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FUNCTIONAL PROPERTIES AND MICROSTRUCTURE OF *Leuconostoc citreum* AND *Lactobacillus casei* SHIROTA EXPOSED TO HABANERO PEPPER EXTRACT AND INHIBITION OF *Staphylococcus aureus*

PROPIEDADES FUNCIONALES Y MICROESTRUCTURA DE *Leuconostoc citreum* Y *Lactobacillus casei* SHIROTA EXPUESTOS A EXTRACTO DE CHILE HABANERO E INHIBICIÓN DE *Staphylococcus aureus*

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Abstract

The effects of habanero pepper extract on *Staphylococcus aureus*, *Lactobacillus casei* Shirota and *Leuconostoc citreum* were studied. The extract contained 8.71 mg/g of capsaicin and 4.72 mg/g of dihydrocapsaicin as well as minor concentrations of gallic, chlorogenic, caffeic, *p*-coumaric acids and quercetin, determined by HPLC. Inhibition of *S. aureus* at 4 h was observed with a death rate of -1.53 h^{-1}. *L. casei* Shirota showed a survival of 4.15×10^5 CFU/mL and *L. citreum* of 2.20×10^5 CFU/mL. When *L. casei* Shirota was exposed to the MRS-50% extract for 12 h, an elongation of the cells was observed and the Feret's diameter increased from 3.3 to 21.6 μm, showing blisters on the cell surface, however, the functional properties of adhesion to mucin and lysozyme resistance were not affected. Regarding *L. citreum*, cell area increased 70% maintaining its circularity; with an improvement in mucin adhesion ability of 4.42×10^5 CFU/mL was found, showing a lower decrease in its viability in bile salts and modifying the synthesis of L36 ribosomal protein, corroborating the presence of the *rpmJ* gene that encodes for this protein.

Keywords: *Leuconostoc citreum*, *Lactobacillus casei* Shirota, *Staphylococcus aureus*, capsaicinoids, morphostructure, functional properties.

Resumen

Se estudiaron los efectos de extracto de chile habanero sobre *Staphylococcus aureus*, *Lactobacillus casei* Shirota y *Leuconostoc citreum*. El extracto contenía 8.71 mg/g de capsaicina y 4.72 mg/g de dihidrocapsaicina, además de concentraciones menores de ácido gálico, clorogénico, caféico, *p*-cumárico y quercetina, determinados por HPLC. Se observó la inhibición de *S. aureus* a las 4 h con una velocidad de muerte de -1.53 h^{-1}. Mientras que, *L. casei* Shirota mostró una supervivencia de 4.15×10^5 UFC/mL y *L. citreum* de 2.20×10^5 UFC/mL. Cuando *L. casei* Shirota fue incubado 12 h en MRS-extracto 50% se observó una elongación de las células y el diámetro de Feret aumentó de 3.35 a 21.66 μm, mostrando ámpulas en la superficie celular, sin embargo, las propiedades funcionales de adhesión a mucina y resistencia a lisozima no fueron afectadas. Con respecto a *L. citreum*, se observó un incremento en el área de las células del 70%, manteniendo su circularidad; también mostró un aumento en la capacidad de adhesión a mucina de 4.42×10^5 UFC/mL y una menor disminución de su viabilidad en sales biliares, se modificó la síntesis de la proteína ribosomal L36, corroborando la presencia del gen *rpmJ* que codifica para esta proteína.

Palabras clave: *Leuconostoc citreum*, *Lactobacillus casei* Shirota, *Staphylococcus aureus*, capsaicinoides, morfoestructura, propiedades funcionales.

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1 Introduction

Food ingredients, particularly phytochemicals, may influence the nutritional and health status of the host by modulating the balance, growth and functional properties of gut microbiota, which include: resistance to acid and bile, adhesion and colonization potential and tolerance to antibiotics (Laparra and Sanz, 2010). In this context, peppers (*Capsicum* spp. fruits) are included in several food products due to their flavor-enhancement effect (Vázquez-Cárdenas *et al.* , 2015). Most of the *Capsicum* fruits contain capsaicin (8-methyl-N-vanillyl-*trans*-6-nonenamide) and dihydrocapsaicin (8-methyl-N-vanillylnonanamide), which are responsible for their pungency. The most pungent *Capsicum* fruits in the world, according to Scoville scale, are the Bhut Jolokia from India, the Red Savina, Carolina Reaper and Scotch Bonet Habanero from USA, as well as the Habanero Jacq. produced in Mexico (Sweat *et al.* , 2016). Some extracts of *Capsicum* spp. have shown antimicrobial activity against pathogenic bacteria (Dorantes *et al.* , 2000). However, there are few studies on the survival of lactic acid bacteria (LAB) when exposed to peppers or their extracts. Cerón-Carrillo *et al.* , (2014), evaluated the antimicrobial effect of a habanero pepper extract, observing a greater inhibition of *Escherichia coli* when compared to *Lactobacillus casei*. Mokhtar *et al.* , (2017), studied the antimicrobial activity of green bell pepper var. biskra extracts against some bacteria; pathogenic bacteria strains were inhibited by the extracts, at the same time *L. acidophilus* CECT 4529 and *Lactobacillus plantarum* CECT 748 remained viable after exposure to the extracts. Additionally, Amund, (2016), emphasizes the importance of relating the technological stress during food processing conditions and its impact on the functionality of beneficial bacteria for health. Some of the most common stressful factors in technological processes are the exposure to heat or cold, osmotic pressure in food and exposure to oxygen during fermentation, as well as the food composition, for example the presence of peppers. Sharma *et al.* , (2013), observed an increase in lactic acid production and glucose consumption of *Lactobacillus acidophilus* during cheese elaboration when red pepper was added to the cheese curd, without affecting the number of viable cells. The study of alterations in the microstructure and morphology and their relation to some characteristics of LAB exposed to pungent pepper extracts may be interesting when positive changes in functional properties occur. Thus, the aim of the present work was to contribute to knowledge of possible changes in viability, microstructure and functional properties of two LAB as compared to a pathogenic bacteria. Particularly, this study aims to select the habanero pepper extract concentration with greater inhibitory effect on *S. aureus* and to evaluate its impact on the growth, glucose consumption, microstructure, survival in bile salts conditions, adhesion to mucin, ribosomal proteomic profile and specific genes associated to the stress response of two LAB. For this study, the probiotic strain *Lactobacillus casei* Shirota (Figueroa-González *et al.* , 2010) and *Leuconostoc citreum* were selected. The utilization of *L. citreum* in this work was based on the fact that this species was isolated from jalapeño pepper fermentation (González-Quijano *et al.* , 2014). Therefore, this organism has a better adaptation potential to peppers extracts.

