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Effect of pH in the survival of *Lactobacillus salivarius* strain UCO_979C wild type and the pH acid acclimated variant

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ABSTRACT

Background: Bacterial acclimation involves cellular changes permitting the survival of a microorganism to prolonged acid pH exposure. The general aim of this work is to support this idea by determining the effect of pH in the survival of the human gastric derived probiotic strain *Lactobacillus salivarius* UCO_979C-1 (wild type) and *L. salivarius* UCO_979C-2 (acclimation to pH 2.6), which possesses anti-*Helicobacter pylori* properties.
Results: To assess this aim, the exopolysaccharide production through the phenol-sulfuric acid method was evaluated. Moreover, morphological and structural changes by transmission and scanning electron microscopy were observed. The bacterial survival was measured by viable count. The results showed that the acclimated variant strain synthesized higher levels of exopolysaccharide (690 ± 0.03 mg/L) more than the wild type (450 ± 0.12 mg/L). In addition, the acclimated variant preserved the viable count at pH 2.6 for 48 h, whereas the wild type strain decreases after 6 h and was non-viable at 24 h.
Conclusion: The results suggest that the acid stress acclimation of the strain *L. salivarius* UCO_979C-1 modified some cellular properties making this strain potentially useful as a gastric probiotic.

1. Introduction

Acid stress not only induces changes on the components of the bacterial membrane, lipids and proteins, but also disturbs the DNA and peptidoglycan components in Gram-positive bacteria. As result of these morphological and phenotypic changes at the cellular level [1,2], and the lack of favorable conditions for bacterial proliferation [1,2,3], one can say that the bacterial viability is unsustainable. More precisely, bacteria presents several mechanisms to avoid stresses, such as, the synthesis of chaperones that act to repair proteins and DNA damage and changes in metabolic pathways to produce alkali and exopolysaccharides (EPS) [1,2,3,4]. Naturally, the bacterial EPS contributes to the formation of biofilms allowing colonization in certain substrates providing an ideal microenvironment for unfavorable conditions [3,4]. Moreover, EPS confers a barrier of protection to acclimated bacteria against acid pH stress preventing proton diffusion at the intracellular medium [5].

Several roles for EPS in lactic bacteria have been noticed, including immunomodulatory properties, anti-ulcer, antioxidant and antibacterial role [6,7,8]. These properties have been demonstrated both *in vitro* and *in vivo*. As an antibacterial, a study *in vivo* showed [6] that the EPS exerts a blocking of specific receptors of the host cell membrane by competing and inhibiting the formation of biofilms by pathogenic bacteria. This has been reported in different strains of *Lactobacillus acidophilus* A4 against *Escherichia coli* O157 enterohemorrhagic, *Salmonella enteritidis*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa* KCCM 11321 and *Listeria monocytogenes* Scott A [6].

In experimental conditions, bacterial strains could be induced to survival even on extreme conditions including acidic environment. Those changes developed by the bacterial strains to survive under adverse conditions are attributed to the phenomenon of acclimatization, which is produced by the expression of different genes [1]. In this process, an individual organism or a whole population alters some aspect of its behavior, morphology and metabolism in response to a signal from the environment [9].

In this context, the idea to induce tolerance to acid pH, emulating features of gastro-intestinal bacterial strains, could be a good option to improve performance of gastro-intestinal probiotics [10].

In this study, the strain *Lactobacillus salivarius* UCO_979C-1 (wild-type) (*L. salivarius* UCO_979C-1), member of the species *L. salivarius* isolated from human stomach, due to its anti-*Helicobacter pylori* [11,12] properties was selected. Furthermore, it was employed that the variant strain of *L. salivarius* UCO_979C-1 previously acclimated

Abbreviations: MRS, De Man, Rogosa and Sharpe; EDTA, Ethylenediaminetetraacetic acid.

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to pH 2.6 for 45 generations, gradually reducing the pH in 0.2 pH units from pH 6.4 to pH 2.6, referred as *L. salivarius* UCO_979C-2 (*L. salivarius* UCO_979C-2). The strain *L. salivarius* UCO_ 979C-1 was first isolated by García *et al.* [11] from gastric biopsy of a Chilean patient. Subsequent studies of this strain showed high hydrophobicity *in vitro*, and a greater production of hydrogen peroxide, which exerts a bactericidal effect causing DNA fragmentation. Another remarkable characteristic of this strain is its ability to produces lactic acid, which can act decreasing the pH in the medium and having an effect on the enzyme urease of *H. pylori*. Urease allows the colonization of *H. pylori* in acidic environment through the catalysis of urea into ammonia and carbon dioxide [11,12].

