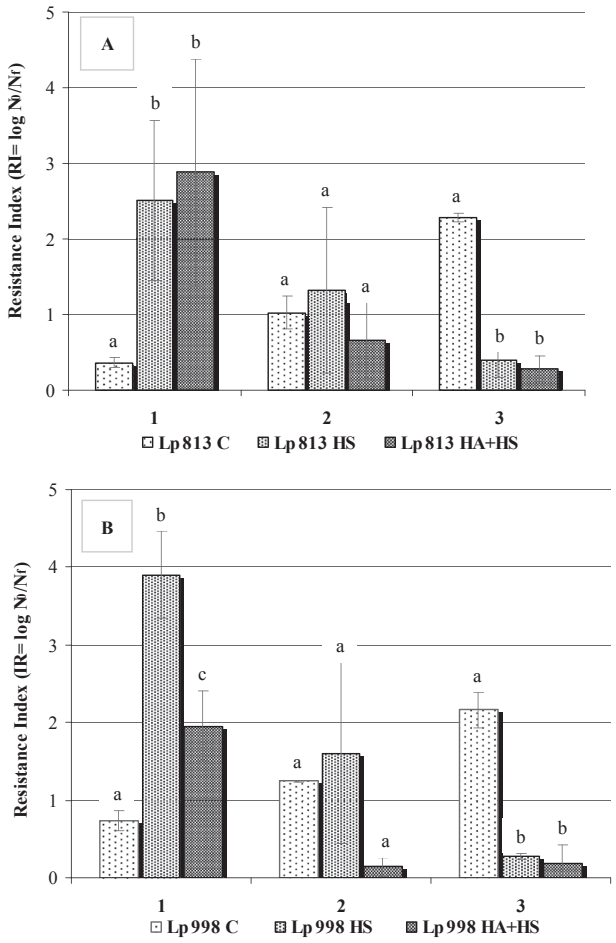

Fig. 1. Resistance Index (RI) of Lp 813 (A) and Lp 998 (B) obtained at each step of simulated gastrointestinal tract (GIT) passage, in control conditions (C) and subjected to heat adaptation (HA: 45 °C − 30 min) and/or heat shock (HS: 55 °C − 15 min) in MRS broth. RI = log N_0/N_f, with N_0 = initial cell count; Nf = final cell count after treatment. Step 1: Saliva + pepsin and pH gradient, Step 2: Bile shock and Step 3: Bile-pancreatin solution. Different letters for each step indicate significant difference ($\alpha = 0.05$, Student t Test).

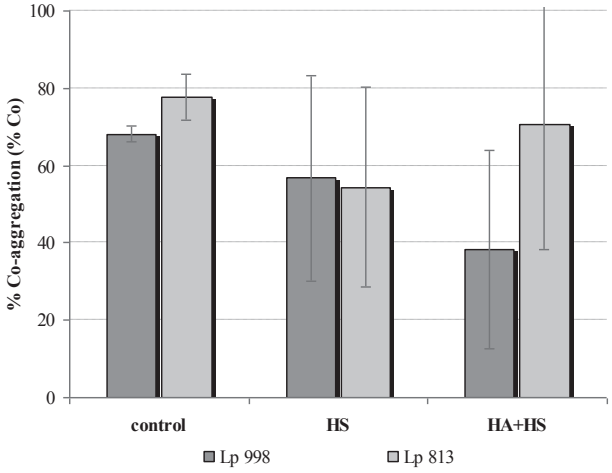

Fig. 2. Co-aggregation percentage (% Co) of *L. plantarum* strains with *S. cerevisiae* in control conditions (C) and subjected to heat adaptation (HA: 45 °C − 30 min) and/or heat shock (HS: 55 °C − 15 min) in MRS broth. % Co = [1 − (N_f/Nc)] × 100, where Nf = final yeast count in the mix supernatant and N_c = final yeast count in yeast auto-aggregation supernatant, both after 1 h of experience. No significant differences were found (one-way ANOVA, $\alpha = 0.05$) between strains neither in each condition nor for each strain in the three conditions.

