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INSIGHT OVER *Lactobacillus plantarum* 299V PHYSICOHEMICAL CHARACTERISTICS OF AGGREGATION KINETICS UNDER STARVATION AND DIFFERENT pH CONDITIONS ESTUDIO SOBRE LAS CARACTERÍSTICAS FISICOQUÍMICAS DE *Lactobacillus plantarum* 299V EN CINÉTICAS DE AGREGACIÓN EN INANICIÓN Y A DIFERENTES CONDICIONES DE pH

G. Melgar-Lalanne[1*], J. Ley-Martínez[1], E. Azuara-Nieto[1], D.I. Téllez-Medina[2], T. Meza[3], C.R. González-González[4], G. F. Gutiérrez-López[2]

[1]*Instituto de Ciencias Básicas. Universidad Veracruzana. Av. Dr. Luis Castelazo Ayala s/n. Col Industrial Ánimas, 91192. Xalapa, Veracruz, Mexico.*

[2]*Escuela Nacional de Ciencias Biológicas. Unidad Profesional Adolfo López Mateos, Av. Wilfrido Massieu Esq. Cda. Miguel Stampa s/n, Col. Zacatenco, C.P.07738. Delegación Gustavo A. Madero. Mexico City. Mexico.*

[3]*Laboratorio de Genómica Humana, Facultad de Medicina, Universidad Veracruzana, Calle Médicos y Odontólogos S/N., Col. Unidad del Bosque, C. P. 91010, Xalapa, Veracruz, Mexico.*

[4]*Instituto Tecnológico Superior de Acayucan. Carretera Costera del Golfo Km 216.4 Colonia Agrícola Michapa, CP. 96100. Acayucan, Veracruz, Mexico.*

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Abstract

Bacterial aggregation is ruled by specific and nonspecific adhesion mechanisms. For the probiotic strain *Lactobacillus plantarum* 299v (*Lp299v*) the biochemistry of adhesion has been previously studied and S-layer proteins have been identified. However, physicochemical mechanisms which ruled the aggregation and adhesion phenomena able to change through the time under different pH conditions have not been completely elucidated. In this study we have described the non-specific adhesion mechanisms (hydrophobicity, zeta potential and morphology) of *Lp299v* auto-aggregates over time (24 h) at different pH levels (from 5.0 to 9.0) under starvation conditions. Auto-aggregation was time-dependent increasing up to 60% after 22 h at pH 5. On the other hand, the acidophilic nature of the *Lp299v* cell surface was confirmed showing higher affinity with chloroform than with n-hexadecane, while zeta potential was negative in all cases. Size and shape parameters were found to be time and pH dependent. A strong Pearson correlation (0.991) was observed between circularity and auto-aggregation. In conclusion, *Lp299v*'s nonspecific adhesion mechanism was found to be more related to time than to pH levels, indicating that van der Waals and hydrophobic forces have the potential to dominate the auto-aggregation of *Lp299v*. Finally, the results showed that lower pH may promote auto-aggregation possibly easing the colonization at the upper level of the digestive system.

Keywords: probiotics, zeta potential, image analysis, aggregation, Feret diameter.

Resumen

La agregación bacteriana se rige por mecanismos de adhesión específicos e inespecíficos. En el caso de *Lactobacillus plantarum* 299v (*Lp299v*), la bioquímica de la adhesión ha sido previamente estudiada y se han identificado algunas proteínas de la capa superficial. Sin embargo, los mecanismos fisicoquímicos que rigen los fenómenos de agregación y adhesión capaces de cambiar a lo largo del tiempo en diferentes condiciones de pH no han sido dilucidados. En este estudio se describieron los mecanismos de adhesión no específicos de los agregados a lo largo del tiempo (24 h) a diferentes niveles de pH (de 5.0 a 9.0) en condiciones de inanición. La auto-agregación incrementó en el tiempo con un nivel máximo de 60% alrededor de las 22 h a pH 5. Por otro lado, se confirmó el carácter acidofílico de la superficie de *Lp299v* al mostrar mayor afinidad al cloroformo que al n-hexadecano, mientras que el potencial zeta presentó carga negativa. Los parámetros de tamaño y forma dependieron del tiempo y el pH. Se observó una fuerte correlación de Pearson (0.991) entre la circularidad y la auto-agregación. En conclusión, el mecanismo de adhesión inespecífica de *Lp299v* se relacionó más con el tiempo que con los niveles de pH, lo que indica que las fuerzas hidrofóbicas y las fuerzas de van der Waals podrian dominar la auto-agregación de *Lp299v* en las distintas condiciones. Finalmente, los resultados mostrados en este estudio sugieren que el pH a bajos niveles podría influir positivamente en la auto-agregación facilitando posiblemente la colonización en la parte superior del sistema digestivo.

Palabras clave: probióticos, potencial zeta, análisis de imagen, agregación, diámetro de Feret.

* *Corresponding author. E-mail*: gmelgar@uv.mx
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1 Introduction

There is a growing field of study focused on beneficial microbes with specific roles. These microbes could be used as probiotics in both, foods and supplements to improve human health to prevent some metabolic diseases and as treatment when they survive the gastrointestinal tract passage and adhere in the intestinal mucosa where produce bioactive metabolites with benefits to the host health. As it has been widely reported, these beneficial effects largely depend on intestinal residence time, regulated by synergic adhesion and aggregation mechanisms. So, aggregation is a complex process involving nonspecific and specific ligand-receptor mechanisms between two surfaces (bacteria-bacteria, epithelial cell-bacteria or intestinal mucosa-bacteria) in which adherence of bacterial cells is directly related to their cell surface physicochemical characteristics (García-Cayuela *et al*., 2014).