2. Methods

2.1. Bacterial cultures and survival curve/growth conditions

The lactobacillus strains were grown using the method described by Kaushik *et al.* [10] with some modifications. They were grown in 10 mL of de Man, Rogosa and Sharpe (MRS) broth (Difco, France) adjusted to pH value of 2.6 for the acclimated variant and 6.4 for the native strain using HCl. Specifically, 10 mL aliquots were used of the MRS broth which were inoculated with 100 μL of 24 h bacterial cultures and incubated under microaerophilic conditions at 37°C. Immediately, 100 μL of each culture were taken and serial dilutions were performed in 900 μL of saline solution (time 0), for bacterial count by the droplet technique [13], spreading 10 μL of each dilution in triplicate (including seeding of undiluted culture drops) in MRS agar plates (Difco, France). Similarly, 100 μL aliquots of each culture were taken after 24 h and 48 h by performing serial dilutions as described before and plated them for bacterial counting microdroplet technique to determine the bacterial colony count. The plates were incubated under microaerophilic conditions (10% CO_2) at 37°C for 48 h. The count was expressed in log colony forming units per mL (log CFU/mL) [13].

2.2. Electron microscopy

The effect of acid stress at the cellular level was evaluated by scanning and transmission electron microscopy using the method described by Anderson [14]. The samples were fixed with 2.5% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.2). Then, the samples were washed in sodium cacodylate buffer and 1% OsO_4 solution for 1 h at 4°C, before being washed with sodium cacodylate buffer and dehydrated in ethanol solutions. Finally, the samples were dried and mounted on a metal grid and covered with a thin layer of gold under vacuum. The samples were observed under transmission electron microscope JEOL JEM-1200 EX II (Jeol Technics Ltd., Tokyo, Japan) and JEOL JEM-6380 LV (Jeol Technics Ltd., Tokyo, Japan).

2.3. Extraction and quantification of exopolysaccharides

Quantification of EPS adhered to the bacterial cell was following the method by Tallon and Bressollier [15]. Cultures 37°C, 24 h in MRS broth adjusted to pH 2.6 for the acclimated variant and pH 6.4 for the native strain, were conducted to EPS extraction by treatment with 0.05 M EDTA for 3 h. The medium was removed and resuspended in 5 mL of EDTA. The resuspended cells were centrifuged and the recovered supernatant was precipitated with 2 volumes of 95% ethanol with continuous stirring for overnight. Then the samples were centrifuged at 8000 *g* for 10 min. The quantification was performed by phenol-sulfuric acid method [16], in which EPS pellet obtained was diluted to 1 mL, and was added 0.5 mL of 5% phenol and 2.5 mL of 98% H_2SO_4. This mixture was kept for 15 m at 95°C and the absorbance was measured at 490 nm. A calibration curve of dextran was performed ranging from 0.1 g/L to 1 g/L to determine the concentration of EPS. All samples were tested in triplicate.

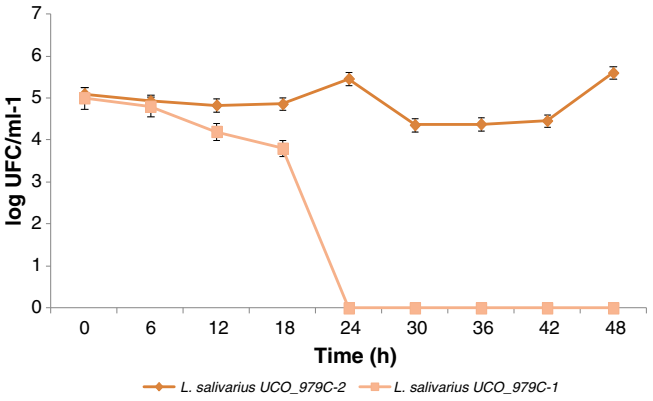

Fig. 1. Growth curves of *L. salivarius* UCO_979C-1 wild type and the acclimated variant *L. salivarius* UCO_979C-2 strains cultivated in MRS broth at pH 2.6.

3. Results and discussion

3.1. Survival curves

L. salivarius UCO_979C-2 (acclimated to pH 2.6) was able to maintain its viability for 48 h, whereas the native strain *L. salivarius* UCO_979C-1 after 6 h of exposure to pH 2.6 showed lower levels of survival and no viable cells were recovered after 24 h (Fig. 1). Interestingly, the acclimated variant strain experienced one logarithm decay between 24 and 40 h to subsequently recover it at 42 h (Fig. 1).