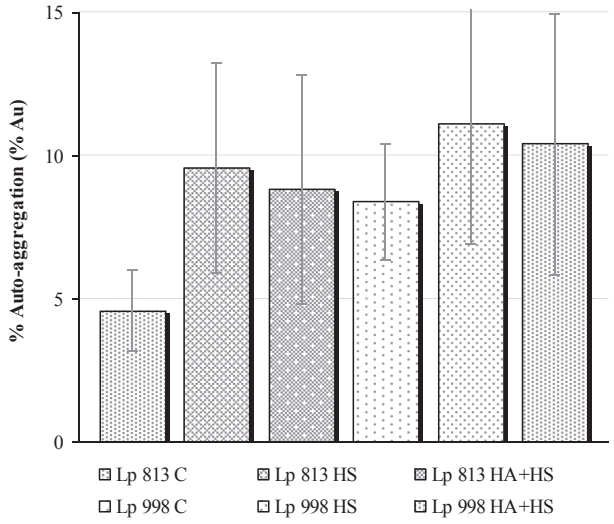

Fig. 3. Auto-aggregation percentage (% Au) of *L. plantarum* strains in control conditions (C) and subjected to heat adaptation (HA: 45 °C − 30 min) and/or heat shock (HS: 55 °C − 15 min) in MRS broth. % Au = [1 − (OD_f/OD_i)] × 100, where OD_i = initial optical density and OD_f = optical density after treatment (5 h). No significant differences were found (one-way ANOVA, $\alpha = 0.05$) between strains neither in each conditions nor for each strain in the three conditions.

pathogens. No significant differences (one-way ANOVA, $\alpha = 0.05$) were found regarding the same pathogen, neither among the same strain of *L. plantarum* nor among the same stress treatments Mean values were of 8.3% and 6.5% for Lp 813, and 11.1% and 7.9% for Lp 998, with *E. coli* and *S. enteritidis* respectively (Fig. 4A and B). On the other hand, the mean auto-aggregation value (% Au) of *E. coli* was higher than the one of *S. enteritidis*, being of 16.5% and 7.5%, respectively (data not shown). It may be due to the fact that both Lp 813 and Lp 998 always showed higher co-aggregation with *E. coli* than with *S. enteritidis*.

3.4. Utilization of prebiotics

All kinetics obtained for Lp 813 and Lp 998 when prebiotics were used instead of glucose showed a longer lag phase. Moreover, the behavior of both strains was similar considering the use of different prebiotics, with or without heat treatments.

Both strains grew very poorly when soluble corn fiber or inulin acted as a carbon source. Heat treatments either reduced or maintained the growth of both strains in the presence of these prebiotics (data not shown).

Under control conditions, strains Lp 998 and Lp 813 were able to grow well by using lactulose, showing a similar growth in relation to glucose after 20 h of incubation, but with a lower growing rate. The $\mu_{máx}$ values for Lp 813 and Lp 998 were 0.68 and 0.66 ΔlnOD/h with glucose, and 0.43 and 0.45 Δ lnOD/h with lactulose. When heat shock was applied, these values (lactulose) diminished to 0.33 and 0.41Δln OD/h for Lp 813 and Lp 998, respectively. When heat adaptation was performed, $\mu_{máx}$ remained almost the same, with values of 0.34 and 0.36 Δln OD/h for Lp 813 and Lp 998, respectively. Moreover, Lp 813 was a little more affected than Lp 998 by heat treatments considering lactulose fermentation (Fig. 5A and B).