There are different types of forces that operate during the adhesion process, depending on the distance between the cells. Thus, specific and molecular mechanisms involve very short distances (< 1 nm), while nonspecific mechanisms mostly involve distances between 5 and 50 nm (Busscher and Van der Mei, 2012). Specific adhesion mechanisms are influenced by the presence of certain physical surface structures, such as flagella and pili, which improve motion. The presence of exopolysaccharides and the presence of specific surface proteins regulate the adhesion at very low distances in a specific way allowing specific ionic, hydrogen and possibly chemical bonds between adhesins and complementary receptors (Laio *et al*., 2015). Sometimes, these specific forces have been categorized as interaction forces, although such forces do not exist from a physicochemical perspective (Busscher and Van der Mei, 2012). The specific adherence characteristics have been revealed by the mannose-binding sites on the mucosa cells in many probiotic strains (Singay *et al*., 2016; Buntin *et al*., 2017). However, along with the physical structures expressed by bacteria and chemically specific ligand-receptors, environmental factors, such as growth phase and state, with the presence of stressors can modify certain chemical characteristics, such as the hydrophobicity and electrostatic charge of the bacterial surfaces. Moreover, some physical characteristics, such as bacterial morphology (cell size and sphericity) can be also affected (Liao *et al*., 2015). In fact, both specific and nonspecific interactions share the same fundamental physicochemical origins and are the sum of every relatively weak pairwise interaction with all atoms in the bacterium adhering to the substrate which yields the final interaction forces (Busscher *et al*., 2008).

The non-specific adhesion interactions are supposed to originate from an interplay between Lifshitz van der Waals forces, attractive or repulsive electrostatic forces, hydrogen bonding and Brownian motion forces which are given in a distance between 5 to more than 50 nm (Busscher and Van der Mei, 2012). These mechanisms are basically ruled by hydrophobic and van der Waals forces and may affect the attachment of bacteria to different surfaces (Kos *et al*., 2003). Strain growth development, survival and production of metabolites may be tightly related to environmental responses that influence the non-specific interactions and so, may affect the beneficial actions of the probiotics strains mainly when the bacteria do not have the proper conditions to produce specific adhesion molecules. The cell-cell interactions during the bacterial aggregation processes at different conditions of time, lack of nutrients and pH over the non-specific forces that rule the physicochemical mechanisms in probiotic aggregation have not been completely elucidated.

Bacterial auto-aggregation is considered a prerequisite for intestinal colonization. The auto-aggregates can adhere to the mucosa surface increasing the probiotic persistence and the cell concentration in the intestine, necessary for their optimum functionality (Collado *et al*., 2007). Hence, the auto-aggregates can inhibit pathogen's adhesion resulting in stopping setting up and colonization. For achieving this, probiotic bacteria struggle in the intestine a stressful environment with drastic pH variations (from 5.0 to 9.0), peristalsis, high osmotic concentrations, the presence of bile salts and enzymes (Gong *et al*., 2012). To survive under these stressful environmental conditions, lactobacilli are able to modify their cell surface membrane resulting in changes in the cell morphology (González-Vazquez *et al*., 2014) and in their fatty acid profile (Haddaji *et al*., 2017) which results in changes in their colloidal surface properties that may modify some adhesion properties.

Microorganisms surface charge properties may influence their physicochemical interactions with interfaces and could be used to estimate the damage or modification in the cell wall that can affect

adhesion and aggregation properties (Cano-Sarmiento *et al*., 2018). These surface charges are affected by complex and nonspecific interactions determined through hydrophobicity and surface charge colloidal properties, which show a correlation with their non-specific adhesion to surfaces (Ubbink and Schär-Zammaretti, 2007). Hydrophobicity has been previously reported through the indirect determination of the bacterial adhesion to hydrocarbons (BATH) test. The BATH test analyzes the relative percentage of bacteria kept in a hydrophobic phase after mixing it with an aqueous phase containing the initial bacterial culture. The cell surface charges can be deduced from the zeta potential measured with zetametry. Zeta potential is an indicator of the stability of the colloidal dispersions and the degree of electrostatic repulsion between adjacent and similarly charged particles in a dispersion (Ayala-Torres *et al*., 2014; Mendoza-Sánchez *et al*., 2018).

The understanding of electric charge interactions is essential to the development of food systems since these interactions can determine the type of particle-particle interactions. Therefore, zeta potential strongly affects the stability, rheological behaviour, sedimentation, redispersion, filtration, shelf life, texture, flavor and color in food systems. Moreover, it can be used to detect changes in the bacterial surface produced when bacteria are in contact with technological and physiological stress factors as well as to predict bacterial adhesion and aggregation (Cano-Sarmiento *et al*., 2018).