2 Materials and methods

2.1 *Preparation and characterization of habanero pepper extract*

Fruits of *C. chinense* Jacq. cv Habanero were obtained from the central area of Yucatán, Mexico. The fruits were harvested at 75-80 days after anthesis. The samples had bright orange color (Chroma=50.9 ± 2.8 and °Hue=53.1 ± 4.2). Once the peduncle was removed, the fruit was washed with tap water and dried with paper towels, then it was frozen at −40 °C until use.

For extract preparation, 250 g of habanero pepper were ground for one minute in a Thermomix (Mod. 3300, Vorwek, Germany). Afterwards, isopropanol (≥ 99.7%, JT Baker, PA, USA) (250 mL) was added to the ground peppers, remaining under stirring for 15 min, then the mixture was heated to boiling point in reflux for 5 min. Pepper pulp residues were removed by paper filtration (Whatman No.1, Maidsone, England).

The filtrate was mixed with activated charcoal at a ratio of 15 g/L, for 10 min; and then the charcoal was eliminated by paper filtration. Afterwards, the excess of isopropanol in the filtrate was separated by vacuum evaporation at 60 °C in rotavapor (V-805, Büchi, Flawill, Switzerland), obtaining an aqueous extract which was centrifuged twice at 3000 x g (Allegra X-

12R centrifuge, Beckman Coulter, CA, USA), for 20 min at 4 °C. The supernatant obtained was preserved in freezing until its use in the subsequent analysis.

The habanero pepper extract (HPE) was analyzed to determine the content of capsaicinoids and phenolic compounds by HPLC (Varian 900-LC, USA, equipped with an UV-vis detector). The capsaicinoids content was determined according to Davis *et al.* , (2007). The phenolic compounds were quantified, injecting aliquots of 20 μL of extract in the HPLC using a C18 column (250×4.6 mm, 5 μm). The mobile phase was composed by solvent A (acetic acid 0.5%) and solvent B (acetonitrile), the absorbance was determined at 280 nm.

The linear gradient elution was performed at 1.6 mL/min and programed as: 0.0-0.1 min 100% A; 0.1-20 min 80% A:20% B; 20-30 min 70% A:30% B; 30-40 min 50% A:50% B; 40-50 min 25% A:75% B; 50-60 min 100% B and 60-65 min 100% A. Each compound in HPE was quantified by comparison with the calibration curve of the corresponding standard.

2.2 *Bacterial strains and growth conditions*

Two LAB were used in this work: *L. citreum* isolated from jalapeño pepper fermentation (González-Quijano *et al.* , 2014) and *L. casei* Shirota isolated from Yakult® (a commercial probiotic beverage). LAB were grown in de Man-Rogosa-Sharpe (MRS) culture broth (Difco, Point-de-Claix, France) at 30 °C for *L. citreum* (Holzapfel *et al.* , 2015), and 37 °C for *L. casei* Shirota. *S. aureus* ATCC 25923 was provided by the Laboratorio de Bacteriología Médica of Escuela Nacional de Ciencias Biológicas of Instituto Politécnico Nacional (ENCB-IPN) and was grown in Mueller-Hinton culture broth (DIBICO, Mexico City, Mexico) at 37 °C.

Stock cultures were prepared from frozen cultures stored at −80 °C in culture broth with glycerol 20% (v/v) (FERMONT, NL, Mexico), performing three consecutive reactivations (24, 18 and 12 h), in the appropriate culture broths with inoculations at 1% (v/v). An initial inoculum of each bacterium was prepared by growing the strains from the stock on solid medium and suspending isolated colonies in sterile NaCl 0.85% (w/v) (Reasol, Mexico City, Mexico) solution, adjusting the concentration of viable cells to 10^7 colony forming units per milliliter (CFU/mL). Triplicates of all analysis were performed.

2.3 *Survival/death kinetics and microstructural analysis of S. aureus exposed to habanero pepper extract*

To carry out this analysis, the HPE obtained was initially sterilized by membrane filtration (Millex-HV, pore size 0.45 μm Merck, Germany). At the same time, a double concentrated Mueller-Hinton culture broth (42 g/L instead of 21 g/L) was sterilized and the HPE and sterilized water were added to obtain final concentrations in the samples of 10, 20, 30 and 50% (v/v). Then, the survival/death kinetics of *S. aureus* exposed to the different HPE concentrations in culture broth were evaluated by viability plate count in Mueller-Hinton agar, during 24 h at 37 °C. For this analysis, each concentration of HPE in culture broth was inoculated at 1% (v/v) with the concentration of viable cells previously prepared (10^7 CFU/mL) as to reach an initial cell concentration of 10^5 CFU/mL. *S. aureus* grown in Mueller-Hinton culture broth without HPE addition was used as control. The effect of HPE on the microstructure was studied by transmission electronic microscopy (TEM). The sample of *S. aureus* exposed to HPE was washed twice with a phosphate buffer solution (PBS) pH 7.2, and fixed 24 h at 4 °C with glutaraldehyde 2.5% in PBS. Once fixed, the sample was stained with osmium tetroxide and embedded in polymerized resin, which was used to obtain the cuts of the sample using an ultramicrotome (Leica Ultracut, Wetzlar, Germany). Later, the cuts were contrasted with uranyl acetate and observed under a transmission electron microscope JEOL (JEM-1010, 60 kV, Electron microscopy Sciences-EMS, Akashima, Japan) (Martínez-Arámburu *et al.* , 2015).

2.4 *Survival and glucose consumption of LAB exposed to habanero pepper extract*

L. casei Shirota and *L. citreum* were exposed to the selected concentration of HPE with greater inhibitory effect on *S. aureus*. The LAB tolerance to HPE was evaluated during the exposure at the optimum growth temperatures by plate count in MRS agar. Based on these results, the growth/death rate (μ) of each bacteria exposed to the HPE was calculated using the Eq. 1 and compared to those of the controls.

$$Growth/death\ rate\ (h^{-1}) = \frac{\log\left(\frac{CFU/mL_{final}}{CFU/mL_{inicial}}\right)}{t} \quad (1)$$

Likewise, the supernatants obtained after centrifugation (3000 *xg* for 15 min at 4°C) of LAB cultures exposed to HPE were used to quantify glucose consumption. Glucose concentration in the supernatants was obtained using the Glucose GO kit (GAGO 20, Sigma, St. Louis, MO, USA), following the manufacturer's instructions. The amount of glucose consumed by LAB exposed to HPE was calculated by subtracting the final concentration in the supernatant to the initial concentration of glucose in the culture broth.

