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Short running head: VAGINAL LACTOBACILLI RESISTANCE TO OSMOTIC STRESS

TECHNOLOGICAL CHARACTERIZATION OF VAGINAL PROBIOTIC LACTOBACILLI: RESISTANCE TO OSMOTIC STRESS AND STRAINS COMPATIBILITY

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ABSTRACT

Aims: The aim was to evaluate the osmotic stress resistance of vaginal beneficial-probiotic strains, their growth kinetics and parameters when growing in salt-added culture media, and their compatibility to go further in the design of a probiotic formula for reconstitution of vaginal microbioma in women.

Methods and results: The resistance to osmotic stress of the lactobacilli was evaluated by determining their growth in MRS (as control) added with NaCl (2 to 8%). The most resistant strains were *L. gasseri* CRL1509, *L. rhamnosus* CRL1332 and *L. reuteri* CRL1327 selected by statistical approaches and growth parameters. Electron microscopy was applied to determine changes. They maintain probiotic properties and viability. Some strains showed incompatibility, then they can not be included in multistrain formulas.

Conclusions: The resistance to different salt concentrations in vaginal lactobacilli is strain-specific, because the behavior is different in strains identified into the same species. The resistance is not related to the metabolic groups.

Significance and Impacts of the Study: The resistance and survival to extreme osmotic resistance is one of the specific requirements of beneficial bacteria after the technological processes for their inclusion in probiotic formulas, in a way to express their beneficial characteristics and exert the effect on the host.

Key words: Vaginal lactobacilli, osmotic stress, resistance, compatibility, vaginal formula

Introduction

Lactobacilli are the predominant bacteria in the healthy women vagina microbioma being *Lactobacillus gasseri*, *L. jensenii, L. crispatus* and *L. iners* those more frequently isolated (Huttenhower *et al.,* 2012). The use of probiotics as preventive or therapeutic agents is applied for women´s health and constitutes a novel alternative. Probiotics were defined as "**Live microorganisms that, when administered in adequate amounts,** confer a health benefit on the host**"** (Hill *et al*., 2014), being administered either by vaginal or oral route, the last to favor the ascending vaginal colonization (Reid 2017). The oral administration is generally used to stimulate the immune system, and more specifically, the urogenital mucosal immunity as target.

The pharmaceutical industry shows an emerging interest in pharmaceutical formulations containing these beneficial microbes, the so-called pharmabiotics, in which specific probiotic strains are included in pharmaceutical products released into the market. The formulas must contain a high number of bacteria, and express their beneficial properties once included in the final product and during storage (Kligler and Cohrssen 2008; Tripathi and Giri 2014; Huang *et al*., 2016). In some specific cases, the probiotic/beneficial strains must be pre-adapted to the technological stress of biomass production.

The resistance to different type of stress is related to a wide variety of factors, reviewed recently: specific genes (Palomino *et al*., 2016, Papadimitriu *et al*., 2016), expression of a set of proteins or quantitative

proteomic, (Schott *et al*., 2017) or glycine-betaine transport (Han *et al*., 2018). In specific strains, the cells can be submitted to pre- stress adaptation (Ferrando *et al*., 2015) in high substrate concentration (Huang *et al*., 2018), or induced regulation of unsaturated fatty acid (Zhang *et al*., 2018) intracellular compatible solute pools (Tian *et al*., 2018) or membrane fluidity (Gandhi and Shah 2016; Meneghel *et al*., 2017). The design of probiotic pharmaceutical and cosmetic formulations with beneficial bacteria requires that the microorganisms remain viable and in high numbers in the final formula, situation in which the water activity (aw) is significantly reduced to help in the cells-viability and storage. In this way, the osmolality of the medium could compromise the physiological functions, viability and expression of beneficial characteristics of microorganisms, then it is required to evaluate the strains resistance to osmotic stress. On the other side, is fundamental to apply some type of statistical studies to evaluate in a faster and more objective way the results obtained through experimental protocols. The use of statistical programs or models allows to evaluate faster and more objectively the beneficial and technological features that are strain-dependent, as indicated by different authors. The CART analysis is capable of detecting complex interactions of variables that might be missed by standard statistical approaches (Breiman *et al*., 1984).