A positive correlation between *in vitro* aggregation ability and hydrophobicity has been reported for some lactobacilli strains, but not in others where both parameters seem to be independents. For example, recently it was concluded that hydrophobic/hydrophilic properties of *L. casei* strains could be independent of the adhesion ability to HeLa cells (Haddaji *et al*.2015a). However, adhesion, aggregation, and surface hydrophobicity are considered phenotypic traits that potentially provide microbial colonization advantages within the intestinal tract (García-Cayuela *et al*., 2014). This phenotypic plasticity of the microbial cells exhibits different subpopulation responses with different growth and stress resistance characteristics. The stress robustness of bacteria that favor the development of the microbial population in the gut has been related with the phenotypic plasticity of the individual microbial cells and stochasticity resulting from the low amount of the biochemical species involved in the stress response pathways (Nguyen *et al*., 2016). Stress factors are responsible for significant changes in the cell composition, morphology, and surface charge of bacteria cell walls, mostly in electrokinetic and hydrophobic forces (Gong *et al*., 2012; González-Vázquez *et al*., 2014) although these factors are poorly studied in probiotic strains. Thus, for example the heat and acid stress can damage the cell envelope of *L. casei* strains modifying the membrane fatty acid composition. So, low pH resulted in saturation levels of fatty acids as a response to the stress exposure (Haddaji *et al*., 2015a; Haddaji *et al*., 201b). The presence of hydronium and hydroxyl ions modifies the ionic forces in the medium affecting the surface interactions and increase the membrane permeability which can result in a loss of viability. Moreover, pH changes can also affect the bacterial morphology, and both phenomena could be responsible of aggregation changes in bacteria to maintain the cell survival (Canzi *et al*., 2005), although it has been poorly studied in probiotic strains. The intestinal pH varies between 5.0 in the proximal duodenum, close to the stomach, to 8.5-9.0 in the colon. Variations in pH also depends form the food matrix and the fat content (Kwon, 2002). The lack of nutrients in the bacterial medium also changes the morphology and the physiology of the bacteria and more coccoid shapes have been reported in some starved bacteria (Parlindungan *et al*., 2018). The size of bacteria, cell surface properties and exopolysaccharides composition are altered by starvation, and the solution chemistry (ion strength and ion types) and the bacteria transport competition is enhanced (Han *et al*., 2013).

The strain *Lactobacillus plantarum* 299v (DSM9843) is a probiotic present in the formulation of some nutraceuticals and probiotic foods which has shown to be effective in the treatment of irritable bowel syndrome, Crohn's disease and colitis and which has previously proved its survival and adhesion thought gastrointestinal gut (Ducrotté *et al*., 2012; Hernández-Rodríguez *et al*., 2016). The auto-aggregation and adhesion to mucin at normal conditions was previously reported (Melgar-Lalanne *et al*., 2013, Buntin *et al*., 2017) as well as some cell surface properties including hydrophobicity to polar and nonpolar solvents, the presence of capsular exopolysaccharides (Melgar-Lalanne *et al*., 2015a) and the influence of the carbon source on the mucin adhesion (Melgar-Lalanne *et al*., 2015b). Studies on cell surface specific and nonspecific mechanisms were also recently reported by Buntin *et al*.2017.

However, the combined influence of pH and time in starvation of aggregation and cell surface

properties, including morphology by scanning electron microscopy and electrokinetic forces (zetametry) have not been previously reported. It is important to note that the adhesion mechanism of *Lp299v* is mediated by surface proteins encoded by genes with LPxTGmotif which may colonize different types of surfaces by modulating the expression level of these genes (Buntin *et al*., 2017).

This study aimed to evaluate the non-specific adhesion mechanisms (hydrophobicity, charge and morphology) of *Lp299v* auto-aggregates thought time (24 h) at simulated intestinal pH conditions and its possible relationships. Also, the kinetic of hydrophobicity, charge and morphological changes in bacterial cell surface properties of auto-aggregated *Lp299v* were explored at pH from 5 to 9. A better understanding of this complex phenomenon may allow for the design of more efficient probiotic vehicles.

2 Materials and methods

2.1 Bacterial strain and growth conditions

The commercial probiotic strain *Lactobacillus plantarum* subs. 299v (Protransitus LP®, Barcelona, Spain) was used for the present reseach. The strain was cultured at 37 °C for 24 hours in Man Rogosa Sharpe (MRS) broth (Difco, Le Point de Claix, France) under aerobic and static conditions (to guarantee the stationary phase) before its experimental use; and preserved frozen at -20 °C with glycerol as cryoprotectant for its conservation between experiments. To estimate the concentration of bacteria, a linear correlation between absorbance in PBS at 600 nm (A_{600nm}) and colony-forming-units (cfu) /mL was obtained by plating serial dilution of the culture in MRS agar at 37 °C for 24 h (R = 0.999; $y\left(\frac{cfu}{mL}\right) = 3 \times 10^8 \times (A_{600nm}) - 7 \times 10^6$). The microbiological count of *Lp299v* in PBS buffer at the three pH tested was ~ 2.9×10^8 CFU/mL.

2.2 Reagents and material

All the reagents used for the experimentation were analytical grade unless otherwise indicated. Material used were the typical for a microbiology laboratory.

2.3 Dispersion solution at different pH

Auto-aggregation, Zeta Potential and hydrophobicty experiments were performed using bacteria suspended in 1X phosphate buffered saline at three different pH. Buffers were prepared as follows: a) buffer PBS at pH 7.0 (8.0 g/L NaCl, 0.2 g/L KCl, 0.541 g/L KH_2PO_4, 0.854 g/L, Na_2HPO_4;) buffer PBS at pH 5.0 (8.0 g/L NaCl, 0.2 g/L KCl, 1.340 g/L KH_2PO_4, 0.021 g/L, Na_2HPO_4); c) buffer PBS at pH 9.0 (8.0 g/L NaCl, 0.2 g/L KCl, 0.008 g/L KH_2PO_4, 1.410 g/L, Na_2HPO_4). All buffer had a calculated ionic strength of 0.177 mol/L.

2.4 Auto-aggregation kinetics

Auto-aggregation was calculated following the methodology proposed by Collado *et al*.2007. Briefly, *Lp299v* was grown statically in MRS broth at 37 ºC for 24 h and then, harvested by centrifugation (Minicentrifuge mySpin6, Thermo Fisher Scientific, Madison, USA) at 10000*xg* for 10 minutes. Finally, the bottom pellet was washed twice with the PBS at the different pH (5.0, 7.0 and 9.0) and suspended in the same buffer. Initial absorbance at 600 nm was adjusted to 1.0 (~ 2.9×10^8 cfu/mL) (Spectrophotometer UV/vis; Cientifica Vela Quim, Mexico City, Mexico). The bacterial suspension was stirred in vortex (Vortex Genie 2 G560, Scientific Industries, USA) for 10 s, incubated at 37 °C in a water bath and absorbance was measured every 2 h during 24 h at the same temperature.