Results obtained with raffinose were particularly interesting (Fig. 6A and B). Kinetics showed atypical behavior compared to sigmoid curves usually obtained for strain development. After 20 h, growth in MRS-raffinose was approx. 63% in relation to glucose (control). Surprisingly, strains with heat treatments developed much better than strains without these treatments. Both strains

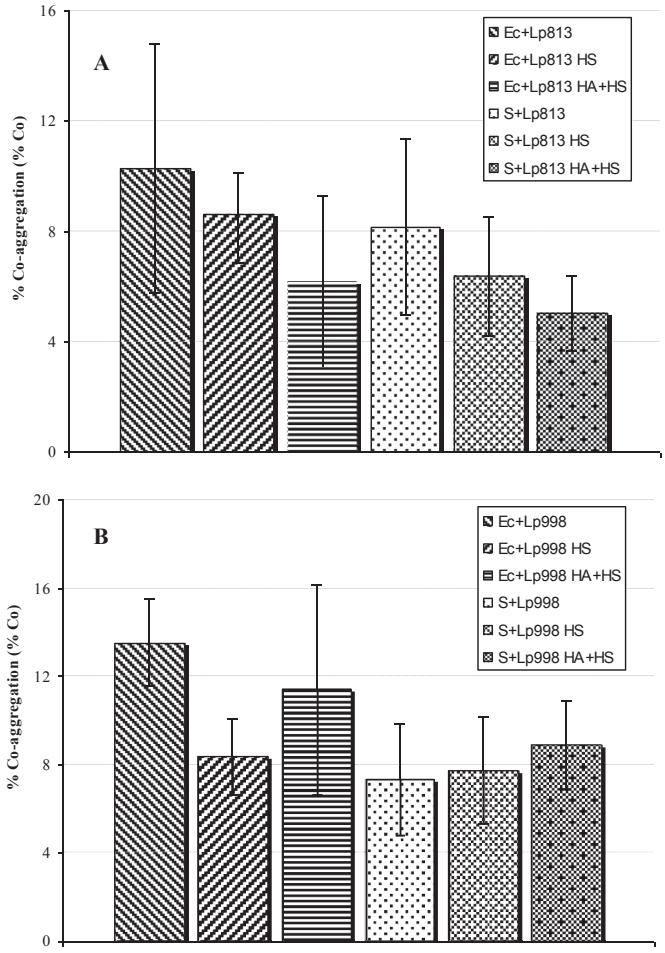

Fig. 4. Co-aggregation percentage (% Co) of *L. plantarum* strains (Lp 813 − A and Lp 998 − B) in control conditions (C) and subjected to heat adaptation (HA: 45 °C − 30 min) and/or heat shock (HS: 55 °C − 15 min) in MRS broth, with *E. coli* (Ec) and *S. enteritidis* (S) strains. % Co = [1 − (OD_f/OD_i)] × 100, where OD_i = initial optical density and OD_f = optical density after treatment (5 h). No significant differences were found (Student *t* Test and one-way ANOVA, $\alpha = 0.05$) for both strains in all conditions with the two pathogens.

grew almost as much as the control of glucose after 20 h of experience ($OD_{570\ mn}$ ~ 1.5). The $\mu_{máx}$ values with raffinose without heat treatments were 0.22 and 0.18 lnOD/h for Lp 813 and Lp 998, respectively. When heat shock and adaptation with further heat shock were applied, these values increased to 0.28 Δ lnOD/h (for both strains) and 0.24 and 0.22 Δ lnOD/h (Lp 813 and Lp 998 respectively).

4. Discussion

High resistance after passage through the gastrointestinal tract (GIT) is essential for any potential probiotic microorganism, since it must reach the intestine showing a large number of viable cells (approx. between 10^5 and 10^6 UFC/g or ml) in order to produce a beneficial effect on the consumers' health (Gobbetti et al., 2010). Results reported by previous studies showed high diversity in response to the GIT passage, depending on diverse factors such as the protocol used to perform the assay, growth strain conditions before assay and, obviously, intrinsic resistance of tested microorganism (Whitehead et al., 2008; Zago et al., 2011; Van Bokhorst-van de Veen et al., 2012; Bove et al., 2013). Both strains used in this study showed a good general resistance to GIT passage, diminishing