Our research group have isolated and identified lactic acid bacteria from women in San Miguel de Tucumán (Argentina) (Ocaña *et al*., 1999a; Juárez-Tomás *et al*., 2011) and evaluated their beneficial properties that include the production of antimicrobial substances (lactic acid, peroxide hydrogen and bacteriocins) (Ocaña *et al*., 1999b, 1999c, 1999d; Vera Pingitore *et al*., 2009; Juárez-Tomás *et al*., 2011; Nader-Macias and Juárez-Tomás 2015) biofilm formation (Leccese Terraf *et al*., 2016; 2017), inhibition of urogenital pathogens (eg, *Candida albicans, Streptococcus agalactiae, Escherichia coli, Staphylococcus aureus*), modulation of the immune system and protection against pathogens in experimental murine model (De Gregorio *et al*., 2014; 2015; 2016; 2019; Nader-Macías and Juárez-Tomás 2015). Also, the technological properties as resistance to lyophilization and storage of concentrated cultures with pharmaceutical excipients in gelatin capsules were studied (Zárate and Nader-Macías 2006; Juárez Tomás *et al*., 2015), either of lactobacilli or combined with salivaricin (Vera Pingitore *et al*., 2015).

The aim of this work was to evaluate the osmotic stress resistance of a list of vaginal beneficial strains previously selected by their probiotic characteristics, their growth kinetics and parameters, through the application of statistical-related methodology when growing in salt-added culture media, and their compatibility to go further in the design of a probiotic formula to be administered for the reconstitution of the vaginal microbioma in women.

Materials and methods

Bacterial strains and media

Thirty Lactobacilli strains isolated from human vagina from women in Tucumán, Argentina (Ocaña *et al*., 1999a; Juárez-Tomás *et al*., 2011) included in the Centro de Referencia para Lactobacilos Culture Collection (CRL, Tucumán, Argentina), and characterized previously by their beneficial properties were used (De Gregorio *et al*., 2019). Frozen aliquots of the strains were maintained at -80 °C in milk-yeast extract (13% nonfat milk, 0.5% yeast extract and 1% glucose; Britania Laboratories, Argentina) supplemented with 20% glycerol (Cicarelli Laboratories, Argentina), subcultured twice in MRS (De Man, Rogosa and Sharpe) broth (Biokar Diagnostics, France) at 37 °C for 16 h in microaerophyllic conditions (5% CO_2, 95% air). The strains used and the metabolic group them belong are included in Table 1.

Resistance to Osmotic stress

The resistance to osmotic stress of the strains was evaluated by using the third subculture (in stationary phase in MRS broth) washed with buffer solution (PBS pH= 6.5) inoculated at 2% (v/v) in MRS broth added with different NaCl concentrations: 1%; 2%; 4%; 6%; 8% (w/v) whose water activity (aw) equivalences are: 0.995; 0.991; 0.977; 0.964; 0.951, respectively. MRS broth pH 6.5 was used as control. This assay was performed in 96 wells polystyrene microplates incubated at 37°C under microaerophyllic conditions for 24 hours, the growth registered at OD_{560nm} (Microplate Reader VERSAmax Tunable). The kinetic parameters were analyzed. Experiments were performed by triplicate in independent trials.

Growth inhibition in stressed conditions

The growth kinetics and parameters (lag phase length, growth rate and maximal OD) were determined in all the growth curves performed under osmotic stressed conditions. Only in those situations where the strains did not grow, these data were not included. The growth of each strain at 10 h. in the osmotic stressed conditions was calculated as percentage of the growth in MRS broth pH 6.5 (used as control). The growth of the strains was compared, determining those able to grow at different osmotic conditions by applying the following formula: $[(OD_s X100)/OD_c]$. OD_s: Optical Density in stress condition, OD_c: Optical Density in control media.