2.5 *Morphometric analysis by laser scanning confocal microscopy (LSCM)*

The bacterial cultures previously exposed to HPE were centrifuged at 3000 x g for 20 min at 4 °C and washed twice with same volume of PBS to remove traces of the culture broth. Once washed, the pellets were fixed for 24 h at 4 °C by adding a solution of glutaraldehyde 2.5% in PBS (Sorenson), to subsequently be stained with 200 μL of 3 mM acridine orange base (Sigma, St. Louis, MO, USA) solution. Then the samples were observed with a confocal microscope (Carl Zeiss LSM 710, Oberkochen, Germany), equipped with an Plan Apocromatic 63X objective (numerical aperture, 1.30). Fluorescent images were obtained after excitation of samples with a laser at 488 nm (González-Quijano *et al*. , 2014).

2.6 *Image analysis*

Binary images of cells exposed to HPE were manually segmented from grayscale images using preloaded Otsu values (25 ± 3 to 255) in the software ImageJ v. 1.49p (National Institute of Health, Bethesda, Maryland, USA). The size (area, perimeter, Feret's diameter) and morphological parameters (circularity, aspect ratio and round) of each bacterium were calculated from the binary images (Perea-Flores *et al*. , 2011).

2.7 *Microstructural analysis by scanning electron microscopy (SEM)*

The bacterial cultures exposed to HPE were used for SEM analysis. The preparation of the samples for SEM microscopy was made according to the methodology proposed by Ávila-Reyes *et al*. (2016). Samples of bacteria exposed to HPE were washed twice with PBS and fixed with glutaraldehyde 3% in PBS for 12 h at 4 °C. Fixed samples were washed with PBS and dehydrated with ethanol at different concentrations (20, 30, 40, 50, 70, 80 and 100%). Then, the dehydrated samples were placed on a copper and carbon tape. Finally, the samples were covered with gold before observation with a scanning electron microscope (JEOL, JSM-7800F, Akashima, Japan).

2.8 *Survival to simulated gastrointestinal tract (GIT) conditions and lysozyme resistance*

L. casei Shirota and *L. citreum* exposed to HPE were centrifuged at 3000 *xg* for 20 min at 4 °C, washed twice and re-suspended with PBS. The bacterial suspension of each LAB was adjusted to an initial count of 10^8 CFU/mL, which is the recommended cell concentration for this analysis (Castro-Rodríguez *et al*. , 2015). The initial count was calculated after inoculation of bacterial suspension in the gastrointestinal tract (GIT) solutions. The bacterial suspension was placed into a flask with NaCl solution adjusted to pH 2 with 6 N HCl and incubated 1 h at 37 °C. At the same time, the bacterial suspension of each strain was also inoculated in NaCl solution supplemented with 0.5% bile salts (Oxgall BD Difco, Sparks, MS, USA) (w/v) and incubated for 3 h at 37 °C. Finally, successive passages through artificial gastric and intestinal juices were performed as follows: a bacterial suspension (10%) was added to artificial gastric juice, which was prepared with 3 mg/mL of pepsin (≥250 U/mg, Sigma, St. Louis, MO, USA) in NaCl solution at pH 2; after 1 h of incubation at 37 °C, 10% of bacterial suspension of the artificial gastric juice was added to the artificial intestinal juice at pH 6.5 with 0.5% bile salts (Oxgall), and 1.9 mg/mL of pancreatin from porcine pancreas (Sigma, St. Louis, MO, USA), remaining in incubation for 3 h at 37 °C. The survival at the end of each one of the simulated GIT conditions was reported as viable cells concentration (CFU/mL) of LAB exposed to HPE, compared to the controls.

For the lysozyme resistance assay, *L. casei* Shirota and *L. citreum* exposed to HPE were washed twice with PBS and then exposed to lysozyme (100 mg/L, >20 kU/mg, egg white lysozyme, AMRESCO, USA), dissolved in a sterile electrolytic solution (0.22 of $CaCl_2$, 6.2 of NaCl, 2.2 of KCl and 1.2 of $NaHCO_3$ g/L). Then, the viability of LAB was evaluated at 30 and 120 min according to the methodology described by Giles-Gómez *et al*. (2016), and the results compared to the controls.

2.9 Assay of adhesion to porcine mucin in vitro

L. casei Shirota and *L. citreum* were exposed to HPE and centrifuged. The bacterial pellets were washed twice with PBS and then re-suspended in a volume previously determined as to obtain an initial inoculum to 10^8 CFU/mL. Adhesion to porcine gastric mucin (Type II, Sigma, St. Louis, MO, USA) assay was performed following the methodology described by Castro-Rodríguez *et al*. (2015). The mucin was dissolved in PBS to a final concentration of 10 mg/mL; and 300 μL of this suspension were immobilized by incubating for 12 h at 4 °C in 96-well cell culture plates (Eppendorf, Germany). After immobilization, the wells were washed twice with 150 μL of PBS and saturated with bovine serum albumin 2% (w/v) solution for 4 h at 4 °C. The wells were washed twice with PBS and the inoculums (150 μL) of each bacterium were placed in wells and incubated 1 h at 37 °C. The unbound bacteria were removed by washing with PBS under agitation; this procedure was repeated three times. Finally, the bound bacteria were treated with 100 μL of Triton X-100 (Sigma-Aldrich, USA) 0.05% solution (v/v) and the plate incubated for 40 min under agitation. For the viability analysis of bound cells, 100 μL of the solution from each well was diluted in NaCl 0.85% solution and plated in MRS agar. Results were compared with the initial concentration of viable cells before the adhesion.

2.10 Ribosomal proteomic analysis

The VITEK MS Plus (bioMérieux, Marcy-l'Etoile, France) equipment was used to obtain the mass spectra of the profile of ribosomal proteins of *L. casei* Shirota and *L. citreum* cultures exposed to HPE, comparing them with those of the controls. First, the cultures exposed to HPE were centrifuged and washed twice with PBS. The pellet of each bacteria was placed on a single well of a disposable, bar code-labeled target slide (VITEK MS-DS; bioMérieux), overlaying the sample with one microliter of saturated solution of α-cyano-4-hydroxycinnamic acid matrix in 50% acetonitrile and 2.5% triflouroacetic acid, and air dried. The mass spectra were acquired in linear positive ion mode at a laser frequency of 50 Hz (50 laser shots per second) with a mass/charge (m/z) ratio from 2,000 to 10,000 Da. For calibration, *E. coli* ATCC 8739 strain was used as reference, according to the manufacturer's specifications. The data were reported as relative intensity using the Research Use-Only (RUO) mode (Rychert *et al.* , 2013). Finally, a bioinformatic search in ribosomal protein database of NCBI (National Center for Biotechnology Information, USA), was conducted relating the value of the m/z ratios of the obtained mass spectra to the molecular weight of the ribosomal proteins reported for each strain in the database.