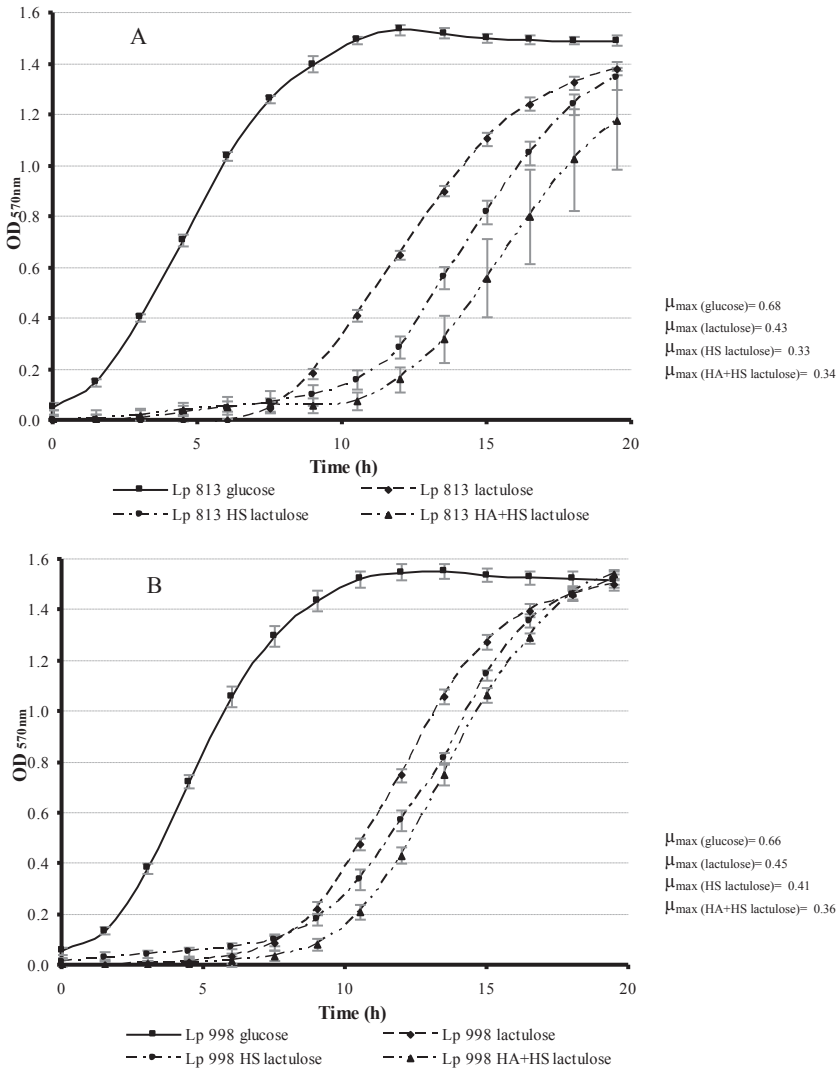

Fig. 5. Kinetics of growth ($OD_{570\ nm}$) of Lp 813 (A) and Lp 998 (B) in modified MRS broth with lactulose (instead of glucose) in control conditions (C) and subjected to heat adaptation (HA: 45 °C − 30 min) and/or heat shock (HS: 55 °C − 15 min) in commercial MRS broth. The μ_{max} values (ln OD_f − ln $OD_0/\theta_f - \theta_0$, where OD_f = final optical density; OD_0 = initial optical density at 570 nm; θ_f = final time; θ_0 = initial time) are also shown.

