Acid stress suggests different determinants for polystyrene and HeLa cell adhesion in *Lactobacillus casei*

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ABSTRACT

Adhesion has been regarded as one of the basic features of probiotics. The aim of this study was to investigate the influence of acid stress on the functional properties, such as hydrophobicity, adhesion to HeLa cells, and composition of membrane fatty acids, of *Lactobacillus* probiotics strains. Two strains of *Lactobacillus casei* were used. Adhesion on polystyrene, hydrophobicity, epithelial cells adhesion, and fatty acids analysis were evaluated. Our results showed that the membrane properties such as hydrophobicity and fatty acid composition of stressed strains were significantly changed with different pH values. However, we found that acid stress caused a change in the proportions of unsaturated and saturated fatty acid. The ratio of saturated fatty acid to unsaturated fatty acids observed in acid-stressed *Lactobacillus casei* cells was significantly higher than the ration in control cells. In addition, we observed a significant decrease in the adhesion ability of these strains to HeLa cells and to a polystyrene surface at low pH. The present finding could first add new insight about the acid stress adaptation and, thus, enable new strategies to be developed aimed at improving the industrial performance of this species under acid stress. Second, no relationship was observed between changes in membrane composition and fluidity induced by acid treatment and adhesion to biotic and abiotic surfaces. In fact, the decrease of cell surface hydrophobicity and the adhesion ability to abiotic surface and the increase of the capacity of adhesion to biotic surface demonstrate that adhesive characteristics will have little relevance in probiotic strain-screening procedures.

Key words: *Lactobacillus casei*, acid stress, hydrophobicity, HeLa cells, fatty acid

INTRODUCTION

Probiotic foods are reported to provide several health benefits, as they help in maintaining a good balance

and composition of intestinal flora and increase resistance against invasion of pathogens. The demand for probiotic functional foods is growing rapidly due to increased awareness of consumers about the effect of food on health. Development of foods with adequate doses of probiotics at the time of consumption is a challenge because several factors during processing and storage affect the viability of probiotic organisms (Tripathi and Giri, 2014).

Lactobacillus species are the major components of starter cultures used in food fermentation or as probiotics (Axelsson, 1998). These bacteria encounter various stresses during processing, production, and gastric transit, including environmental stresses such as heat and cold exposure during processing and high hydrostatic pressure during packaging (Upadrasta et al., 2011). The phenomenon of adhesion to epithelial cells has been considered a priority for the selection of probiotics and it is mediated by the physicochemical properties of the bacterial surface such as the microbial cell surface hydrophobicity, known as one of the determining factors in microbial adhesion, but also by specific molecular interactions (Upadrasta et al., 2011).

Lactobacilli are of significant importance to food industries due to their involvement in the production of various fermented dairy, meat, and vegetable foods. Also important to the health industry, *lactobacilli* are used as probiotics due to their health-promoting effects when consumed. One of the frequently exploited activities used to screen probiotic candidates is the adhesive power, which is presumed to be requisite for sufficient host-interaction to confer health benefits (Ouwehand et al., 2002). However, *Lactobacillus casei* is one of the major components of starter cultures used in food fermentation or as probiotics (Axelsson, 1998).

The changes in lipid composition enable the microorganisms to maintain membrane functions in the face of environmental fluctuations. Appropriate proportions can be reached by increasing the degree of FA unsaturation, branching, or decreasing the FA chain length, although a great diversity of responses has been observed (Suutari and Laakso, 1994).

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However, the regulation of the biosynthetic routes of FA in the majority of microorganisms has been poorly studied. In particular, the way in which the FA composition of membrane lipids is altered in response to growth temperature appears to depend on the mechanism of unsaturated fatty acid (UFA) synthesis used (Keweloh and Heipieper, 1996). In bacteria, both anaerobic and aerobic mechanisms are responsible for the synthesis of UFA (Aguilar et al., 1998). In certain bacteria and eukaryotes, the introduction of double bonds into SFA is catalyzed by oxygen-dependent desaturation of the full-length FA chain, on either an acylthioester or a phospholipid FA moiety, and requires a specific electron transport chain. Under stress, bacteria can change the composition of their cell membrane to maintain proper membrane fluidity (homeoviscous adaptation; Sinensky, 1974) to adapt to stress conditions (Cronan, 2002; Veerkamp, 1971). Proper fluidity of the membrane is essential for the mobility and functionality of embedded proteins and lipids, diffusion of proteins and other molecules across the membrane, and the appropriate separation of membranes during cell division. It has been reported that bacterial membrane composition changes during acid shock (Chang and Cronan, 1999; Kim et al., 2005), osmolarity fluctuations (Vargas et al., 2005), freeze-drying (Muñoz-Rojas et al., 2006), and exposure to suboptimal growth temperatures (Guerzoni et al., 2001).