2.11 Sequencing of the genes responsible for the synthesis of ribosomal proteins

Cultures of *L. citreum* and *L. casei* Shirota, grown in control conditions and exposed to HPE, were used for DNA extraction. The extracted DNA was used as a template for PCR amplification of the genes responsible for synthesis of ribosomal proteins affected by HPE addition. The methodology proposed by Molina-López *et al*. (2011), was modified for the amplification of genes, increasing the final reaction volume to 50 μL. PCR was performed with a Biometra T-Gradient thermocycler (Göttingen, Germany), under the following conditions: denaturalization for 5 min at 95 °C, 35 cycles of 30 s at 95 °C, 1 min at 55 °C and 2 min at 72 °C, with a final elongation at 72 °C for 10 min. The electrophoresis of PCR products, loaded on 1.5% agarose gel, was performed at 50 volts. After electrophoresis the gel was stained with ethidium bromide and observed under ultraviolet light in a gel documentation system (BioSens SC 645, Shanghai, China). Subsequently, the PCR products were purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific, MA, USA), to later be sequenced and compared to the NCBI GenBank by BLAST (Basic Local Alignment Search Tool), translating nucleotide to protein. The sequencing of the purified PCR products was performed in the Instituto de Fisiología Celular (IFC) of the Universidad Nacional Autónoma de México (UNAM), in Mexico City.

2.12 Statistical analysis

The analysis of variance (ANOVA) was performed on the resulting data, followed by a Tukey's test with a significance level of 5% ($P \leq 0.05$) and the confidence intervals were calculated from means of triplicates with a confidence level of 95%, using the statistical software Minitab 16 (Minitab Inc. State College, PA, USA). For graphics design, the software Kaleidagraph v. 4.0 (Synergy Software, PA, USA) was used.

3 Results and discussion

3.1 *Habanero pepper extract characterization*

Two capsaicinoids were identified and quantified in the HPE: 8.71 mg/g dry weight (d.w.) of capsaicin and 4.72 mg/g d.w. of dihydrocapsaicin; equivalent to a total capsaicinoids concentration of 13.43 mg/g d.w. in HPE. This concentration is similar to the one reported by Sweat *et al*. (2016), who extracted 15.7 mg/g d.w. of capsaicinoids (10.4 mg/g d.w. of capsaicin and 5.33 mg/g d.w. of dihydrocapsaicin), from habanero pepper. The difference between the obtained and reported concentrations of capsaicinoids may be due to the affinity of the solvent used for each extraction, since in this study isopropanol was used, while Sweat *et al*. (2016), carried out the extraction using toluene.

A concentration of 1.45 mg/g d.w. of phenolic compounds was quantified in the HPE, in which gallic (0.12 mg/g d.w.), chlorogenic (0.26 mg/g d.w.), caffeic (0.21 mg/g d.w.) and *p*-coumaric (0.40 mg/g d.w.) acids, as well as quercetin (0.46 mg/g d.w.) were identified. These concentrations of phenolic compounds were similar to those reported by Troconis-Torres *et al*. (2012), detecting also the presence of protocatechuic, *o*-coumaric, sinapinic and *t*-cinnamic acids, as well as catechin, rutin and vainillin.

3.2 *Survival/death kinetics and microstructural analysis of S. aureus exposed to habanero pepper extract*

The maximum growth of *S. aureus* in the control was $2.50\times10^8 \pm 0.16\times10^8$ CFU/mL after 12 h incubation. There was no significant difference ($P \leq 0.05$) in the viable cells count of *S. aureus* grown in Mueller-Hinton culture broth with 10% of habanero pepper extract (HPE). The concentration of 20% HPE in the media prolonged the adaptation stage up to 12 h and the maximum growth decreased 1 log CFU/mL. *S. aureus* was inhibited after 8 h of exposure to 30% of HPE in culture broth, while at 50% HPE *S. aureus* was inhibited at 4 h (Fig. 1.Sa). Additionally, the effect of the highest concentration of HPE in culture broth (50%) on the microstructure was evaluated by TEM microscopy, observing a complete lysis of *S. aureus* cells after 4 h of exposure (Figs. 1.SaC and 1.Sa+HPE).

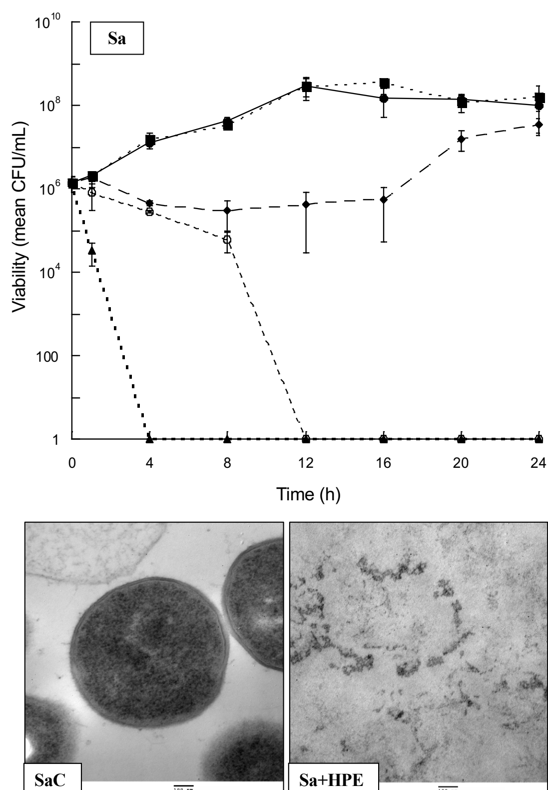

Fig. 1. Survival/death kinetics of *Staphylococcus aureus* at 37°C for 24 h with habanero extract in Mueller-Hinton culture media (Sa). Habanero extract concentrations: 0 (●), 10 (▪), 20 (♦), 30 (○) and 50% (▲). Standard deviation (SD) and mean of triplicates. TEM micrographs of *Staphylococcus aureus* cells grown in MRS culture medium (SaC) and exposed 4 h to 50% of habanero pepper extract in Mueller-Hinton culture medium (Sa+HPE).

Considering these results, it may be concluded that a dose response effect was obtained when adding HPE to the culture broth of *S. aureus* . The antibacterial effect of HPE may be attributed mainly to capsaicin, since the phenolic compounds concentration is very low in the habanero pepper extract. Capsaicin has shown antibacterial effect against *S. aureus* and *Streptococcus mutans*, in capsaicin-chitosan films observing an increase in inhibition zones when increasing the amount of capsaicin (Akyus *et al.* , 2018).

These authors also suggest that a possible mechanism by which capsaicin causes antibacterial effect may include binding to the cell wall, disruption of cytoplasm and finally, cell lysis.

Table 1. Viability and growth/death rate of *Staphylococcus aureus*, *Lactobacillus casei* Shirota and *Leuconostoc citreum* exposed habanero pepper extract.