Auto-aggregation (A) was calculated using equation [1]:

$$A(\%) = \left(1 - \frac{A_s}{A_0}\right) \times 100 \quad (1)$$

Where A_s is the absorbance of the supernatant at 600 nm at each time and A0 is the absorbance at 600 nm at the initial time. The absorbance is directly related with the turbidity of the media and so, with the bacterial aggregation (Collado *et al*., 2007).

2.5 Determination of cell surface hydrophobicity (CSH)

The bacterial adhesion to solvents (BATH) assay was used to determine CSH following the methodology of Kos *et al*.(2003). In brief, bacteria were growth in MRS broth during 24 h, and then were washed and diluted to an initial A_{600nm} = 1.0 in PBS buffer previously adjusted to pH 5.0, 7.0 and 9.0. The cell

suspension was mixed with n-hexadecane (as a non-polar solvent) or chloroform (as an electron acceptor or acidic solvent) in a 4:1 (v/v) ratio. The mixture was gently mixed in a vortex for 2 min and the tubes could stand for 1 h in a water bath at 37 °C. Absorbance of the aqueous phase was determined in a spectrophotometer (Velab VE-5600UV, Velquim, Mexico City, Mexico). Then, bacteria were adhered to the non-polar solvent thought hydrophobic forces. The hydrophobicity (H) of each solvent was calculated with equation [2]:

$$H(\%) = \left(1 - \frac{A_f}{A_0}\right) \times 100 \tag{2}$$

2.6 Zeta potential

The measurement of the zeta potential was performed on a Zeta Plus Analyzer (Zetaplus Brookhaven Instruments, Holtsville, New York, USA) following the methodology proposed by Ayala-Torres *et al*.2014 with some modifications. *Lp299v* was grown as previously indicated, harvested by centrifugation (10000xg, 15 min), washed with PBS and adjusted to A600 nm 1.0 with the same buffer. The temperature of samples was controlled at 37 °C with a water bath (BÜCHI Heating Bath B-490, Switzerland). Zeta potential was monitored at 0, 2, 4, 16, 20 and 24 h at pH 5.0, 7.0 and 9.0. The blank used to measure zeta potential was PBS without the bacteria adjusted to the different pH tested. Results were analyzed with the software zetapw32 5.58 (Brookhaven Instruments Corporation, Holtsville, New York, USA).

2.7 Scanning Electron Microscopy

Samples were treated in PBS buffer at pH 5.0, 7.0 and 9.0 at 0, 4 and 24 h of auto-aggregation kinetics. For that, 10 μL of sample were fixed with a glutaraldehyde solution (2.5%, v/v) for 4 h and washed with Sorensen buffer (0.2M; pH 7.4;81 mL of 0.2M NaH_2PO_4 (35.61 g/L) to 19 mL 0.2 M of Na_2HPO_4 (31.21 g/L)). Then, samples were centrifuged at 2,500 g, 10 min and 10 °C in a microcentrifuge (Eppendorf 5424, Hamburg, Germany). The precipitate was gradually dehydrated in crescent ethanol/water solutions from ethanol 10% to absolute ethanol. The precipitate was suspended in each ethanol solution with a vortex (Genie 2 Scientific Industries Model G560, USA) and centrifuged (3,500 g, 5 min, 10 °C) three times. Samples were finally functionalized for microscopy analysis with a sample holder previously cleaned, polished and ultrasonicated at 20 kHz and amplitude of 20 μm (Ultrasonicator, Leo B20Q, Taiwan) for 5 min. Finally, samples were dried by using a critical point dryer (Quorum K850, England). Then, samples were received in a gold metal plated with an ionizer (Quorum Q150R S, England) and kept into a desiccator (Scienceware Z553395, U.S.A.) at 15% relative humidity, until observation under a scanning electron microscope (SEM) (FEI Quanta 250 Feg, USA) at magnification of 5000x.

2.8 Morphological descriptors

SEM micrographs obtained at 5000x (720X480 pixels resolution) were processed by digital image analysis (DIA) to obtain morphological descriptors from the auto-aggregated cells to measure the following descriptors chosen to estimate aggregate morphology:

- The 'maximum Feret's diameter' which is the maximum distance between any two points on the perimeter of the particle.
- The 'circularity' which measures how similar the object shape is to a circle independently of the object size. A value of 1.0 indicates a perfect circle; as the value approaches 0.0, it indicates an increasingly elongated shape. It is calculated as equation [3]:

$$Circularity = 4\pi \frac{[area]}{[perimeter]^2} \tag{3}$$

- The 'aspect ratio' (AR) which is defined as the ratio of the Feret's minimum length to the Feret's maximum length. Thus, as the width and length of the shape approach the same value, the aspect ratio approaches to one, but it doesn't mean that the shape is circular; often very symmetric shapes also have a very high aspect ratio (square, regular octagon, equilateral triangle, etc.).

DIA was performed by using the software ImageJ v1.51a (http://imagej.nih.gov/ij) (National Institutes of Health, Bethesda, Maryland, USA) as González-Vázquez *et al*.2014. The scale for the measurements was calibrated at 26 pixels / μm; thus, the measuring tools of the software package were directly used to determine the parameters mentioned. All cells from each individual micrograph were selected to extract the morphometric data.