Viability and maintenance of beneficial traits after the stress applied

The resistance, in terms of viability of the selected strains, to the osmotic stress (at 4% NaCl in MRS medium) was determined by the number of CFU/ml before (CFUB) and after (CFUA) the osmotic stress, by the plate dilution method in MRS agar plates. The growth at optimal conditions was considered as control and the CFU numbers after the stress were expressed as survival rate, calculated as follows: CFUA/CFUB at the same time of culture.

The beneficial properties of the strains, as hydrophobicity and self-aggregation were evaluated before and after the stress. Selected strains were grow in MRS medium (as control) and in MRS-4% NaCl, as previously described. Cells in late exponential phase were collected by centrifugation at 10000 xg for 15

min, and cell pellets washed with 0.85% NaCl saline twice. The hydrophobicity was determined by measuring the variation of the optical density at 600 nm (OD_{600nm}) of cell suspensions in physiological solution after partition with organic solvents adding xylene and toluene as indicated by the technique described by Ocaña *et al*., (1999). Self-aggregation of washed bacteria was monitored using a method modified in our laboratory (Ocaña and Nader-Macías 2002). The degree of hydrophobicity and self-aggregation was calculated using the score: high (71–100%), medium (36–70%), and low (0–35%).

Scanning electronic microscopy after the stress conditions

Scanning electronic microscopy was used to determine the changes of the bacterial morphology when they were subjected to osmotic-stressed conditions. Cultured cells from stressed and no stressed conditions were collected at the late growth exponential phase by centrifugation at 8000 x g for 10 min and washed 3 times with PBS buffer. 200 microliters of the bacterial suspension previously fixed in Karnovsky fixative (Phosphate buffer pH 7.2, Glutaraldehyde 1.7% and Paraformaldehyde 2.7%) were placed for 2 hours on 1.2% agar layer prepared on a coverslip. Then the dehydration was performed in alcohol drums starting with 30%, 50%, 70%, 90%, 100% alcohol until reaching 100% acetone in passages of 10 min each step. Critical drying point was produced on Denton Vacuum brand equipment (model DCP-1). The samples were mounted on aluminum supports (stubs) and then coated with gold (coating) in a JON-ION Sputter equipment (model JFC-1100). Finally, they were placed and observed in a Scanning Electronic Microscope (SEM) (Zeiss model SUPRA 55VP).

Statistical evaluation

The bacterial growth parameters were estimated applying the 4-parameter modified Gompertz-model:

$$OD = N_0 + A*\exp\{-\exp[(\mu*e/A)*(\lambda-t) + 1]\},$$

where OD is the optical density at time t (time of growth in hours), N_0 the OD at t = 0, A the difference between the final and the initial ODs, μ the maximum specific growth rate (h^{-1}), λ the lag phase time in hours and e the base of the neperian logarithm.

For the estimation of the parameters constrained nonlinear regression was performed. This method uses a sequential quadratic programming algorithm. For the parameter standard errors and confidence intervals the method of Bootstrapping was applied, using repeated samples from the original data set. For each growth curve 100 bootstrap samples were taken.

To evaluate the multivariate effects of the different conditions, NaCl concentrations on the growth parameters, the nonlinear mixed-effects model as proposed by Lindstrom and Bates (1990) was applied using restricted maximum likelihood.

To identify subgroups of combinations of the strains with different NaCl concentrations with optimal growth, a Classification and Regression Tree (CART) analysis was performed. The estimated growth

parameter A was included as independent variable and the parameters strain and NaCl concentration were included as predictors in the analysis. Only experiments with an estimated parameter A (O.D.) of at least 0. 8 and an estimated lag phase shorter than 6 h were selected for the CART analysis.

For the analyses and graphical presentations, the statistical programs SPSS 25, S-Plus 8.1 and STATISTICA 12 were used. The CART analysis was performed with Salford Predictive Modeler 8.2.