HPE in broth (%)	Viable cells concentration (CFU/mL) 0 h	4 h	μ(h^{-1})
Staphylococcus aureus			
0	13.9 ± 2.8×10^5	1.27 ± 0.16×10^7	0.49
50	13.9 ± 2.8×10^5	0	−1.53
Lactobacillus casei Shirota			
0	4.84 ± 1.01×10^5	2.22 ± 0.55×10^6	0.16
50	4.84 ± 1.01×10^5	4.15 ± 0.26×10^5	−0.01
Leuconostoc citreum			
0	4.70 ± 0.71×10^5	4.32 ± 0.46×10^6	0.24
50	4.70 ± 0.71×10^5	2.20 ± 0.21×10^5	−0.08

HPE: Habanero pepper extract. μ: Growth/death rate at 4 h of exposure to HPE. Results of viable cells counts expressed as the means of triplicates with 95% confidence interval.

The inhibition of *S. aureus* in food is important, since this organism is potentially pathogen and may produce hemolysins and leukotoxins responsible for causing various diseases in consumers (Otto, 2014). Based on the results of Akyus *et al*. (2018), and the present study the application of HPE as a coating in cheese and sausages may be suggested, in order to avoid surface contamination with *S. aureus*, minimizing the modifications on the sensory properties inside the food product. Mokhtar *et al*. (2017), reported the inhibition of seven *S. aureus* strains by green bell pepper var. biskra using the agar disk diffusion method. Also, in the present study the culture broth method was selected to improve the contact of the antimicrobial agent with the bacteria, as well as to relate the concentration of the extract with the viable cells count. The highest inhibitory concentration of *S. aureus* was 50% HPE, and was selected to be tested in *L. casei* Shirota and *L. citreum* cultures analyzing its effects on the growth of LAB, some functional properties, microstructure and ribosomal proteins synthesis.

3.3 *Survival and glucose consumption of LAB exposed to habanero pepper extract*

Bearing in mind that *S. aureus* was completely inhibited after 4 h of incubation in Mueller-Hinton culture broth added with 50% of HPE, it was decided to study the survival of LAB under this condition. There was a slight decrease (15%) in the population of *L. casei* Shirota exposed to 50% of HPE after 4 h of incubation. When exposed to 50% HPE in culture broth, the survival of *L. citreum* was 54% of the initial count. In these conditions, negative values in the growth rates of *L. casei* Shirota and *L. citreum* were observed; however, the decrease in viability was lower than those observed in *S. aureus*, (Table 1), indicating that both LAB are more resistant to the HPE than *S. aureus*. Similar results have been observed by Cerón-Carrillo *et al*. (2014), who compared the antibacterial activity of habanero pepper extract against *L. casei* and *E. coli*, reporting a greater resistance to the extract by *L. casei* compared to *E. coli*. The higher survival of LAB exposed to HPE compared to *S. aureus* may be related to their resistance to osmotic stress (González-Quijano *et al*., 2014), since it has been reported that a high concentration of capsaicin can induce cell lysis as a result of osmotic stress, generated by the interaction of capsaicin with some receptor on the cell membrane that changes the ionic potential (Sharma *et al*., 2013; Kurita *et al*., 2014).

Once the resistance of LAB to the HPE after 4 h of exposure was observed, changes in growth of both LAB along 24 h were studied, monitoring their viable cell concentrations every 4 h. In *L. citreum*, the survival decreased to 58% at 12 h, but remained constant up to 24 h. For *L. casei* Shirota, a survival of 36% was observed in cells exposed to HPE for 12 h, while at 24 h no viable cells were found. Because the survival of *L. casei* Shirota remains constant for 12 h and inhibited after 24 h of exposure, it was decided to perform the subsequent analyses of functional properties, microstructure and ribosomal proteins profile of both LAB after 12 h of exposure to 50% HPE.

The glucose consumed by each LAB exposed 12 h to 50% HPE was quantified. The initial concentration of glucose in culture broth was 2.04 ± 0.13 mg/mL. The residual glucose concentration in culture broth was determined and the quantification of consumed glucose per CFU/mL was calculated for *L. casei* Shirota and *L. citreum* (Table 2).

Table 2. Glucose consumption per CFU/mL of *Lactobacillus casei* Shirota and *Leuconostoc citreum* exposed to habanero pepper extract.

HPE in broth (%)	Glucose consumption (ng/μL) *Lactobacillus casei* Shirota	*Leuconostoc citreum*
0	2.81 ± 0.15 ×10^{-6a}	2.74 ± 0.15×10^{-6x}
50	5.56 ± 0.89×10^{-3b}	6.54 ± 0.70×10^{-4y}

Means ± Standard deviation (SD). ^{a,b} and ^{x,y}: Different letters the in the columns indicate significant difference ($P \leq 0.05$) by Tukey's test. HPE: Habanero pepper extract.

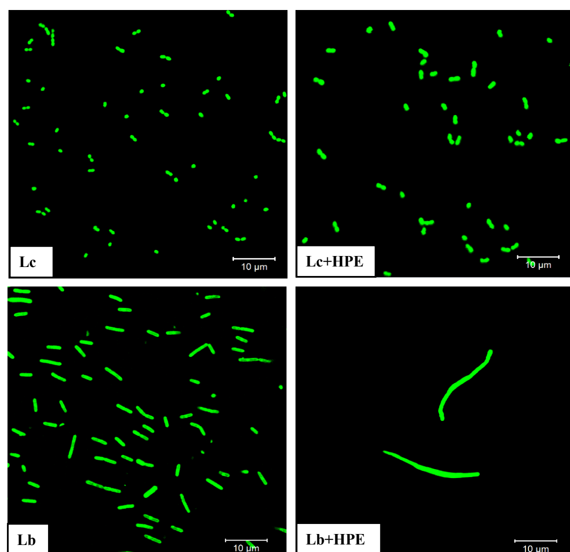

Fig. 2. Fluorescent images of *Leuconostoc citreum* and *Lactobacillus casei* Shirota grown 12 h in MRS culture medium (Lc and Lb) and exposed 12 h to 50% of habanero pepper extract in MRS culture medium (Lc+HPE and Lb+HPE).

The consumption of glucose by both bacteria was the same in the controls. It was observed that the glucose consumed by *L. casei* Shirota exposed to HPE for 12 h was significantly enhanced as compared to the control; this effect was also observed in the culture of *L. citreum* under the same stressful conditions. These results agree with the observed by Sharma *et al.* (2013); who report a significantly increase ($P \leq 0.05$) in glucose consumption of *L. acidophilus* exposed to an aqueous extract of red pepper during cheese curdling. These authors report that *L. acidophilus* incubated with red pepper extract showed an increase in its metabolic activity. The comparisons between the amount of glucose consumed by the controls and bacteria exposed to pepper extracts suggest an increase in its metabolic activity as a response to stressful conditions.