The aim of our study was to investigate the influence of acid stress on the functional properties, such as hydrophobicity, adhesion to HeLa cells, and composition of membrane FA, of *L. casei* probiotics strains. HeLa cells were chosen because they are commonly used to study the adhesion of lactic acid bacteria (Milon et al., 1990) and their culture is relatively easy.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

The strain first used in our study, S1, was *L. casei*, a potential probiotic, obtained from the Laboratory for Analysis, Treatment and Valorisation of Environmental Pollutants and Products, Faculty of Pharmacy (Monastir, Tunisia) collection; this strain was identified by sequencing the 16S rRNA gene (BankIt1773923 BL2 KP123430). The second strain, S2, was *L. casei* ATCC 393. All strains were stored on de Man, Rogosa, Sharpe (MRS; Lab M, Bury, UK) broth added with 15% (vol/vol) sterile glycerol at -80°C . Working cultures were grown at 37°C in modified MRS broth at pH of 6.4 for 24 h. The pH of the modified MRS was 6.4 and, unless otherwise stated, it was maintained constant by addition of 1 M NaOH.

In our study, the cells were grown at 37°C on MRS for 24 h. The MRS microcosms (100 mL) were incubated at pH 3.2, 3.5, 3.8; the pH was adjusted by hydrochloric acid (Sigma-Aldrich, St. Louis, MO) using a pH meter (Bio-Rad, Hercules, CA) and autoclaved (121°C for 20 min) in 100-mL Erlenmeyer flasks. *Lactobacillus* cells were washed 3 times by centrifugation ($15,890 \times g$ for 10 min at 20°C) with MRS and then suspended in 10 mL of MRS. The microcosms (100 mL) were inoculated with these suspensions (approximately 10^9 cfu/mL) and then incubated at 37°C .

Cell Surface Hydrophobicity

Hydrophobicity was measured by the hexadecane partitioning method of van Loosdrecht et al. (1987). Bacterial cells grown overnight in MRS were washed with PBS, resuspended in 4 mL of PBS, and the optical density (OD) was determined. One milliliter of hexadecane (Sigma-Aldrich) was added to each cell suspension and equilibrated for 10 min. Each suspension was reincubated at 37°C for 30 min. The aqueous layer was removed and aerated to remove all traces of hexadecane, and absorbance (OD) was measured against a hexadecane-extracted PBS blank. The hydrophobicity index was expressed as the ratio of absorbance of the hexadecane-extracted sample to absorbance of the sample before extraction.

Adherence Assay to Measure Biofilm Production by *Lactobacillus* Cells Under Stress

Biofilm production by *Lactobacillus* strains grown in MRS with different conditions was determined using a semiquantitative adherence assay on 96-well tissue culture plates (Nunc, Roskilde, Denmark), as described previously by Chaieb et al. (2007) with some modifications. An overnight culture grown in MRS at 37°C was diluted to 1:100 in MRS supplement with 2% (wt/vol) glucose and different pH, as reported elsewhere (Rachid et al., 2000). A total of 200 μL of each cell suspension was transferred in a U-bottomed 96-well microtiter plate. Each strain was tested in triplicate. Wells with sterile MRS alone served as controls. After incubation for 24 h at 37°C , the culture was removed and the plates were washed 3 times with 200 μL of PBS (7 mM Na_2HPO_4 , 3 mM NaH_2PO_4 , and 130 mM NaCl at pH 7.4) to remove nonadherent cells and then dried in an inverted position. Adherent cells were fixed with 95% ethanol and were stained with 100 μL of 1% (wt/vol) crystal violet (Merck, Kenilworth, NJ) for 5 min. Then, unbound crystal violet was removed and the wells were washed 3 times with 300 μL of sterile distilled water. The water was then cleared and the

microtiter plate was air-dried for 3 to 4 h. The optical density of each well was measured at 570 nm using an automated Multiskan reader (GIO, De Vitae, Rome, Italy). Biofilm formation was categorized as highly positive ($OD_{570} \geq 1$), low-grade positive ($0.1 \leq OD_{570} < 1$), or negative ($OD_{570} < 0.1$).