Compatibility between lactobacilli strains

The compatibility between fourteen beneficial lactobacilli strains was determined by the agar plate diffusion method described previously by Juárez-Tomás *et al*. (2011). Different concentrations of lactobacilli (10^6 and 10^7 CFU/ml) were inoculated in MRS agar plates, each strain was used both as an indicator and as producer of inhibitory substances. Then, 20 µl of lactobacilli supernatants were added to 4 mm wells on the agar plates and dry off during 5 h at room temperature and then incubated 24 h at 37°C in microaerophyllic conditions. Inhibition of the growth of indicator strains by supernatants was evidenced by a zone of inhibition around the well and expressed in millimeters of the inhibition halo.

Results

Resistance to osmotic stress

The stress resistance of the lactobacilli strains evaluated in this work have shown that the growth at different salt concentrations is dependent of each specific strain, even if they were identified into the same genus or metabolic group. The NaCl concentration has shown a significant effect on the A and µ parameters (nonlinear mixed model) in all the strains under evaluation, indicating also that the degree of inhibition is dependent on each specific strain. As examples, the growth kinetics of different strains, some of them identified into the same species, are included in Fig 1.

L. gasseri CRL 1509 shows to grow between 1% NaCl (aw: 0.995) and 8% NaCl (aw: 0.951) (Fig. 1A), while *L. gasseri* CRL 1264 does not grow at 4% NaCl (aw: 0.977) (Fig. 1B), and *L. gasseri* CRL 1290 grows only in MRS with no salt added (control) (Fig. 1C). In the case of *L. reuteri* CRL 1327 (Fig. 1D), it grows in all the conditions assayed at different growth rates, while *L. reuterii* CRL 1324 (Fig. 1E) and *L. mucosae* CRL 1508 (Fig. 1F) do not grow at NaCl 6% (aw: 0.964).

When evaluating *L. rhamnosus* strains, *L. rh.* CRL 1332 (Fig. 1G), *L. rh.* CRL 1511 (Fig. 1H) and *L. paracasei* CRL 1512 (Fig. 1I) even though they are classified as obligate heterofermentative, they grow in all the conditions assayed at different growth rates and maximal O.D. In this group, CRL 1332 is the strain showing a higher growth at 4% NaCl (aw: 0.977).

When *L. jensenii* strains are compared, *L. je.* CRL 1313 (Fig. 1J) grows better than *L. je.* CRL 1333 (Fig.1K) and *L. je.* CRL 1317 (Fig.1L).

The behavior of all the strains with the different salt concentrations assayed is summarized in Fig. 2, where the osmotic resistance is expressed as the growth in salt-added liquid media (as percentage) referred to the growth in MRS broth (as control) at 10 h incubation at 37°C. The results have shown that the response to the increased salt concentration in liquid media, or the osmotic resistance of the different vaginal lactobacilli strains under evaluation is again strain-dependent, because the sixteen *L. gasseri* strains show different degree of growth inhibition, being *L. gasseri* CRL 1509 and CRL 1311 the most resistant to NaCl. Looking at the *L. rhamnosus* strains, they were a little more resistant to the osmotic stress, and higher than *L. paracasei* CRL 1512. These results also evidence that there is no correlation of the fermentative capability of the strains, indicated by the metabolic group where they are included, or to their genetic information referred to the osmotic resistance.

Evaluation of the Resistance to stressed conditions by CART (Classification and regression tree analysis) analysis

With the experimental data obtained, the growth parameters of each one of the strains at different salt conditions was calculated. The cut points established were O.D.: 0.8, lag phase shorter than 6 h, and longer incubation time 24 h. The statistical evaluation by applying the CART (Classification and regression tree analysis) software, allowed defining the following:

The CART analysis selected 63 plate growth assays in which the 30 lactobacilli strains showed a higher growth of O.D.: 1.298 (Fig. 3). In this way, the two areas separated show at the left 20 growth curves in which the lactic acid bacteria (*L. ga.* CRL 1252*, L. ga.* CRL 1255, *L. ga.* CRL 1270, *L. ga*. CRL 1311, *L. jo.* CRL 1292, *L. ga.* CRL 1320, *L. re.* CRL 1324, *L. re.* CRL 1327) have shown a growth of O.D.= 1.100 ("Terminal Node 1"), while at the right are included 43 assays with a maximum O.D.= 1.391 where the strains (*L. je.* CRL 1313, *L. ga.* CRL 1265, *L. ga.* CRL 1307, *L. ga.* CRL 1314, *L. ga.* CRL 1322, *L. ga.* CRL 1509, *L. mu.* CRL 1508, *L. pa.* CRL 1512, *L. sa.* CRL 1296, *L. sa.* CRL 1328, *L. fe.* CRL 1287, *L. ga.* CRL 1256, *L. ga*. CRL 1261, *L. ga.* CRL 1264, *L. rh.* CRL 1332*, L. rh.* CRL 1511) are selected.

After the revision of the growth parameters of all the strains under evaluation, and complemented with the CART analysis results, a high variability between the different responses to the salt concentration stress was observed. Table 2 shows only the parameters of the selected strains. *L. gasseri* CRL 1509, *L. rhamnosus* CRL 1332 and *L. reuteri* CRL 1327 were the strains with a higher resistance to the salt conditions, because they grow at 6 to 8 % NaCl (indicated in grey color in the table).

The behavior of all the strains showing to grow at 4% NaCl plotted together is summarized in Figure 4, where is very clear which are the strains surviving in osmotic environments. In this way, is easier to select those strains than could survive to a higher osmotic stress in the technological process applied further.

Viability and Maintenance of beneficial properties

The effect of high salt concentration on the viability of strains selected is shown in Table 3, a slightly lower survival rate was observed for the strains grow in MRS at 4% of NaCl than the growth in MRS as control. Referred to the maintenance of beneficial properties, most of the strains showed no significant differences after the stress conditions applied. Only *L. gasseri* CRL 1509 modified the self-aggregation of 11.67% before to 43.3% after the stress. Referred to other strains, as examples: *L. rhamnosus* CRL 1332 showed 11.6% self-aggregation before and after the stress, and *L. reuteri* CRL 1327 a value of 9.1% before and 11.86% after the stress. The hydrophobicity of *L. gasseri* CRL 1509 in toluene was 35% before and 33 % after.

Scanning electronic microscopy evaluation

Scanning electronic microscopy of the stressed resistant strains have evidenced modifications in the morphology of the bacterial cells. *L. gasseri* CRL 1509 grown at 4% NaCl (Fig. 5A) shows shorter bacilli compared to MRS control (Fig. 5B). *L. rhamnosus* CRL 1332 appears as curved and spiral chains (Fig. 5C) referred to control (Fig. 5D), while *L. reuteri* CRL 1327 (Fig. 5E) looks like more ovoid shape than control when replicating (Fig. 5F).

Compatibility between resistant osmotic strains

Most of the beneficial lactobacilli strains were compatible, which means that they can be combined for the design of a pharmaceutical formula. The strains that showed no-compatibility are included in Table 4, and an example is included in Figure 6. *L. reuteri* CRL 1327*, L. rhamnosus* CRL 1332 and *L. gasseri* CRL 1509, the most resistant strains to osmotic conditions, are compatible between them. However, other strains with a good performance in osmotic conditions, as *L. paracasei* CRL 1512 and *L. salivarius* CRL 1296 are not compatible with *L. jensenii* CRL 1333 indicating they cannot be included in the same formula.

Discussion

Lactic acid bacteria (LAB) have been used as starter for the food industry from ancient times, and their stress physiology studied strongly. On the other side, the requirement of the survival of probiotic LAB in the host have intensified interest in the field, being different that the technological stress (Amund 2016).