At least three fields or each triplicate aggregation kinetic sample were taken for the microscopic analysis and several algorithms were analyzed to calculate the analyzed parameters (Tijani *et al*., 2015). To measure single cells and not chains, cell fragments or image noises, circularity was adjusted from 0.25 to 0.75 and aspect ratio (AR) lower than 3.0 (Krieger *et al*., 2013).

2.9 Statistical analysis

Statistical analysis was performed using Sigma Plot 12.5 (Systat Software, USA). Analysis of variance (two-way ANOVA) was performed for the effect of pH and time on the auto-aggregation, zeta potential, size and shape parameters as responses. Tukey post-hoc analysis was performed to compare means between groups. The significance level was set at $2\alpha = 0.05$. Graphics were made in R-project (R Core Team, 2017) using ggplot2 package for boxplots (Wickham, 2009) and GeoR (Ribeiro and Diggle, 2016) and lattice (Sarkar, 2008) for surface plot by using a 2nd degree polynomial regression fitting for prediction of auto-aggregation and zeta-potential as dependent variables, and pH between 5 and 9 (3 levels) and time from 0 to 24 h (13 levels) as independent variables (Cleveland, Grosse and Shyu, 1992). All experiments were performed in triplicate.

3 Results and discussion

3.1 Kinetics of auto-aggregation at different pH levels

This experiment consisted in evaluating the response of auto-aggregation, zeta potential, and size and shape parameters of *Lp299v* as dependent variables and pH from 5 to 9 and time from 0 to 24 hours as independent variables under starvation conditions at the same ionic strength (0.177 mol/ L) at 37 ºC. Prior to the experiments, the viability of bacteria at the three pH values was tested at both the starting point (0 h) and after the end point (24 h). As expected in inanition conditions, no significant variations on viable cell counts were found ($\sim 2.9 \times 10^8$ cfu/mL).

The auto-aggregation kinetics (%) of *Lp299v* at $A_{600nm} = 1.0$ at different pH levels are shown in Figure 1. Auto-aggregation increased with the time, reaching its highest at 22 h and pH 5.0 (61.367 ± 0.305).

Table 1. Contribution (%) in function of pH and time (Two-way ANOVA) of analyzed parameters auto-aggregation (%), zeta potential (mV) size parameters (area and Feret diameter) and shape parameters (circularity and Aspect Ratio).

Parameter/Factor	Contribution(%)*		
	time	pH	Time × pH
AA (%)	90.73	1.28	7.94
Zeta potential (mV)	35.50	41.27	22.71
Area (μm^2)	30.39	40.00	29.41
Feret diameter (μm)	38.88	28.88	32.22
Circularity	13.83	38.30	47.83
AR	40.22	22.56	37.20

$*contribution(\%) = \frac{Sum\ of\ squares\ of\ a\ factor}{total\ sum\ of\ squares}$

According to the ANOVA output, showed in Table 1, pH had a smaller effect than time, however, there was a significant difference on auto-aggregation after 24 h ($P < 0.01$), indicating that lower pH enhanced auto-aggregation. Furthermore, a Pearson correlation ($P > 0.899$) was found for auto-aggregation with time.

3.2 Cell surface properties

The hydrophilic and acidic affinity of the cell surface of *Lp299v* was confirmed as the cells repelled n-hexadecane ($5.0 - 9.0$) but not chloroform across the pH range tested (Table 2). The electrostatic charge of the cell surfaces was measured as the zeta potential for 24 h at pH levels of 5, 7 and 9 in the PBS buffer (Figure 2). Results revealed that *Lp299v* cells maintained their negative charge under all the conditions tested. The most negative zeta potential resulted at pH 9.0 and 18 h (-17.310 ± 0.578 mV), and the highest was found at pH 5.0 and 24 h (-0.215 ± 0.4172). Zeta potential depended on both the level of pH and time with the strongest contributing factor being pH (41.27%). There are however significant contributions made by the interaction of both pH and time (22.71%) (Table 1).

3.3 Size and shape parameters

SEM micrographs of auto-aggregated *Lp299v* in PBS were taken prior the preparation of the samples to observe the auto-aggregates (*micrographs not shown*).

Table 2. Hydrophobicity (%) to n-hexadecane (apolar) and chloroform (acidophilic) of *L. plantarum* 299v at pH 5.0, 7.0 and 9.0. Results from three independent experiments performed in triplicate.

pH	Hydrophobicity (%) (n-hexadecane)	Hydrophobicity (%) (chloroform)
5.0	17.033 ± 0.493	87.192 ± 1.099
7.0	12.921 ± 0.275	83.499 ± 0.731
9.0	13.401 ± 0.359	79.646 ± 0.681

For the morphometric analysis a prepared sample which involved the complete separation of individual cells was performed to have a better segmentation of each cell for the determination of individual size and shape parameters. The process is explained above in the section 2.7 of materials and methods. Micrographs showed the typical coccoid-rod form of *L. plantarum* (Figure 3). More significant results were found at times 0, 4 and 24 h and pH levels of 5.0, 7.0 and 9.0 which are presented in Figure 3. However, all results were considered for statistical analysis, and DIA of size and shape parameters.

Results of particle size and shape distribution obtained at times 0, 4 and 24 h are presented in Figure 4. The bacterial length increased at 4 h but not at 24 h for pH 7.0 and 5.0 respectively. The area ranged from 0.540 to 0.678 μm^2 whereas the Feret's maximum diameter from 1.183 to 1.503 μm. All micrographs were analyzed and significant differences in area were found across both pH and time ($p \leq 0.05$); however, the Feret's maximum diameter displayed significant differences between pH levels ($p \leq 0.05$) as is possible to observe in Figure 4.