Quantification of Viable Cells in the Biofilm by XTT Assay

The 2,3-bis [2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide (**XTT**) assay was used to quantify bacterial biofilm (Pettit et al., 2005). It measures the reduction of a tetrazolium salt [2, 3-bis (2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide] and can be easily quantified colorimetrically (Moriarty et al., 2005; Pettit et al., 2005). The XTT (Sigma-Aldrich) solution (1 mg/mL) was prepared in PBS (7 mM Na_2HPO_4 , 3 mM NaH_2PO_4 , and 130 mM NaCl at pH 7.4), filter-sterilized, and stored at $-80^\circ C$. Menadione (Sigma-Aldrich) solution (1 mM) was prepared in acetone and sterilized immediately before each assay. An overnight culture grown in MRS at $37^\circ C$ was diluted to 1:100 in MRS supplement with 2% (wt/vol) glucose. A total of 200 μL of these cell suspensions was transferred in a U-bottomed 96-well microtiter plate. Each strain was tested in triplicate. Wells with sterile MRS alone served as controls. Following incubation for 24 h at $37^\circ C$, the biofilms were first washed 5 times with 200 μL of PBS and then 100 μL of PBS and 12 μL of XTT-menadione solution (12.5:1 vol/vol) were added to each of the prewashed wells and the control wells. The plate was then incubated for 3 h in the dark at $37^\circ C$. Following incubation, 100 μL of the solution was transferred to fresh wells and the color change in the solution was measured with a multiskan reader at 492 nm (Bio-Rad).

Analysis of FA

To analyze the total cellular FA, starved and control cells recovered from 10 mL of each cell suspension were pretreated following the MIDI protocols (Sasser, 1990). All reagents for saponification, methylation, extraction, and washing were dispensed with autopipets into this same tube, making the hands-on time minimal. Next, the final extracts were analyzed by GC (column = 30 m $\times$ 0.25 mm HP-Innowax; flame ionization detection temperature = $280^\circ C$; carrier gas N_2 at 1 mL/min; injector temperature = $270^\circ C$; oven temperature programmed from 130 to $230^\circ C$) using a Hewlett-Packard HP 5890 capillary gas chromatograph linked to an HP Chemstation integrator (Hewlett-Packard, Palo Alto, CA). The identification of FAME was performed by

external standards (all purchased from Sigma-Aldrich) submitted to the same processes of manipulation as the analyzed biological samples. A known quantity of heneicosanoic acid methyl ester (C21:0) was used as an internal injection standard. The values of FA are presented as area percentage of total FA. Total SFA, total UFA, and total cyclic FA were used to determine the differences among membrane FA of lactobacilli cells grown under the different conditions. The UFA-to-SFA ratio was used as an indirect indicator of the membrane fluidity.

HeLa Cells Adherence Assays

Quantitative adherence assays of stressed and control *L. casei* cells was performed with laryngeal cancer cells (HeLa) obtained from the American Type Culture Collection (CCL2) as described by Chatti et al. (2007). HeLa cells were seeded at a concentration of 2×10^5 cells/mL and grown overnight in minimal essential medium with Earle's salts and 10% fetal bovine serum in 24-well microtiter plates at $37^\circ C$ with 5% CO_2 . Each lactobacilli strain was grown overnight in MRS at $37^\circ C$. The bacterial cells were washed 3 times by centrifugation at $7,500 \times g$ for 15 min at $4^\circ C$ with minimal essential medium without serum and resuspended in the same medium. The number of bacteria in the suspension was adjusted to 10^8 cfu/mL. The monolayers of human cells were inoculated, for each tested strain, with 10^8 cfu/mL and incubated at $37^\circ C$ in 5% CO_2 for 3 h. The cells were washed 3 times with PBS buffer and fixed in methanol for 5 min at room temperature. Staining of HeLa cells and adherent bacteria was achieved with a Giemsa solution diluted 1:20 for 30 min at room temperature. Finally, the slides were washed 3 times with PBS, dried, and mounted on slides for microscopic observation. All experiments were performed in triplicate.