The results have shown that the native strain *L. salivarius* UCO_979C-1 was unable to survive the acid conditions after 6 h of exposition. Previous studies by Maragkoudakis *et al.* [17] and Ryan *et al.* [18], reported that certain strains of *Lactobacillus rhamnosus* subjected to pH 2.0, were subject to a rapid viability decline after 24 h obtaining zero percent survival. At the contrary, the acclimated variant *L. salivarius* UCO_979C-2 remained viable for a longer period. Some investigations in stress response have shown that microorganisms, which have been subjected to acidic conditions and are adapted to low pH, can withstand better the subsequent acid stress [19].

3.2. Electron microscopy

The electron microscopy images show the two bacterial variants with different morphological features (Fig. 2). The acclimated variant strain *L. salivarius* UCO_979C-2 shows a decrease in cell size and a thinner thickness of the peptidoglycan layer (PG). Furthermore, an appropriate extracellular matrix of EPS was observed (Fig. 2). The image of scanning electron microscopy shows two cellular morphotypes: the native strain *L. salivarius* UCO_979C-1 shows straight and curved rods, whereas the acclimated variant strain *L. salivarius* UCO_979C-2 displays single straight rods and also wastes of exopolysaccharide fiber EPS (Fig. 3).

The bacterial cell envelope is a protection barrier against environmental conditions. There are evidences supporting that the wall and cell membrane change during the stress response. Therefore, the fact that the cells are growing in an acid pH environment may have an impact on cell morphology. It has been reported that *Lactobacillus casei* grown at low pH and a change in the morphology of the cytoplasmic membrane were observed [20]. The regulation of genes involved in the biogenesis of the cell wall and lipid metabolism was the reason of persistence. In another study, *Lactobacillus reuteri*

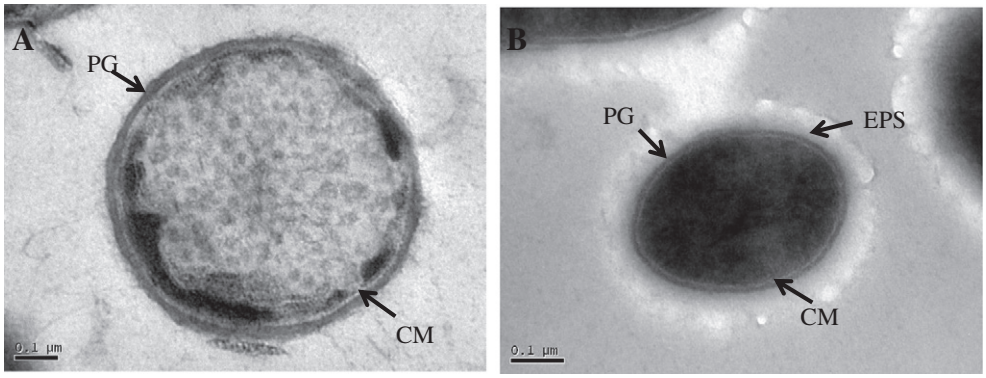

Fig. 2. Transmission electron microscopy of the *L. salivarius* UCO_979C-1 (wild type) isolated from human gastric tissue and its acclimated variant to acid pH; *L. salivarius* UCO_979C-2. (B) Strain *L. salivarius* UCO_979C-1. (B) Strain *L. salivarius* UCO_979C-2. CM: cytoplasmic membrane, PG: peptidoglycan, EPS: exopolysaccharide.

has confirmed that acid pH influences gene expression and the alterations in the cell envelope. These adaptations are vital to the stress response [21].

The electron microscopy images indicate that the viscous layer on the outer surface of the acclimated variant strain *L. salivarius* UCO_979C-2 fits to EPS and they are not the S-layer found in other bacteria. In line with our results, Wasko *et al.* [22] have shown that the S-layer proteins present different morphology by electron micrographs. The S-layer is less dense and more translucent than the EPS. In contrast, the EPS shown in this study, it is thicker and deducted that is a viscous product extending to nearby cells.