3.5–4.0 log orders of viable cell count, thus remaining approx. 10^6–10^5 UFC/ml. The first GIT step involves strain resistance against acidic environment and enzymes presence (mainly pepsin). Zago et al. (2011) studied the lysozyme resistance (100 mg/l − 30 min–37 °C) of 27 *L. plantarum* strains and reported for 15 of them, a resistance higher than 68% and, among them, Lp 813 and Lp 998 were included. Acid-stress response in lactobacilli is a process that mobilizes a large spectrum of different cellular functions. As acknowledged, the three main mechanisms, which regulate the homeostasis of the intracellular pH (pHi) and the proton − translocating in lactobacilli, are the F0F1-ATPase proton pumps, amino acid decarboxylation/catabolism and the expression of general stress proteins (GSPs) and chaperones that repair or degrade damaged DNA and proteins (Cotter and Hill, 2003; De Angelis and Gobbetti, 2004). However, some authors found that F0F1-ATPases were less abundant in acid-stressed strains than in the strain without acid stress (Hamon et al., 2013, Heunis et al., 2014). Also, small heat shock proteins (Hsp1 and 3) and chaperonins (DnaK, GrpE, GroEL, and GroES), were detected in acid stressed strains (Serrazanetti et al., 2009; Heunis et al., 2014). The identity of these induced heat shock proteins can vary from one species to another (De Angelis and Gobbetti, 2004). Some authors (Zago et al., 2011; Van Bokhorst-van de Veen et al., 2012; Bove et al., 2013; Turchi et al., 2013) reported good resistance to acid step on GIT for *L. plantarum* strains and suggested that this resistance strongly depends on the final pH value reached on the assay. These authors also reported that, if the final pH was between 3.0 and 2.3, cell viability diminished approx. 0–3 log orders when applied in cells in stationary phase. In our study, Lp 813 and Lp 998 showed high survival at this step, in which the final pH was 2.3. Regarding bile shock, our strains showed good resistance, diminishing cell count in approx. 1 log order. This resistance could be due to bile salt hydrolase activity (bsh) that deconjugates bile salts and inactivates its powerful antimicrobial action (Van de Guchte et al., 2002). The *bhs* gene and its expression (active hydrolase) have been reported for 27 *L. plantarum* strains, including Lp 813 and Lp 998, in a previous work (Zago et al., 2011). Finally, when reaching the intestine, probiotics must resist the action of pancreatic enzymes that can have an effect on the cell wall or membrane components affecting their viability or activity. Lp 813 and Lp 998 diminished approx. 2 log orders their cell count at this GIT step. Zago et al. (2011) reported good survival (>4 10^5 UFC/ml) after bile-pancreatin exposure only