The shape parameters at 0, 4 and 24 h are shown in Figure 4. Circularity varied from 0.524 within the initial conditions to 0.686 at a pH of 9.0 and 24 h. Both pH and time were found to be significant ($p \leq 0.05$). The AR ranged from 2.059 to 2.471 and was found to be significantly different from time ($p \leq 0.05$) but not from the pH level. Moreover, circularity and area were the morphological parameters that showed statistically differences across the conditions tested followed by the Feret's maximum diameter. In the present work, the nonspecific adhesion mechanisms of *Lp299v* were studied in an auto-aggregated form at different pH under starvation conditions. The *in vitro* models are essential to understand the mechanisms of adhesion and provide an important information regarding strains differences (García-Cañuela *et al*., 2014). Here it has designed an *in vitro* model to study of some factors of importance such as pH and time in under starvation to investigate the changes the cell may have per se in its physicochemical properties that may provide important information about the applications for a better delivery design of probiotics and to enhance their efficacy. For this purpose, a more complex *in vitro* model than usual with a starvation media, at low and constant ionic strength levels, comprising auto-aggregated *Lp299v* at three different pH levels (5.0, 7.0 and 9.0) over time (24 h) was defined. PBS buffer is a low ionic buffer that allows to maintain the adequate salinity of the media to avoid osmotic stress for bacteria with a low interference in the potential zeta measurement. Cell starvation can influence bacterial cell characteristics and hence alter some properties such as the transport behavior and hence the aggregation.

However, there is a gap in knowledge about probiotics of how the starvation conditions alter the cell surface characteristics and influences the aggregation (Haznedaroglu *et al*., 2008). The lack of nutrients for the bacteria allowed that the cell surface charges resulted from the intrinsic ability of the strain to confront the environmental conditions; the low ionic strength permitted that the ionic forces were for the pH changes determined in the buffer composition for the experiments. During the experiments conducted at pH 5.0, 7.0 and 9.0 for 24 h all the *in vitro* nonspecific adhesion mechanisms (hydrophobicity, charge and morphology) showed significant changes in the auto-aggregates (Fig. 1 and Table 1).

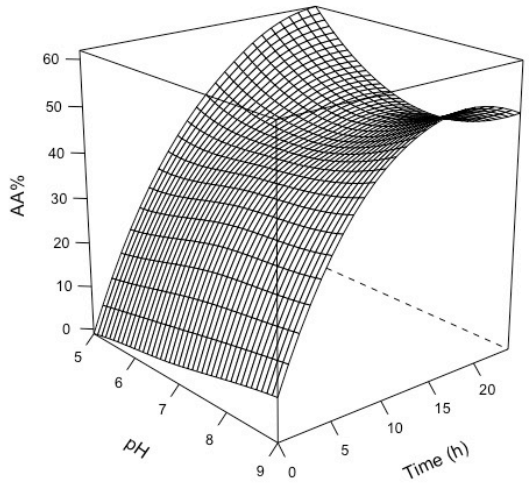

Fig. 1. Auto-aggregation kinetics of *L. plantarum* 299v measured at 600 nm in PBS buffer in function of pH (5.0 - 9.0) and time (0-24 h). Method: surface response; Residual Standard Error: 3.834; Number of replicates per 3.

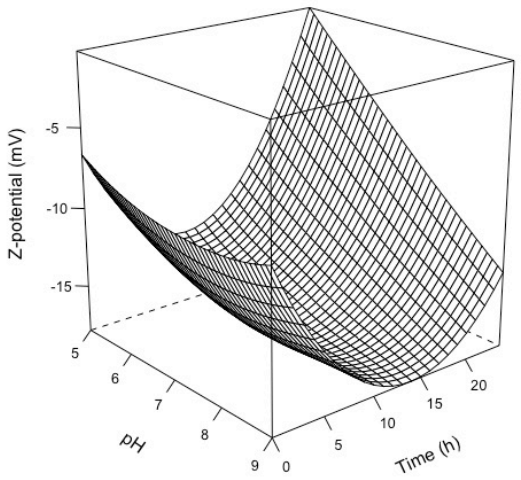

Fig. 2. Surface response of zeta potential (mV) of auto aggregation kinetics in *L. plantarum* 299v in function of time (0-24 h) and pH (5.0-9.0). Method: surface response; Residual Standard Error: 1.349. Number of replicates PER 3.

Lp299v was able to survive across a range of pH levels from 2.0 to 9.0 over 24 h in MRS broth and gastrointestinal simulation (Melgar-Lalanne *et al*., 2014). In this study has been demonstrated that *Lp299v* can survive at pH levels from 5.0 to 9.0 for 24h at starvation conditions without viability loss. Therefore, the strain can be considered in a stationary phase close to a dormancy state (Prakash *et al*., 2013). In this particular state, bacteria are in a thermodynamic stable state in which does not change their physiological or genetic features, and their vital components are protected by a physical arrest that is independent of the cell's energy consumption (Minsky *et al*., 2002). Thus, under these experimental conditions, auto-aggregation is ruled more by non-specific mechanisms than by the mannose-specific receptors of the bacteria.