Statistical Analyses

The statistical analysis was performed on SPSS v.17.0 statistics software (SPSS Inc., Chicago, IL). The statistical differences and significance were assessed by ANOVA test; $P < 0.05$ was considered significant. The statistical analysis was performed between the control cell and the stressed cell.

RESULTS

Adherence to a Polystyrene Microplate

Biofilm formation of *Lactobacillus* strains was evaluated in a 96-well plate with MRS at different pH. The results of the OD_{570} test presented in the Table 1 showed that S1 and S2 were able to form biofilm (OD_{492} was

Table 1. Adherence to polystyrene microplate, to HeLa cells, quantification of viable cells in the biofilm by 2,3-bis [2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide (XTT) assay, and hydrophobicity of *Lactobacillus casei*

Strain ¹	Hydrophobicity (%) $\pm$ SD	Adherence (OD ₅₉₅ ² $\pm$ SD)	Biofilm viability (XTT OD ₅₉₅ $\pm$ SD)	HeLa cell adhesion (cells/HeLa $\pm$ SD)
S1	23 $\pm$ 0.2	0.726 $\pm$ 0.012	0.450 $\pm$ 0.014	28.66 $\pm$ 1.52
S1i	65* $\pm$ 0.12	0.119* $\pm$ 0.043	0.125* $\pm$ 0.015	16.66* $\pm$ 2.51
S1ii	62* $\pm$ 0.15	0.095* $\pm$ 0.0006	0.066* $\pm$ 0.024	5.66* $\pm$ 0.57
S1iii	50* $\pm$ 0.3	0.1* $\pm$ 0.01	0.278* $\pm$ 0.005	11.66* $\pm$ 2.08
S2	25 $\pm$ 0.17	0.43 $\pm$ 0.046	0.427 $\pm$ 0.019	6.33 $\pm$ 0.57
S2i	70* $\pm$ 0.32	0.051* $\pm$ 0.006	0.286* $\pm$ 0.0005	4.33* $\pm$ 1.52
S2ii	68* $\pm$ 0.11	0.061* $\pm$ 0.0011	0.099* $\pm$ 0.003	12.33* $\pm$ 2.88
S2iii	30* $\pm$ 0.14	0.053* $\pm$ 0.0008	0.156* $\pm$ 0.01	5.33* $\pm$ 0.57

¹S1 = *Lactobacillus casei* of collection laboratory; S2 = *Lactobacillus casei* ATCC 393; i = strains incubated at pH 3.2; ii = strains incubated at pH 3.5; iii = strains incubated at pH 3.8.

²Optical density at 595 nm.

* $P < 0.05$: significant difference between stressed strain and control strain.

0.726 and 0.43, respectively); these values decreased significantly ($P < 0.05$) at low pH.

Quantification of Viable Cells in the Biofilm by XTT Assay

Two strains of *Lactobacillus* were adhesive to a polystyrene 96-well microtiter plate at different degrees. The optical density values of XTT reduction estimated at 492 nm were 0.45 for S1 and 0.427 for S2 (Table 1). After the application of acid stress, the ability of starved cells to form biofilm decreased significantly ($P < 0.05$). These results correlated with the result of the adhesion to polystyrene microplate.

Effect of Stress on Surface Hydrophobicity

Affinity of bacteria to solvents was performed to evaluate the surface properties of each strain and acid stress had a significant effect on the surface hydrophobicity of *L. casei* (Table 1). The percentage of bound cells to hexadecane was used to evaluate the hydrophobicity. According to Chae et al. (2006), cells could be strongly hydrophobic when the bound cells to hexadecane were higher than 55%, moderately hydrophobic (30–54%), moderately hydrophilic (10–29%), and strongly hydrophilic (<10%). The S1 and S2 strains showed a moderately hydrophobic character. The stressed cells showed a significant increase ($P < 0.05$) in surface hydrophobicity at low pH; 65, 62, and 50% with pH 3.2, 3.5, and 3.8, respectively, for S1, and 70, 68, and 30% with pH 3.2, 3.5, and pH 3.8, respectively, for S2.