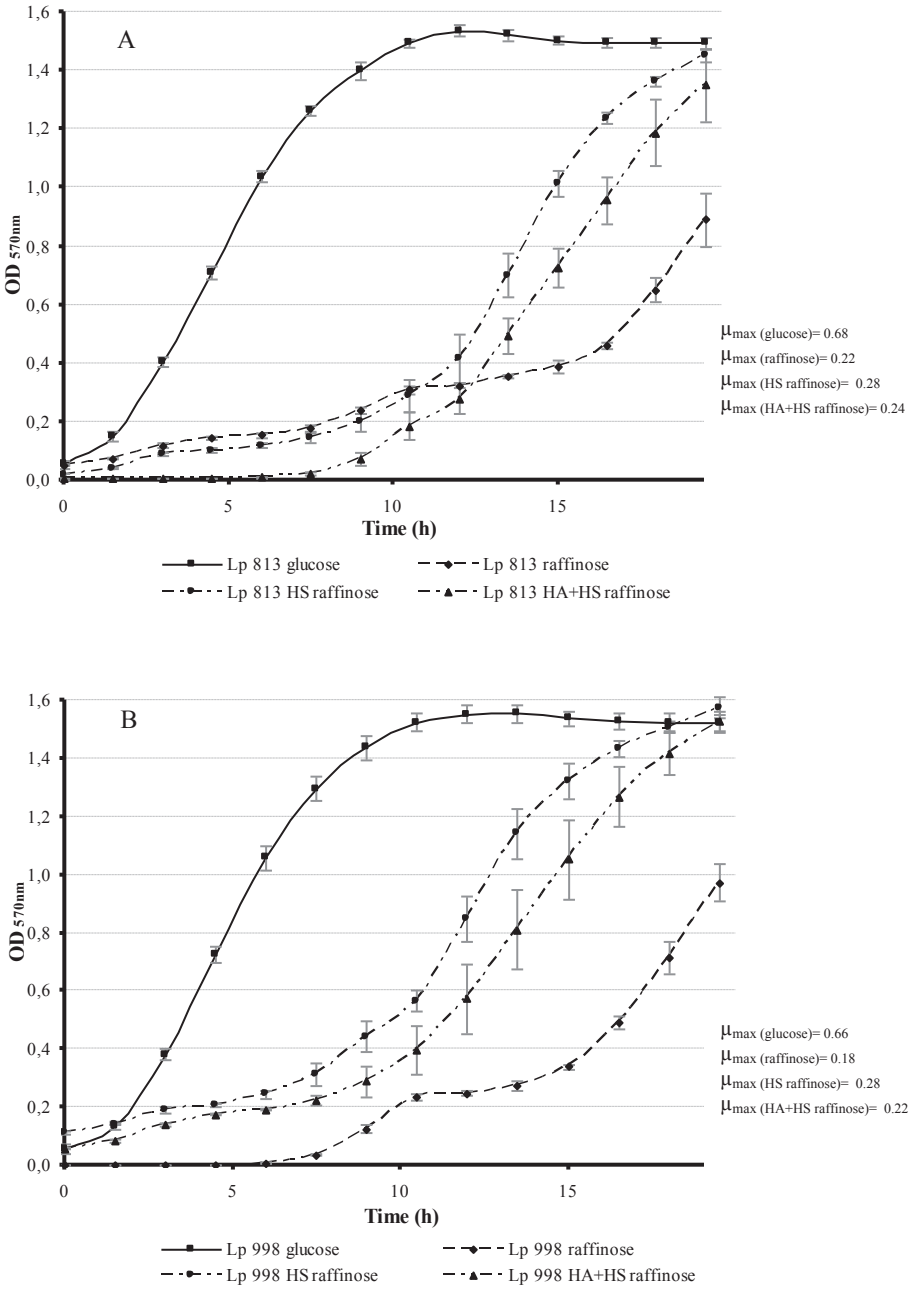

Fig. 6. Kinetics of growth ($OD_{570\ nm}$) of Lp 813 (A) and Lp 998 (B) in modified MRS broth with raffinose (instead of glucose) in control conditions (C) and subjected to heat adaptation (HA: 45 °C − 30 min) and/or heat shock (HS: 55 °C − 15 min) in commercial MRS broth. The μ_{max} values ($\ln OD_f - \ln OD_0/\theta_f - \theta_0$, where OD_f = final optical density; OD_0 = initial optical density at 570 nm; θ_f = final time; θ_0 = initial time) are also shown.

for 7 *L. plantarum* strains from a total of 27 strains. As for *L. plantarum* WCFS1, Van Bokhorst-van de Veen et al. (2012) informed that stationary phase cells died less than 1 log order after bile-pancreatin stress (bile salt 0.5% w/v and 3.0% w/v of porcine pancreatin at pH 6.5 during 1 h). On the other hand, Bove et al. (2013) demonstrated that *L. plantarum* WCFS1 did not diminish their cell count in presence of bile − pancreatin (0.3%–0.1%) when the pH of the acidic step was between 4.0 and 6.0, while for lower pH values, a slightly recovering of the cells was observed.

After heat shock, both strains showed higher sensitivity to the acid step, with cell death increasing in 2–3 log orders compared with controls, probably due to destabilization of cell wall and membrane, together with denaturalization of proteins and other macromolecules, as a result of heat stress (Van de Guchte et al., 2002). The response to bile shock was not affected by heat shock, probably because it involves proteins which are more specific to this stress, such as bile salt hydrolase. Golowczyc et al. (2011) reported that *Lactobacillus kefir* 8321 and *L. kefir* 8348 subjected to spray drying (where heat shock is the main stress factor) did not show a different response to bile in relation to the controls. On the other hand, viability after intestinal stress increased in cells previously subjected to heat shock. It could be possible that the combination of heat shock, plus acid and bile stress through the GIT passage activated the expression of certain stress related proteins, generating an adaptive response to further stress such as the one due to pancreatic enzyme activity.