3.4 Morphometric analysis by LSCM

Fig. 2 shows the fluorescent images obtained by confocal microscopy for both LAB exposed 50% HPE and the controls. Images of *L. citreum* exposed to 50% HPE (Fig. 2.Lc+HPE) show a significant increase ($P \leq 0.05$) in area of 1.32 μm^2, in perimeter of 1.33 μm and in Feret's diameter of 0.38 μm, as compared to the control (Table 3). However, the morphological parameters (circularity, aspect ratio and round) were not significantly affected by HPE addition (i.e. the cells increased its size but retained their original shape). Meanwhile, the images of *L. casei* Shirota exposed to 50% of HPE (Fig. 2.Lb+HPE), showed a significant longitudinal cell elongation ($P \leq 0.05$) of 18.31 μm in the Feret's diameter and significant differences ($P \leq 0.05$) in the morphological parameters as compared to cells grown in the control (Table 3). The bacterial cells elongation has been observed in cells of LAB species exposed to different stress factors such as: low nutrient availability, low pH and high osmotic pressure (Gong *et al.*, 2012; Parlindungan *et al.*, 2018). Taheri-Araghi *et al.* (2015), indicate that this elongation is the result of cells segmentation restriction during the cell division cycle, being not a size increase of a single bacterium, but a set of them that have not completed their division cycle.

Table 3. Size and morphometric parameters calculated for *Lactobacillus casei* Shirota and *Leuconostoc citreum* exposed 12 h to 50% of habanero pepper extract.

Parameter	*Leuconostoc citreum*		*Lactobacillus casei* Shirota	
	Control	Exposed to HPE	Control	Exposed to HPE
Area (μm^2)	1.86 ± 0.33^{a}	3.18 ± 0.49^{b}	7.11 ± 0.70^{x}	68.42 ± 41.30^{y}
Perimeter (μm)	3.18 ± 0.26^{a}	4.51 ± 0.28^{b}	8.89 ± 0.59^{x}	52.14 ± 26.27^{y}
Feret's diameter (μm)	1.14 ± 0.08^{a}	1.52 ± 0.10^{b}	3.35 ± 0.26^{x}	21.66 ± 9.72^{y}
Circularity	0.71 ± 0.21^{a}	0.66 ± 0.09^{a}	0.41 ± 0.04^{x}	0.15 ± 0.12^{y}
Aspect ratio	1.44 ± 0.05^{a}	1.49 ± 0.06^{a}	3.72 ± 0.33^{x}	7.73 ± 2.48^{y}
Round	0.71 ± 0.02^{a}	0.69 ± 0.02^{a}	0.33 ± 0.04^{x}	0.16 ± 0.07^{y}

Means ± SD. ^{a,b} and ^{x,y}: Different letters indicate significant difference ($P \leq 0.05$) among the parameters of the same LAB grown in the control and exposed 12 h to 50% habanero pepper extract (HPE).

Papadimitriou *et al*. (2016), consider the bacterial cell elongation as a homeostatic response to the osmotic, pH, temperature or toxic stress; being a reversible mechanism that bacteria employs to ensure the viability of future generations, recovering their original size when the stress conditions are removed.

3.5 Microstructural analysis by SEM

Likewise, in SEM micrographs of *L. citreum* exposed to HPE (Fig. 3.Lc+HPE), it was observed that the surface of the cells changed from smooth in the control to rough, possibly due to changes of exopolysaccharides produced by the bacteria (Fig. 3.Lc). The loss of exopolysaccharide has been related to the inactivation of the extracellular enzymes (glycosyltransferase), responsible for its synthesis. This inactivation has been reported in cells of *Leuconostoc mesenteroides* subsp. *mesenteroides* incubated in increasing salts concentrations (Harutoshi, 2013). This finding suggests that HPE may also inactivate the exopolysaccharide production through an osmotic stress produced by capsaicin. The SEM micrograph of *L. casei* Shirota exposed to 50% HPE showed elongation and the presence of blisters on the cell wall (Fig. 3.Lb+HPE).

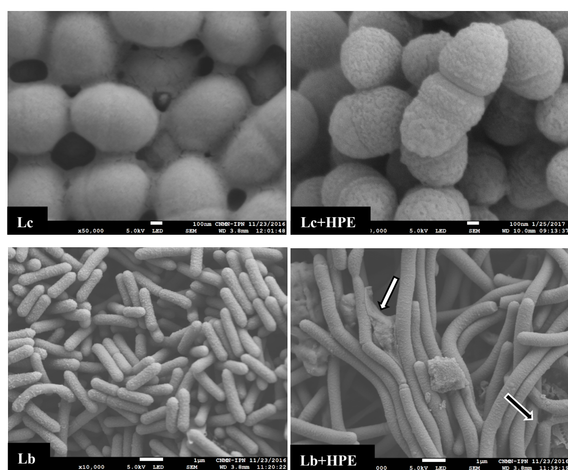

Fig. 3. SEM micrographs of *Leuconostoc citreum* (50000×) grown 12 h in MRS culture medium (Lc) and exposed 12 h to 50% of habanero pepper extract (Lc+HPE). SEM micrographs of *Lactobacillus casei* Shirota (10000×) grown 12 h in MRS culture medium (Lb) and exposed 12 h to 50% of habanero pepper extract (Lb+HPE), both in MRS culture medium. White and black arrows indicate cytoplasmic inclusions/blistering and damaged/lysed cells, respectively.

Besides the elongation of *L. casei* Shirota cells, the addition of HPE facilitated the formation of curls and overlapping of cells. Booyens and Thantshat, (2013) related the effect of an extract of garlic with the increase of membrane permeability in three different *Bifidobacterium* species (*B. lactis*, *B. longum* and *B. bifidum*); facilitating leakage of cytoplasmic material and deterioration of the enzymatic system, culminating in cell inhibition. The presence of blisters in the cell surface of *L. casei* Shirota exposed to HPE may be due to a defense mechanism by which stressed bacteria try to confine the antimicrobial agent to avoid its activity against cell viability. The elongation of cells suggests a morphological adaptation to stress by which the bacteria increase their contact surface in order to consume higher amounts of substrate, in order to adapt and survive (Papadimitriou *et al*., 2016). Sharma *et al*. (2013), have considered that the mechanism by which capsaicinoids interact with *L. acidophilus* include the presence of the ATP-binding cassette (ABC) transporters in the cell wall, with a similarity to the transient receptor potential vanilloid 1 (TRPV1), which is the ion channel responsible for the sensation of pungency or heat activated by capsaicin.