Auto-aggregation is an important requirement for probiotic bacteria to gain enough bacterial cell mass to secrete a significant quantity of beneficial metabolites to the intestinal lumen. The more a strain can auto-aggregate, the higher the cell mass present. Moreover, aggregation and biofilm formation are considered a defensive bacterial strategy against predators or even stressful conditions, making bacteria more inaccessible and maintaining them entirely or partially alive (Young, 2007). The autoaggregation depends on the environmental conditions present in the lumen (Gong *et al*., 2012). Under these parameters, the intestinal pH range along with high osmolarity and the presence of enzymes and bile salts have been considered as determinants of cell adhesion (Wilson *et al*., 2001). However, low osmotic stress (< 0.3M) did not interfere with *Lactobacillus* growth and biofilm formation (Aoudia *et al*., 2016) but high NaCl concentrations (0.8 M) increased the auto-aggregation levels and decreased hydrophobicity in *L. salivarius* strains (Gong *et al*., 2012). In this work, the low osmolarity (0.177 M) decided for all the experiments did not affect in the auto-aggregation levels. In fact, autoaggregation increased over time at the three pH levels tested. This increase was higher at pH 5. Moreover, at higher times, the auto-aggregation at pH 7.0 was slightly lower than at pH 5.0 and 9.0, which showed the importance of the ionic charge of the media where the bacteria are suspended in the aggregative occurrence. Although the change is not significant, lower pH seems to boost auto-aggregation. This behavior has been previously observed using enriched media in other culture (Juarez-Tomas *et al*., 2005) and was attributed to changes in the microbial surface charges such as the decrease of Coulomb forces (Vandevoorde *et al*. 1992). This may also imply that auto aggregation may be promoted at the lower stomach or duodenum or even the caecum levels where the pH is between 5-6. However, more research is needed to know the behavior of *L. plantarum* at lower pH, i.e. < 3, to know the potentiality of *L. plantarum* to colonize the upper part of the stomach.

All the non-specific adhesion mechanisms studied (hydrophobicity, charge and morphology) change in the auto-aggregates along time and pH.

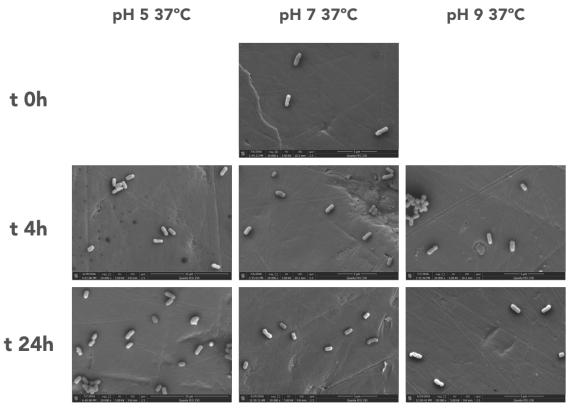

Fig. 3. Scanning Electron Microscopy (SEM) of *L. plantarum* 299v at different pH and time at 20,000 X in auto-aggregation kinetics with sample preparation to facilitate image analysis at different pH (5.0, 7.0 and 9.0) and times (0, 4 and 24 h).

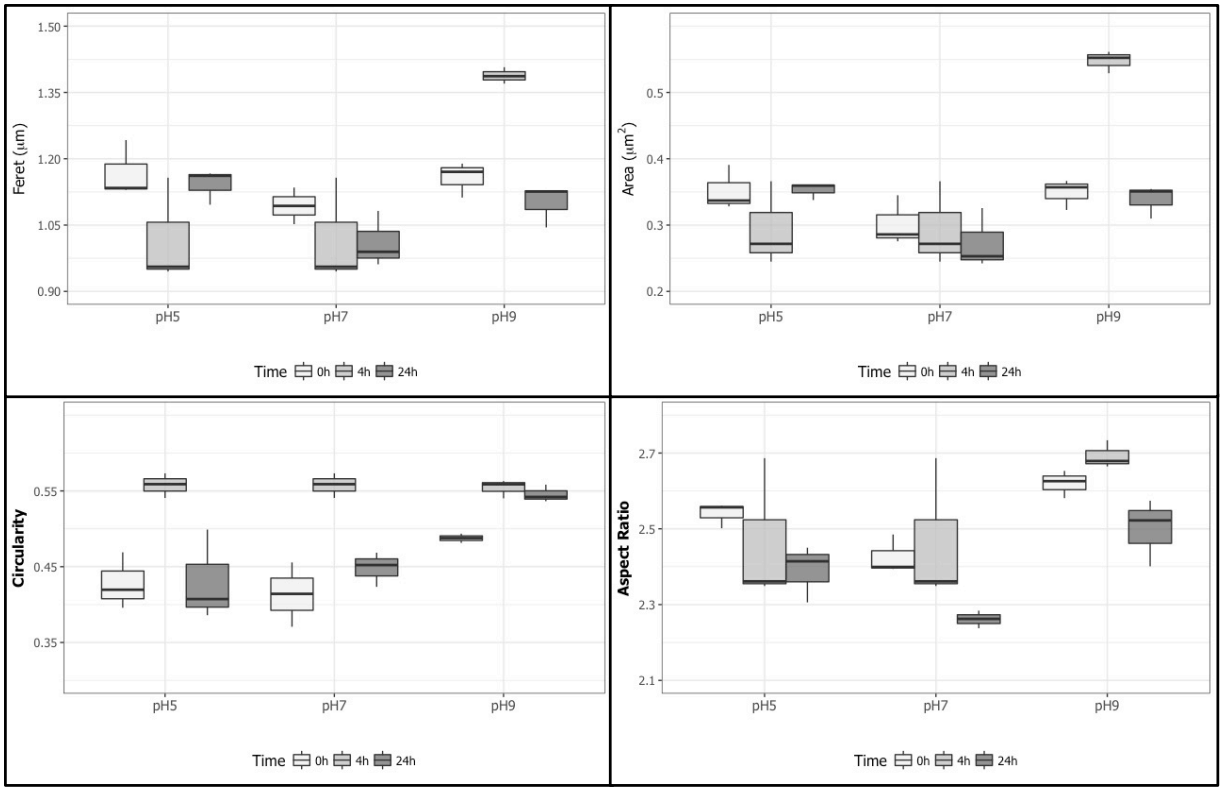

Fig. 4. Boxplot of size parameters: a) Feret diameter (μm), b) area (μm^2); and shape parameters: c) circularity, and d) aspect ratio of *L. plantarum* 299v cells at selected conditions of pH (5,7 & 9) and time (0, 4, & 24 h) (n=3).