Differences in the behavior of Lp 813 and Lp 998 were observed at the acid step when heat adaptation was applied prior to heat shock. Lp 813 cells showed similar viability when heat was applied, with or without heat adaptation, whereas Lp 998 improved survival to this step due to adaptation, increasing 2 log orders of cell survival. Heat stress induces the synthesis of proteins, including chaperone ones, which are also present when the cells are stressed by others factors as acidity, high salt concentration and starvation (De Angelis et al., 2004; Arena et al., 2006, Zotta et al., 2008; Serrazanetti et al., 2009). The induction of General Stress Proteins (GSPs) by heat should protect the cells also against low pH values and would be predictable a better viability after the acid step. Nevertheless, this effect was only observed for Lp 998 strain. In agreement with this result, De Angelis et al. (2004) reported that DnaK and GroEL were induced only from exponential phase cells but not from stationary phase cells of *L. plantarum* DPC2739. It seems that the improvement of cell survival against GIT conditions by means of heat adaptation is strain dependent. Step 2 and 3 did not modify the cell viability of either strain, with or without adaptation.

Auto- and co-aggregation with pathogens or yeasts are superficial properties related with the adhesion to intestinal epithelia (auto- and co-aggregation with yeasts) and their ability to stick to enteric pathogens, reducing their possibility of adhesion to intestinal epithelia (Servin and Cocconier, 2003; Jankovic et al., 2012; Sengupta et al., 2013). In particular, co-aggregation with *S. cerevisiae* specie was proposed by Adlerberth et al. (1996) due to the presence of a specific adhesin reported for *L. plantarum* (*msa*), which binds with mannose residues present in intestinal cell lines, such as HT29. Due to the fact that these residues are also present in *S. cerevisiae* cell wall, this yeast was used as an *in vitro* model to simulate intestinal adhesion of Lp 813 and Lp 998 (Zago et al., 2011).

Auto-aggregation values obtained for Lp 813 and Lp 998 were determined at 5 h and ranged from 5 to 10%. Even if this interaction could be considered low, it is expected to increase with longer experimental time. In fact, Janković et al. (2012) reported, for *L. plantarum* strains, auto-aggregation values from 20 to 30% for 5 h and from 80 to 90% after 24 h. Abdulla et al. (2014) informed auto-aggregation values similar to our results (from 12 to 22% after 5 h of incubation) for *Lactobacillus paracasei*, *Lactobacillus acidophilus* and *L. plantarum* strains isolated from dairy products. The capacity of *Bifidobacterium* spp. strains of human origin to adhere to Caco-2 and HT29 cell lines has been associated to high auto-aggregation and hydrophobicity values (Pérez et al., 1998; Del Re et al., 2000; Servin and Cocconier, 2003). Lp 813 and Lp 998 showed high co-aggregation values with *S. cerevisiae* (77% and 68%, respectively). Out of a total of 33 strains of *L. plantarum* evaluated, Turchi et al. (2013) found that 21 of them were positive to this test. These authors, as well as Zago et al. (2011), used a simple microscopic observation to perform the assay, thus obtaining subjective results. We developed a quantitative test, obtaining highly objective values, suitable to comparisons.

The co-aggregation values of Lp 813 and Lp 998 with *E. coli* and *S. enteritidis* ranged between 5 and 14% after 5 h of experience. Both strains showed higher % Co values with *E. coli*, possibly due to the higher auto-aggregation (16.5%) obtained for this pathogen, approx. twice as much as *S. enteritidis* (7.5%). Janković et al. (2012) reported, after 5 h of experience, values between 9 and 17% for *L. plantarum* strains with *Salmonella enterica*, *E. coli* and *Listeria monocytogenes* whilst, after 24 h, these values increased up 30–42%. Abdulla et al. (2014) informed a co-aggregation value of 19% between *L. plantarum* and *S. typhi*, after 24 h of interaction. Golowczyc et al. (2007) demonstrated that the co-aggregation of *L. kefir* 8321 with *S. enterica*, mediated by surface layer (S-layer) proteins, protected Caco-2/TC-7 cells from the invasion of this pathogen.