The stress response is not related with a specific strain or metabolic group. Up to date, more than 200 lactobacilli strains were identified (Sun *et al.*, 2015; http://www.bacterio.net/Parte 2018). The results obtained in our work indicate that the response is directly dependent on the strain, even though all of them were isolated from women vagina. The strains with a higher resistance to the osmotic conditions evaluated were not into the same specie, because they were identified previously as *L. gasseri* CRL 1509, *L. rhamnosus* CRL 1332 and *L. reuteri* CRL 1327. These results support the use of specific strains with very well defined beneficial properties, to determine if they can survive to the technological process. Most of

the studies published are consequence of different experimental protocols applied mainly to strains used as probiotics for gastrointestinal tract, and their resistance to the stress produced under different areas of this tract, either acid, bile salts, or enzymes released to the mucosal areas (Amund, 2016). Hill *et al.* (2017) publish the differences in the stress response of *L. casei* group, constituted by *L. casei, L. paracasei and L. rhamnosus*. Senan *et al.* (2014) have shown in *L. helveticus* different stress-responsive gene sets required to adapt to gut and dairy niches, indicating a niche adaptation. Arnold *et al*. (2018) have recently showed that intra-specie genomic and physiological variability impact stress resistance in strains of probiotic potential. But, there is no studies describing which are the mechanisms related to the stress resistance in strains isolated from the urogenital tract, and this aspect supports the originality of our results.

On the other side, stress responses have been correlated with specific phenotypes, in a way that can be induced in a controllable and reproducible manner. Under environmental stress conditions, LAB change metabolic and energy fluxes, modify the rate of growth, and adapt the metabolism of carbon sources to the new environment by modifying the synthesis of enzymes and metabolites. LAB modulate the synthesis of specific transporters depending on the type of carbohydrate available (Muscariello *et al*., 2011; Siragusa *et al*., 2014). Some other mechanisms include the production of L-lactic acid in a *L. paracasei* strain (Tian *et al.,* 2014), intracellular accumulation of polyphosphate of *L. casei* BL23 grown in hyper concentrated (30% dry matter) sweet whey (Huang *et al.*, 2018), an increased synthesis of inorganic polymer polyphosphate, the role of Poly-P as a molecule that modulates host-signaling pathways (Alcantara *et al.*, 2018), or the addition of poly-Y glutamic acid (Bhat *et al*., 2015). Different studies performed previously on the technological applications of lactobacilli in our group (not published), have shown some type of correlation between the metabolic ways that support their classification in the homo or heterofermentative groups, related mainly with their temperature resistance (Garrity *et al*., 2015). The results obtained in this work do not provide information on the relationship between the stress resistance and the classification in a physiological group (homofermentative, heterofermentative) that indicates which are the main metabolic pathways responsible of carbohydrates degradation or intermediate-final metabolites produced in stressed conditions, information that would be of main importance to help in some technological-relates decisions.

The results summarized in this work were obtained from a very long list of experimental data, which were evaluated in an easier way through the application of the CART analysis combined with statistical programs, contributing then to a best tool to select those strains able to grow and survive at the osmotic-stress conditions produced by the addition of NaCl (from 2 and 8%) in the culture media. Then, in the strains able to survive to the higher NaCl concentration their growth parameters were calculated, to decide the most suitable strains to be included in a vaginal probiotic formula, because they could resist

then to the technological process applied. The resistance of the strains to a simulated environment was evaluated previously determining the maintenance of their viability in a media similar to the vaginal tract (Juárez-Tomás and Nader-Macías 2007). At the end, the strains with a higher resistance pattern determined by the application of this method were the same than those selected by the direct evaluation of the experimental results, that took a very long-time.