Interestingly, auto-aggregation appears to be independent from zeta potential since no correlation could be found (< 0.1). Thus, while in auto-aggregation time was the contributor parameter, (99.73%), in zeta potential the contribution was due pH and time in the electrokinetic response of cells surfaces (Table 1). Results of *Lp299v* on the zeta potential demonstrated the contribution of pH and time in the electrokinetic response of cells surfaces. It has been seen that *Lp299v* modified their surface charges to suit the levels of pH over time. The sub-lethal alterations on the surface of the strain can improve the aggregation properties. The negative zeta potential may indicate the presence of surface proteins (Schär-Zammaneti *et al*., 2005, Buntin *et al*., 2017) and the recorder low hydrophobicity and high affinity to chloroform may indicate the presence of acid exopolysaccharides, in the external cell membrane of *Lp299v* (Melgar-Lalanne *et al*., 2015). *Lp299v*'s surface charges may be ascribed to anionic compounds such as lipoteichoic acids, acidic polysaccharides and proteins present in the cell surface (Jiang *et al*., 2016). During the kinetics, all samples displayed normal negative surface charges commonly presents on bacteria under these physiological conditions (Wilson *et al*., 2001) as survival rates of the bacteria showed. So, the experimental conditions of pH levels over time which have been tested here cannot be considered determinant stressors concerning of electrokinetic charges.

Traditional microbiology focused in quantify the number of microorganisms (individual cells or colony forming units) and/or biomass in different environment. However, such measures provide little information about what the microorganism is doing or how are they spatially distributed. So, microorganisms often exhibit different morphologies under different environmental responses and can vary some geometrical parameters, mainly size and shape, to adapt to the environment stressors (Heinrich *et al*., 2015). This phenomenon was observed in *Lactobacillus* spp. Submitted to osmotic stress (Gong *et al*., 2012), acid, ethanol, heat and cold (Shao-Ji *et al*., 2008) but modifications in size and shape

parameters of the bacteria had not been quantified. To carry out this quantification, Digital Image Analysis (DIA) allows the interpretation of digital analysis by processing algorithms in different food matrixes (Kumar and Mittal, 2009). It was also successfully used to describe quantitatively inter and intra specific differences within colonies (in solid media) and even within own cells (in liquid media) (González-Vázquez *et al*., 2014).

As auto-aggregation is time dependent in addition to geometric aspects, kinetic changes should be considered to understand the complexity of the phenomena. Since the three-dimension information of the samples was lost with the images taken by SEM, only individual geometrical parameters were analyzed, and not the auto-aggregated sizes and shapes. DIA revealed individual variations in cells under all the conditions tested (Table 3).

These results are similar to those previously reported by González-Vázquez *et al*.2014 in *Lactobacillus casei* Shirota who found that Feret's maximum parameter and circularity were the most significant parameters. Moreover, data presented here showed a significant heterogeneity regarding of size and shape of individual bacterial cells. This individual heterogeneity has been related with the gene expression of each bacteria and with the phenotypic individuality observed thought microscopy in growth, survival and inactivation responses (Koutsoumanis *et al*., 2017). Bacteria adopt a more coccoid form at the end of the experiment time at pH 7.0 ($C = 0.647 \pm 0.196$) and 9.0 ($C = 0.686 \pm 0.045$) but this form seems to be dependent both from pH and time (Table 1). This coccoid form might act in the auto-aggregation of bacteria although, adhesion experiments at more pH are necessary to conclude this perception. Moreover, a strong Pearson correlation between auto-aggregation and circularity at the different conditions tested could be described.

This found supports the hypothesis that bacteria shape may be related with the auto-aggregation phenomena as a non-specific mechanism. Probably these morphological changes affect the aggregation and adhesion of the bacterial cells. As expected, two-way ANOVA analysis showed that all size and shape parameters were time and pH dependent, like zeta potential but contrary to auto-aggregation that is dependent form time but not from the pH tested (Table 1). This statistical analysis indicated that *Lp299v* cells were able to modify their size and shape both for the presence of hydronium ions and time at starvation.

Conclusions

Non-specific adhesion mechanisms though time at different pH levels in auto-aggregated *Lp299v* were evaluated and resulted more related with the time of exposition than with the pH levels. This indicates that the van der Waals and hydrophobic forces might rule the auto-aggregation more than ionic-forces. Time was the contributor parameter in auto-aggregation. So, *Lp299v* changed its hydrophobicity, charge and morphometric parameters and improves the auto-aggregation along the time of the experiment. Moreover, time was positively correlated with auto-aggregation and a decrease in electronegativity (zeta potential). Evidence showed that under starvation conditions *Lp299v* might change their length and shape to maintain their survival optimizing the auto-aggregation at simulated intestinal pH (from 5.0 to 9.0). A strong correlation could be observed between auto-aggregation and the circularity parameter in *Lp299v* strain under the conditions tested. According to this model, the pH-time factor may play an important role promoting auto-aggregation at lower levels predicting better adhesion in the upper parts of the digestive system or the caecum where the pH is lower than 6 and time longer than 4 hours of digestive transit. This may support the hypothesis of the importance of the individual cell morphology in the auto-aggregation phenomenon. This is likely in order to deal with stress factors and to preserve the life of the bacterial colony. To the best of our knowledge, this is the first study to report the influence of pH level on non-specific adhesion mechanisms (hydrophobicity, charge and morphology) of *Lp299v* though time.

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