Heat adaptation and shock did not significantly affect neither auto-aggregation nor co-aggregation properties of Lp 813 and Lp 998. In general, heat treatments affected the variability of results but not the mean values. Turchi et al. (2013) also reported no modifications in the co-aggregation capacity of *L. plantarum* strains with *S. cerevisiae* after stress due to lysozime, acidity and bile salt exposure. Golowczyc et al. (2011) did not find any variation in the adhesion of *L. plantarum* 83114 and *L. kefir* 8321 to Caco-2 cells before and after spray drying (thermal, osmotic and oxidative stress). Servin and Cocconier (2003) also found that *L. acidophilus* LB showed an efficient adhesion to Caco-2 cells before and after lethal heat treatment.

Many commercial functional foods employ a combination of probiotics and prebiotics within their formulation. Prebiotics are selectively fermented by probiotics and commensal microorganisms but not used by enteric pathogens, promoting the growth of beneficial intestinal microflora along with the production of healthy metabolic compounds (Patel and Goyal, 2012; Hardy et al., 2013). The sequence analysis of *L. plantarum* WCFS1 provided invaluable information about the high potential of this strain to import and metabolize a large number of carbohydrates, including prebiotics. A large proportion of the genes encoding sugar transport and utilization, as well as genes encoding extracellular functions, would be important for the interaction of *L. plantarum* to the environment and would constitute a lifestyle adaptation region in the chromosome (Kleerebezem et al., 2003; Siezen and van Hylckama Vlieg, 2011). In this work, both strains studied showed a longer lag phase when growing in presence of prebiotics, in comparison with glucose utilization, probably due to the need to activate the expression and activity of proteins involved in carbohydrate metabolism. Some authors (Plumed-ferrer et al., 2008; Siragusa et al., 2014) reported, for several *L. plantarum* strains, that these microorganisms are able to simultaneously use diverse carbohydrates during the early exponential phase of growth, in spite of the presence of glucose. This behavior is particularly interesting because, for the majority of LAB, the presence of glucose inhibits the expression or activity of proteins involved in other carbohydrates metabolism (Plumed-Ferrer et al., 2008). Lp 813 and Lp 998 used better lactulose and raffinose, in a percentage between 60 and 100% in comparison with glucose. These results were consistent with those reported by Zago et al. (2011) who had previously evaluated the growth of these strains obtaining similar final values. Instead, others studies informed a better use of inulin, followed by lactulose and raffinose for different species of *Lactobacillus* of industrial and human origin (Pan et al., 2009; Kunova et al., 2011). Heat treatments affected in a different way depending on the prebiotic analyzed. In general, all prebiotics were similarly or less used by both strains after heat adaptation and shock. However, both strains used better raffinose after heat treatments, possibly due to activation of proteins general stress involved in this carbohydrate metabolism (Plumed-Ferrer et al., 2008). This result is particularly interesting since it raises the potential to include raffinose in functional foods, which production involves heat stress (eg. Spray drying) of probiotic strains of *L. plantarum*, where this prebiotic could help achieve an adequate cellular recovery after stress. In fact, protective or stabilizer effect of prebiotics has been reported on diverse *Lactobacillus* species, improving heat, oxidative and phenol response and some functional properties such as the production of short − chain fatty acids and hydrophobicity (Pan et al., 2009; Nazzaro et al., 2012).

The results obtained in this study could be very useful when it comes to the selection of microorganisms to be used and conserved in processes andmethodologies that involve high temperatures as a stress factor (spray drying or cheese elaboration).

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