3.3. Quantification of exopolysaccharides

Exopolysaccharides (EPS) are high molecular weight sugars that are secreted by bacteria. EPS establish a biofilm under stress conditions, such as acid stress, that helps to protect the bacteria [3]. To evaluate the presence and feature of EPS in the acclimated variant strain *L. salivarius* UCO_979C-2 was tested to acid pH. The results have shown EPS production after 24 h at pH 2.6 of 690 ± 0.03 mg/L compared to the native strain *L. salivarius* UCO_979C-1 that was of 450 ± 0.12 to mg/L at pH 6.4. An increase of 240 mg/L of EPS was observed in the acclimated variant strain. The measure of EPS production of the native strain was at pH 6.4, since pH 2.6 is not viable. Our results suggest that the increase in EPS production may have influenced the survival of the acclimated variant strain. Liu *et al.* [7] performed the biomass EPS quantification for different species of *Lactobacillus*, these authors reported that EPS production ranges from 4.10 mg/L to 29.20 mg/L. Between the *Lactobacillus* species and *L. salivarius* strain CRCB 14759 showed the highest production of EPS with a biomass of 29.20 mg/L. Other reports had shown that EPS production in *Lactobacillus* species differs depending on the strain and species studied, detecting a production of 250 mg/L in some cases [23]. Our results are over the average of previous studies; however, EPS production varies depending on the environmental conditions in which bacteria are grown, the composition of the culture medium, temperature and strain origin. All of the previous conditions mentioned, are major variations in the production rate of EPS [7,8]. EPS are synthesized for the formation of bacterial biofilms [5], one of the proposed function could be to allow greater adhesion to the gastrointestinal epithelium, thus resulting in a longer residence in the gastrointestinal cavity increasing its probiotic role [4].

4. Concluding remarks

The results presented herein have shown that the strategy exhibited by some bacteria to tolerate an acid stress environment or acclimation allows the generation of new bacteria variants, which may have application in the development of probiotics for gastric use. The acclimated variant strain *L. salivarius* UCO_979C-2 analyzed in this study, seems to be a perfect candidate for future directions in our research group and has opened new avenues in the investigation of possible alternative treatment in the eradication of *H. pylori*.

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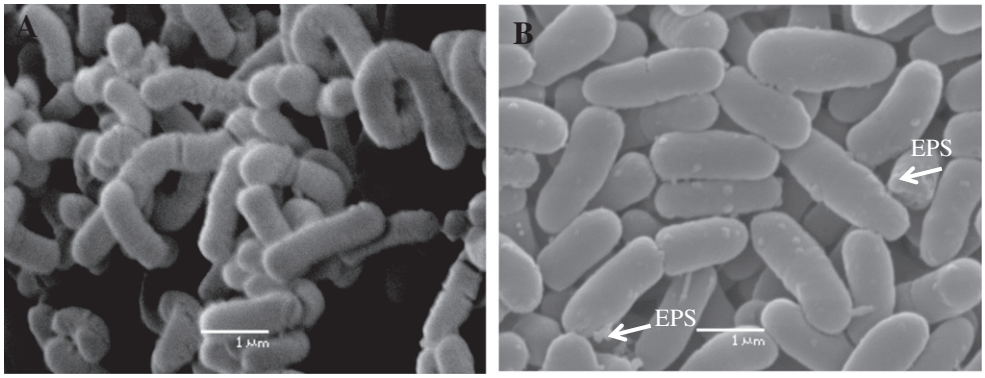

Fig. 3. Scanning electron microscopy of wild type *L. salivarius* UCO_979C-1 from human gastric origin and the acid pH acclimated variant *L. salivarius* UCO_979C-2. (A) Strain *L. salivarius* UCO_979C-1. (B) Strain *L. salivarius* UCO_979C-2.

References

[1] De Angelis M, Gobbetti M. Environmental stress responses in *Lactobacillus*: A review. Proteomics 2004;4:106–22. http://dx.doi.org/10.1002/pmic.200300497.

[2] Mayorga-Reyes L, Bustamante-Camilo C, Gutiérrez-Nava A, Barranco-Florido F, Azaola-Espinoza A. Crecimiento, sobrevivencia y adaptación de *Bifidobacterium infantis* a condiciones ácidas. Rev Mex Ing Quim 2009;8:259–64.

[3] Lee Yuan K, Salminen S. Handbook of Probiotics and Prebiotics. 2nd ed. New Jersey: Johns Wiley & Sons; 2009.

[4] Sutherland IW. Structure–function relationships in microbial exopolysaccharide. Biotechnol Adv 1994;12:393–448. http://dx.doi.org/10.1016/0734-9750(94)90018-3.

[5] Stack HM, Kearney N, Stanton C, Fitzgerald GF, Ross RP. Association of beta-glucan endogenous production with increased stress tolerance of intestinal Lactobacilli. Appl Environ Microbiol 2010;76:500–7. http://dx.doi.org/10.1128/AEM.01524-09.