Adherence to HeLa Cells

Quantitative adherence to HeLa cells of S1 and S2 strains of *L. casei* was assessed before and after acid

shock (Figure 1). Indeed, the control strain cells were slightly adherent to HeLa cells (28.66 and 6.33 cells/HeLa). Our results showed that, after incubation at pH 3.2, 3.5, and 3.8, both strains were adherent. However, S1 showed much higher adherence rate (Table 1; Figure 1). The S2 strain demonstrated different behavior, as the capacity of interaction with HeLa cells increased significantly ($P < 0.05$; 12.33 cells/HeLa) at pH 3.5. To the contrary, adherence was significantly decreased ($P < 0.05$) for S1.

Membrane FA Analysis

The membrane FA composition of *Lactobacillus* before and after incubation in the different pH was determined using a chromatographic method (Tables 2). Three predominant SFA (C14:0, C16:0, C18:0) accounted for over 50% of total FA and their proportions changed after environmental acidification.

Irrespective of pH, palmitic acid (16:0) was the major SFA, whereas palmitoleic acid (C16:1n-7), vaccenic acid (C18:1n-7), and linoleic acid (C18:1n-9) were the major UFA found in both strains (Table 3). The palmitic acid proportion of the starved cells was significantly ($P < 0.05$) higher than that observed in the control cells. Conversely, the palmitic acid (16:0) proportion of *Lactobacillus* decreased from 47 (control) to 44% (of stressed cells) after pH variation.

The proportion of other FA found in the stressed cells of *Lactobacillus* did not differ significantly ($P < 0.05$) from that observed in the control cells. It was also noted that SFA proportion is higher, whereas the UFA proportion is lower in the control cells compared with that in the starved cells. The pH treatment resulted in an increased ratio of UFA to SFA in *Lactobacillus* cells (Table 3).

Figure 1. Adherence of *Lactobacillus casei* to HeLa cells observed with optical microscope. S1 = *Lactobacillus casei* of collection laboratory; S2 = *Lactobacillus casei* ATCC 393; i = strains incubated at pH 3.2; ii = strains incubated at pH 3.5; iii = strains incubated at pH 3.8 (magnification $\times 100$). Color version available online.

DISCUSSION

With the great interest on health-oriented nutritional habits, the food industry is requested to provide more and more functional foods containing healthful components. By the recently adopted definition that probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001), probiotic bacteria seem to be such components.

To exert positive health effects, the lactobacilli should resist the stressful conditions of the stomach and upper intestine that contain bile (Chou and Weimer, 1999) and the potential for the lactic acid bacteria to provide probiotic benefit, after exposure to the stressful conditions of the gastrointestinal tract, remains intact (Amund et al., 2014). Acidity is believed to be the most detrimental factor affecting growth and viability of lactobacilli, because their growth was down significantly below pH 4.5 (Lankaputhra and Shah, 1995; Lankaputhra et al., 1996). In the present study, it was observed the influence of pH medium on the many properties of the probiotics strains *L. casei*, such as hydrophobicity, adhesion to host cells, and composition of FA membrane. In fact, several studies showed that *L. casei* ATCC 393 has a poor ability to adhere to intestinal epithelial cells (Huseh et al., 2010). This strain changes its membrane biochemical composition as a function of the external environment. Most of these variations compensate each other to maintain membrane fluidity. However, the proton permeability

and packing of lipid vesicles were altered in response to the external ionic strength of the medium. These changes are likely to be critical to cope with the stress conditions produced by the osmotic imbalance between the cells and their environment (Machado et al., 2004).