3.6 Survival to simulated GIT conditions and lysozyme resistance

The inoculum of LAB exposed and unexposed to HPE was adjusted to a viable cells concentration of 10^8 CFU/mL, from which the following analyses were performed. *L. casei* Shirota exposed to HPE did not show viability at pH 2 and in simulated GIT conditions. However, this microorganism was viable when exposed to HPE, and to bile salts in the control conditions. *L. casei* Shirota grown in the control showed a decrease of 0.24 log CFU/mL after 3 h of exposure to bile salts, whilst the same bacteria exposed 12 h to 50% of HPE decreased 4.13 log CFU/mL, under the same conditions. Likewise, *L. citreum* did not show viability at pH 2 and in simulated GIT conditions. Meanwhile a decrease of 5.38 log CFU/mL was observed for the control of *L. citreum* submitted to bile salts for 3 h; however this organism decreased 4.47 log CFU/mL after being exposed for 12 h to 50% of HPE and treated under the same bile salts conditions (Table 4).

The survival to GIT conditions, such as pH 2 and bile salts is one of the criteria for the pre-selection of potential probiotic strains (Silva *et al*., 2017).

Table 4. Functional properties of *Lactobacillus casei* Shirota and *Leuconostoc citreum* exposed 12 h to 50% of habanero pepper extract and the controls.

HPE in broth (%)	Survival to bile salts conditions		Lysozyme resistance		Adhesion to porcine mucin	
	Initial (CFU/mL)	Final (CFU/mL)	Initial (CFU/mL)	Final (CFU/mL)	Initial (CFU/mL)	Final (CFU/mL)
Lactobacillus casei Shirota						
0	$4.00 \pm 2.58{\times}10^{8a}$	$2.30 \pm 0.02{\times}10^{8a}$	$1.43 \pm 0.41{\times}10^{9c}$	$1.55 \pm 0.53{\times}10^{9c}$	$4.87 \pm 1.37{\times}10^{7e}$	$1.00 \pm 0.71{\times}10^{3f}$
50		$2.92 \pm 0.56{\times}10^{4b}$	$4.75 \pm 0.80{\times}10^{5d}$	$3.80 \pm 2.54{\times}10^{5d}$	$4.30 \pm 0.68{\times}10^{7g}$	$6.00 \pm 4.96{\times}10^{3h}$
Leuconostoc citreum						
0	$6.40 \pm 0.28{\times}10^{8i}$	$0.16 \pm 0.06{\times}10^{4j}$	$1.70 \pm 0.09{\times}10^{9k}$	$1.40 \pm 0.39{\times}10^{9k}$	$9.35 \pm 2.03{\times}10^{7m}$	0^{n}
50		$1.35 \pm 0.43{\times}10^{4j}$	$4.00 \pm 0.89{\times}10^{5l}$	$3.93 \pm 0.17{\times}10^{5l}$	$9.85 \pm 0.63{\times}10^{7o}$	$4.42 \pm 0.92{\times}10^{5q}$

Means ± SD. $^{a-q}$: Different letters in the same line indicate significantly difference ($P \leq 0.05$) by Tukey's test, among the initial and final viable cells counts for each analysis. HPE: Habanero pepper extract.

However, both LAB (controls and exposed to 50% of HPE) did not show resistance to pH 2, which is the most extreme condition along the GIT system. The control of *L. casei* Shirota maintained its initial viability in bile salts conditions, which proves its probiotic character. However, when *L. casei* Shirota was exposed to 50% HPE, it was more susceptible to the action of bile salts, showing a decrease in population from 10^8 to 10^4 CFU/mL. Meanwhile, *L. citreum* grown in the control was more sensitive to the bile salts than *L. casei* Shirota. When exposed to 50% HPE, *L. citreum* showed a lower decrease in viability in bile salts conditions. Mendoza-Madrigal *et al*. (2017), also observed viability loss in *Bifidobacterium* in a serrano pepper matrix after 30 min of GIT simulation. Likewise, Silva *et al*. (2017), compared the protective effect of a simulated standardized food (containing lipids, protein, carbohydrates and water) and saline solution on the survival of *L. citreum* and *Weissella cibaria*, during their exposure to stomach and intestinal conditions. These authors reported an increase of 5 log CFU/mL in the survival of both bacteria when the standardized food was used as food matrix, compared to the saline solution. The former indicates that the protective effect depends not only on the presence or absence of the food matrix, but also on the type of matrix used.

Results of lysozyme resistance at 30 and 120 min for *L. casei* Shirota and *L. citreum* are presented in Table 4. The concentrations of viable cells remained constant throughout the analysis for both LAB. *L. citreum* exposed to HPE and grown in the control showed a lysozyme resistance > 90%; this same result was observed for *L. casei* Shirota exposed to HPE and grown in the control. The results of lysozyme resistance obtained in this study agreed with those reported for LAB. Giles-Gómez *et al*. (2016) reported a lysozyme resistance greater than 80% in *Leuconostoc mesenteroides* P45 isolated from pulque (a traditional Mexican beverage).

Similarly, García-Ruíz *et al*. (2014) also observed a resistance of 80% for *Lactobacillus plantarum* CIAL-49, *Pediococcus pentosaceus* and *L. casei*, even at 120 min of exposure to the enzyme. These bacterial strains, including those used in this study, have mechanisms associated to changes in the cell wall, particularly $N-$ and $O-$ substitutions in the peptidoglycan structure, by which are able to tolerate the antimicrobial effect of lysozyme (Giles-Gómez *et al*., 2016).

3.7 Assay of adhesion to porcine mucin in vitro

The results of this analysis are reported as concentration of viable cells adhered to porcine mucin (Table 4). The viability of the adhered cells of *L. casei* Shirota exposed to 50% of habanero pepper extract was the same as the viability of the cells grown in the control. An increase in viability of adhered cells was observed in *L. citreum* exposed to 50% of HPE, in which a concentration of $4.42{\times}10^5$ CFU/mL was observed. Meanwhile, viability of adhered cells of *L. citreum* was null in control samples. The adhesion to porcine mucin is one of the most important characteristics of probiotic strains, being a fundamental requirement for colonization of the GIT in which probiotic bacteria are located and do not allow the adhesion of other undesirable bacteria. The adhesion ability is influenced by the hydrophobicity of the cell wall and the ability to form cell aggregates, as well as the formation of hydrogen bonds for the reception or donation of electrons between the surface of the cell wall and mucin glycoproteins; although it is important to consider that these characteristics are particular of each strain (Nishiyama *et al*., 2016).