[6] Kanmani P, Satish Kumar R, Yuvaraj N, Paari KA, Pattukumar V, Arul V. Probiotics and its functionally valuable products—A review. Crit Rev Food Sci Nutr 2013;53: 641–58. http://dx.doi.org/10.1080/10408398.2011.553752.

[7] Liu CT, Hsu IT, Chou CC, Lo PR, Yu RC. Exopolysaccharide production of *Lactobacillus salivarius* BCRC 14759 and *Bifidobacterium bifidum* BCRC 14615. World J Microbiol Biotechnol 2009;25:883–90. http://dx.doi.org/10.1007/s11274-009-9965-x.

[8] Mozzi F, Vaningelgem F, Hebert E, Van der Meulen R, Moreno MRF, De Valdez GF, et al. Diversity of heteropolysaccharide-producing lactic acid bacterium strains and their biopolymers. Appl Environ Microbiol 2006;72:4431–5. http://dx.doi.org/10.1128/AEM.02780-05.

[9] Rainey PB, Travisano M. Adaptive radiation in a heterogeneous environment. Nature 2004;394:69–72. http://dx.doi.org/10.1038/27900.

[10] Kaushik JK, Kumar A, Duary RK, Mohanty AK, Grover S, Batish VK. Functional and probiotic attributes of an indigenous isolate of *Lactobacillus plantarum*. PLoS One 2009;12:1–11. http://dx.doi.org/10.1371/journal.pone.0008099.

[11] García A, Henríquez P, Retamal C, Pineda S, Delgado C, González C. Propiedades probióticas de *Lactobacillus* spp aislados de biopsias gástricas de pacientes con y sin infección por *Helicobacter pylori*. Rev Med Chile 2009;137:369–76. http://dx.doi.org/10.4067/S0034-98872009000300007.

[12] García A, Sáez K, Delgado C, González CL. Low co-existence rates of *Lactobacillus* spp. and *Helicobacter pylori* detected in gastric biopsies from patients with gastrointestinal symptoms. Rev Esp Enferm Dig 2012;104:473–8. http://dx.doi.org/10.4321/S1130-01082012000900005.

[13] Herbert RA. Methods for enumerating microorganisms and determining biomass in natural environments. Methods Microbiol 1990;22:1–39. http://dx.doi.org/10.1016/S0580-9517(08)70238-1.

[14] Anderson TF. Techniques for the preservation of three-dimensional structure in preparing specimens for the electron microscope. Trans NY Acad Sci 1951;13: 130–4. http://dx.doi.org/10.1111/j.2164-0947.1951.tb01007.x.

[15] Tallon R, Bressollier P. Isolation and characterization of two exopolysaccharides produced by *Lactobacillus plantarum* EP56. Res Microbiol 2003;154:705–12. http://dx.doi.org/10.1016/j.resmic.2003.09.006.

[16] Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350–6. http://dx.doi.org/10.1021/ac60111a017.

[17] Maragkoudakis PA, Zoumpopoulou G, Miaris C, Kalantzopoulos G, Pot B, Tsakalidou E. Probiotic potential of *Lactobacillus* strains isolated from dairy products. Int Dairy J 2006;16:189–99. http://dx.doi.org/10.1016/j.idairyj.2005.02.009.

[18] Ryan KA, Jayaraman T, Daly P, Canchaya C, Curran S, Fang F, et al. Isolation of lactobacilli with probiotic properties from the human stomach. Lett Appl Microbiol 2008;47:269–74. http://dx.doi.org/10.1111/j.1472-765X.2008.02416.x.

[19] Cotter P, Hill C. Surviving the acid test: Responses of Gram positive bacteria to low pH. Microbiol Mol Biol Rev 2003;67:429–53. http://dx.doi.org/10.1128/mmbr.67.3.429-453.2003.

[20] Fozo EM, Kajfasz JK, Quivey RG. Low pH-induced membrane fatty acid alterations in oral bacteria. FEMS Microbiol Lett 2004;238:291–5. http://dx.doi.org/10.1016/j.femsle.2004.07.047.

[21] Wall R, Fitzgerald G, Hussey S, Ryan T, Murphy B, Ross P, et al. Genomic diversity of cultivable *Lactobacillus* populations residing in the neonatal and adult gastrointestinal tract. FEMS Microbiol Ecol 2007;59:127–37. http://dx.doi.org/10.1111/j.1574-6941.2006.00202.x.

[22] Wasko A, Polak MB, Kuzdralinski A, Skrzypek T. Variability of S-layer proteins in *Lactobacillus helveticus* strains. Anaerobe 2014;25:53–60. http://dx.doi.org/10.1016/j.foodchem.2005.11.033.