To check whether potential correlation exists between a given surface of *L. casei* and its ability to interact with a target intestinal tract, we studied the adhesion of our strains to HeLa cells. As a result, we cannot conclude the direct involvement of the physicochemical properties of the surface on the adhesion of *L. casei*. Indeed, we observed that the surface hydrophobicity increased significantly with decreasing pH values, whereas adhesion ability to HeLa cells decreased for S1. At pH 3.5, S2 had an elevated adhesive power with an elevated hydrophobicity, whereas the S1 strain, having a hydrophobicity bred to this pH, exhibits a very low adhesion. Similar results have been described by Wu et al. (2012), who indicated that alteration in membrane fluidity, FA distribution, and cell integrity are common mechanisms used by *L. casei* to withstand severe acidification and to reduce the deleterious effect of lactic acid on the cell membrane. Other studies have made similar observations on some strains of lactobacilli (Savage, 1992; Schillinger et al., 2005), as they did not find any correlation between hydrophobicity surface and adhesion capacity of some lactobacilli to mucus and epithelial cells. Schillinger et al. (2005) conducted a study on a set of *Lactobacillus acidophilus* strains and *L. casei* and reported that strains with strong surface hydrophobicity generally exhibit a significant adhesion

Table 2. Fatty acids composition of *Lactobacillus casei* strains grown at pH 3.2, 3.5, and 3.8¹

Strain ²	C10:0	C12:0	C14:0	C15:0	C16:0	C16:1	C16:ln9	C17:0	C18:0	C18:ln7	C18:ln9	C18:2	C19:0	C20:0
S1	1.11	0.12	10.22	0.24	45.01	1.36	3.2	0.72	5.93	19.2	2.16	6.6	0.77	0.6
S1i	1.1	0.77	11.31	0.62	42.7	1.14	5.92	0.69	7.63	19.3	1.87	5.44	0.3	0.7
S1ii	1.17	1.3	13.41	0.18	38.23	1.39	6.03	0.14	7	20.1	2.23	5.74	0.36	1.3
S1iii	1.16	0.32	10.07	0.7	41.19	1.77	6.2	0.1	10.06	19.5	1.97	4.93	0.62	1.2
S2	1.14	1.3	12.45	0.12	47.31	1.89	2.1	0.26	8.22	18.1	1.69	4.5	0.42	0.08
S2i	1.12	0.32	10.12	0.48	48.29	1.47	1.8	0.88	9.32	18.2	1.66	5.2	0.53	0.05
S2ii	1.22	0.43	10.36	0.2	46.72	1.87	2.6	0.09	7.16	17.9	2.23	5.44	0.25	1.9
S2iii	1.32	0.05	8.12	0.85	44.22	1.47	3.6	0.69	5.07	22.5	1.28	5.58	0.22	1.1

¹C10:0 = capric acid; C12:0 = lauric acid; C14:0 = myristic acid; C15:0 = pentadecylic acid; C16:0 = palmitic acid; C16:1 = palmitoleic acid; C16:ln9 = hexadecenoic acid; C17:0 = margaric acid; C18:0 = stearic acid; C18:2 = linoleic acid; C18:ln7 = vaccenic acid; C18:ln9 = oleic acid; C19:0 = nonadecanoic acid; C20:0 = arachidic acid.

²S1 = *Lactobacillus casei* of collection laboratory; S2 = *Lactobacillus casei* ATCC 393; i = strains incubated at pH 3.2; ii = strains incubated at pH 3.5; iii = strains incubated at pH 3.8.

Table 3. Effect of acid stress on the FA composition (% of total FA) of *Lactobacillus casei*

Strain ¹	FA ²		
	SFA	UFA	UFA/SFA
S1	71.32	24.56	0.34
S1i	71.26	27.09	0.38*
S1ii	68.83	28.36	0.41*
S1iii	70.35	27.67	0.39*
S2	75.8	21.89	0.28
S2i	76.31	21.66	0.28*
S2ii	73.77	22.73	0.30*
S2iii	67.22	27.38	0.4*

¹S1 = *Lactobacillus casei* of collection laboratory; S2 = *Lactobacillus casei* ATCC 393; i = strains incubated at pH 3.2; ii = strains incubated at pH 3.5; iii = strains incubated at pH 3.8.

²UFA = total unsaturated FA; UFA/SFA = unsaturated to saturated FA ratio.