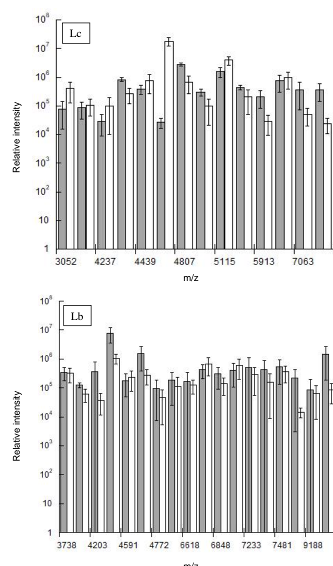

Fig. 4. Relative intensity of ribosomal proteins of *Leuconostoc citreum* (Lc) and *Lactobacillus casei* Shirota (Lb), grown 12 h in MRS culture medium (■) and exposed 12 h to 50% of habanero pepper extract in MRS culture medium (□). Means ± SD of triplicates obtained.

Based on this information, the increase in mucin adhesion of *L. citreum* may be related to modifications observed on cell wall surface generated by HPE addition.

3.8 Ribosomal proteomic analysis

Fig. 4 shows the mass spectra of each LAB exposed for 12 h to 50% of HPE, comparing to those grown in the controls. The mass spectra of *L. citreum* showed 14 repeated signals in the control and in samples exposed to HPE, from which it was possible to identify a significant increase ($P \leq 0.05$) in the relative intensity of a signal with m/z=4675 (Fig. 4.Lc). According to the bioinformatic search, the signal detected in *L. citreum* mass spectra could correspond to the 50S ribosomal protein L36 with a molecular weight of 4670 Da. This ribosomal protein is made up of 39 amino acids being the smallest bacterial ribosomal protein of 50S ribosomal subunit, codified by the *rpmJ* gene. The upregulated synthesis of this ribosomal protein has been related to size increase and slow growth of *E. coli* cells incubated in starving environments (Maeder and Draper, 2005). The previous information suggests that the growth behaviour during exposure to HPE observed in *L. citreum* may be due to the change in the synthesis of ribosomal protein L36. Secondly, *L. casei* Shirota showed 18 repeated signals in the mass spectra of the cells grown in the control and exposed to 50% of HPE (Fig. 4.Lb). The exposure of *L. casei* Shirota to HPE significantly decreased ($P \leq 0.05$) the relative intensity of the ribosomal protein with m/z=4696, compared to the control.

The comparison of the value of the m/z ratio of the signal with the molecular weight of the ribosomal proteins in database suggests that the signal detected correspond to the cold shock protein *cspA* with a molecular weight of 4640 Da. The cold shock *cspA* ribosomal protein of 42 amino acids has been reported in several *L. casei* strains. This ribosomal protein helps control the effects of cold in bacteria by binding to nucleic acids maintaining the linear structure of RNA and DNA, allowing an efficient conservation of genetic information once the thermal stress has ended (Bisht *et al*., 2014). Cold-shock ribosomal proteins are encoded by the gene *cspA*, which has been extensively studied in LAB such as *Lactococcus lactis*, *L. acidophilus* and *L. plantarum*, due to their use as starter cultures that experience low temperatures. Although *cspA* protein plays an important role during the cold response, recent studies have shown that this protein has a varied participation in several stress responses (Keto-Timonen *et al*., 2016).

At physiological temperature (37 °C), this protein helps improve nutrient consumption during the adaptation stage, and its synthesis decreases when increasing the metabolism of bacteria (Bucka-Kolendo and Sokolowska, 2017). Capozzi *et al*. (2011), have observed a cold shock-like response at 37 °C, when sub-lethal amounts of chloramphenicol were added to *Bacillus subtilis* cultures. This finding suggests that there may be a correlation between a cold shock response and the exposure of the bacteria to antimicrobial substances, even at an optimum growth temperature. Based on these results, it was decided to amplify the genes *rpmJ* (for *L. citreum*) and *cspA* (for *L. casei* Shirota), responsible for the synthesis of the ribosomal protein related to the size increase and to the behaviour during the exposure to 50% HPE for 12 h observed in both LAB.

3.9 *Sequencing of the genes responsible for the synthesis of ribosomal proteins*

Once the genes to be amplified were identified, the primer pairs used in PCR were designed using the Primer3 software (www.bioinformatics.nl/primer3plus). The designed primers were synthetized in the IFC of UNAM in Mexico City, according to the nucleotide sequences presented in Table 5.

Table 5. Sequences of designed primers for amplification of *rpmJ* and *cspA* genes in lactic acid bacteria exposed 12 h to 50% of habanero pepper extract.

Gene	Primer sequence (5'→ 3')	Product size (bp)
Lactobacillus casei Shirota		
cspA	Forward: GGGTTACGGCTTCATCACTG	111
	Reverse: TCGTAGGAAACAGCCTGACC	111
Leuconostoc citreum		
rpmJ	Forward: AGCGCGTTGCTTATGCTTAG	111
	Reverse: ATGAAGGTACGCCCATCAG	110

PCR amplification probably showed the presence of *rpmJ* gene (expected size ~120 bp) in *L. citreum* and *cspA* gene (expected size ~136 bp) in *L. casei* Shirota, in the controls and those exposed to HPE. After obtaining the sequences of amplified genes, they were edited with the Chromas Lite 2.6.4 software (Technelysium Pty Ltd., QLD, Australia). The edited sequences were used to find the highest similarity to the ribosomal protein database using the NCBI blastx (translating from nucleotide to protein). The gene sequence of *L. citreum* grown in the control showed a 100% similarity with 50S ribosomal protein L36 (GenBank accession number: WP002816013.1); while a 94% similarity to this same ribosomal protein was observed with the sequence of *L. citreum* exposed to HPE. For *L. casei* Shirota grown in the control a similarity of 83% to major cold-shock protein (GenBank accession number: AAC80244.1) was observed; while *L. casei* Shirota sequences exposed to HPE showed a similarity of 59% with the same ribosomal protein. However, the low percentages of similarity observed in *L. casei* Shirota exposed and unexposed to HPE, were not enough to confirm that the ribosomal protein *cspA* is present in *L. casei* Shirota cells.

Conclusions

The exposure of *S. aureus* to 30% of the habanero pepper extract caused inhibition at 8 h, while when exposed to 50% of the extract this pathogenic bacteria was inhibited at 4 h; in both cases the lysis of *S. aureus* cells was observed in the micrographs. In the same conditions, the viability of *L. casei* Shirota, a probiotic strain, was maintained in 85% with respect to the inoculum cell concentration. It was observed and elongation of the cells of this probiotic bacteria as an adaptation response, however, the concentration of cells adhered to mucin and the lysozyme resistance were not affected, compared to the controls, which are important functional properties for the survival of probiotics in the gastrointestinal tract. *L. citreum* cells showed a 54% of survival and a size increase when exposed to 50% of HPE; however, the adhesion to mucin was improved and the resistance to bile salts showed a smaller decrease when compared to the control. Although the ribosomal proteomic profile of both lactic acid bacteria showed some modifications, a positive relationship between the exposure to the habanero pepper extract and two functional properties of the two lactic acid bacteria was observed.

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