* $P < 0.05$: significant difference between stressed strain and control strain.

ability to epithelial cells HT-29 MX. However, other strains belonging to these groups, characterized by a very low hydrophobicity around 2%, are able to adhere to HT-29 cells (MX membership 40% capacity). Schillinger et al. (2005) concluded that the hydrophobic surface may be useful for adhesion, but is clearly not the only prerequisite or necessary factor involved in bacterial adhesion process. In fact, the hydrophobicity of the surface is one of physicochemical properties that facilitate the initial contact between microorganisms and host cells. It is involved in the initial phase of nonspecific adhesion that allows approximation of their target bacteria, which gives way to the second stage of specific adhesion, involving protein molecular entities or nonanchored protein at the bacterial cell wall (Granato et al., 1999; Rojas et al., 2002; Roos and Jonsson, 2002). Other previous studies support the absence of a direct correlation between hydrophobicity and adhesion capacity (Wadström et al., 1987; Vinderola et al., 2004), but a recent study (Valeriano et al., 2014) showed a significant correlation between adhesion, hydrophobicity of cell surface, and autoaggregation. This study gives basic knowledge for the elucidation of the adhesion mechanism of *Lactobacillus* spp. and prediction of its adherence in specific host models (Valeriano et al., 2014). In fact, surface treatment with proteases showed that adhesion to hexadecane was dependent on the cell wall proteins, whereas adhesion to epithelial cells required both proteins and nonproteinaceous compounds present at the surface of *Lactobacillus* (Deepika et al., 2009). Further supporting this view, inactivation of different sortases in *L. casei* selectively impaired hexadecane affinity or epithelial cells adhesion (Muñoz-Provencio et al., 2012). Conversely, it has previously been suggested that lipoteichoic acids (LTA) are involved in biofilm formation of *Lactobacillus*

and adhesion to human enterocyte-like cells or mouse gastric epithelium (Lebeer et al., 2007; Mohamadzadeh et al., 2011). Lactobacilli genes putatively involved in stress resistance or in adhesion studied by mutant analysis showed the influence of LTA and D-alanylation on probiotic capacity (Lebeer et al., 2008). A putative function of the modified LTA structure is compatibility with growth under salt-stress conditions and with the overall envelope modifications taking place during this stress condition (Palomino et al., 2013).

Based on our results, it appears that acid stress caused changes in membrane FA composition of *L. casei*. However, the results obtained in our study clearly identify an increase in saturation level of FA as a response to exposure to pH decrease. It is noticeable that our strain had high concentrations of SFA and cyclic FA as compared with data available for other species. This behavior suggests that desaturase activation or hyperinduction plays an important role in the response to pH stress at least in this strain. Moreover, on the basis of our results it can be hypothesized that, as a response to the physicochemical stresses applied, the oleic acid associated with phospholipids could competitively be converted to linoleic acid (and subsequently to vernolic acid; Guerzoni et al., 2001). By comparing the FA composition of cells obtained without acidification (pH 6) and after 30 min of acidification at pH 5.25, SFA and cyclic FA concentrations slightly changed (Muller et al., 2011). According to Teixeira et al. (2002), reducing UFA and cyclic FA concentrations allowed membrane fluidity to slightly decrease as a result of an enhanced solid-to-fluid transition temperature (Teixeira et al., 2002). Other authors observed an increase of UFA content of *Streptococcus thermophilus*, thus leading to a higher membrane fluidity when the pH of the culture was reduced (Wang et al., 2005a). This was further confirmed with *L. acidophilus* RD758 (Wang et al., 2005a,b). The discrepancy found between our results and the literature can be explained by 3 different factors: the microorganisms employed, different approaches used to study acid adaptation (acidification at the end of the culture instead of fermentation at acidic pH), and the different methods of evaluation. The high UFA and cyclic FA contents of the cells may also explain these observations. Moreover, as variations in membrane FA composition were small, it could be hypothesized that the short time of adaptation was not long enough to induce the higher changes that were observed by Wang et al. (2005b).

CONCLUSIONS

Our data support that the cell envelope is the first main structure to be damaged by physicochemical

stress. The cell membrane itself plays an important role in stress resistance because of its composition, which can change to adapt to harsh conditions. However, the adhesion capacity to abiotic surfaces and the surface hydrophobicity of *L. casei* were affected under heat stress, which was able to change the composition of membrane FA. Despite these changes in surface properties, adhesion to HeLa cells was enhanced in heat-stressed bacteria. This study also showed that hydrophobic or hydrophilic properties of *L. casei* could be independent from the adhesion ability to HeLa cells. The exact molecular mechanism for adaptation of bacteria under stress conditions should be the focus of additional studies.

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