Maintaining the integrity of the cell wall under stress conditions is a matter of life or death for bacteria. The cell envelope is also a major cellular organelle with several physiological functions. The Scanning electron microscopy of three lactobacilli resistant strains selected in this work shows morphological and conformational modifications on the cell shape, length of the cells cultured under the higher salt concentration where they have survived. It was demonstrated that LAB, like other bacteria, closely monitor the integrity of the cell envelope and that specialized repair mechanisms are induced in case of damage (Jordan *et al*., 2008). When *L. casei* is placed under osmotic stress, its cell wall conformation changes. High salt concentrations led to an increase of the size of the cells and to sensitivity to antimicrobial peptides targeting PG, such as nisin (Piuri *et al*., 2005). Osmotic stress induced by a high salt concentration in *L. delbrueckii* subsp. *lactis* led to an increase of autolytic activity and survival following lyophilization (Koch *et al*., 2007). Exposure of *Lc. lactis* subsp. *lactis* to osmotic stress favour the expression of *murF* and *murG* genes, which are involved in PG biosynthesis (Xie *et al*., 2004). We did not explore which are the genes expressed or overregulated under the experimental assays performed, because we were interested in the selection of the most suitable strains to continue different type of studies. Further research is urgently required to determine the relationships between the probiotic properties and the exposure to stress of probiotic to be applied in the vaginal tract.

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Conflict of interest

There is no conflict of interest

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Table 1. *Lactobacillus* strains used in this work.

Metabolic group	*Lactobacillus* strains
Obligate homofermentative	*L. gasseri* CRL1252
	L. gasseri CRL1255
	L. gasseri CRL1256
	L. gasseri CRL1261
	L. gasseri CRL1263
	L. gasseri CRL1264
	L. gasseri CRL1265
	L. gasseri CRL1268
	L. gasseri CRL1270
	L. gasseri CRL1290
	L. gasseri CRL1307
	L. gasseri CRL1311
	L. gasseri CRL1314
	L. gasseri CRL1320
	L. gasseri CRL1322
	L. gasseri CRL1509
	L. jensenii CRL1313
	L. jensenii CRL1317
	L. jensenii CRL1333
	L. jensenii CRL1349
	L. johnsonii CRL1292
	L. salivarius CRL1296
	L. salivarius CRL1328
Facultative heterofermentative	*L. paracasei* CRL1512
	L. rhamnosus CRL1332
	L. rhamnosus CRL1511
Obligate heterofermentative	*L. fermentum* CRL1287
	L. reuteri CRL1324
	L. reuteri CRL1327
	L. mucosae CRL1508

Table 2. Growth parameters of the strains selected by their resistance to osmotic conditions.

		Metabolic group	Obligate homofermentative				**F**acultative heterofermentative		Obligate heterofermentative
		Strain	*L. ga.* CRL 1322	*L. ga.* CRL 1509	*L. je.* CRL 1313	*L. sa.* CRL 1296	*L. pa.* CRL 1512	*L. rh.* CRL 1332	*L. re.* CRL 1327
Control	MRS	Lag phase (h)	3.74±0.89	2.93±0.93	5.08±0.36	3.03±0.37	3.48±1.11	3.99±0.70	2.99±0.85
		OD (560nm)	1.47±0.10	1.45±0.15	1.42±0,03	1.52±0.06	1.46±0.16	1.46±0.06	1.31±0.18
		$\mu_{máx.}$	0.22±0.04	0.27±0.05	0.19±0.04	0.28±0.02	0.21±0.04	0.23±0.04	0.51±0.28
Salt concentration (%)	1% NaCl	Lag phase (h)	3.29±0.66	3.46±0.82	6.49±0.70	3.13±0.56	3.91±0.99	3.98±1.04	3.19±0.93
		OD (560nm)	1.47±0.07	1.40±0.09	1.30±0.04	1.46±0.05	1.39±0.11	1.38±0.11	1.26±0.19
		$\mu_{máx.}$	0.17±0.02	0.27±0.05	0.22±0.04	0.26±0.08	0.19±0.03	0.21±0.04	0.46±0.28
	2% NaCl	Lag phase (h)	4.76±0.98	3.14±1.11	6.85±0.71	3.22±0.34	4.28±0.91	4.16±1.27	3.19±1.09
		OD (560nm)	1.44±0.06	1.39±0.11	1.32±0.05	1.48±0.03	1.35±0.07	1.35±0.10	1.19±0.07
		$\mu_{máx.}$	0.20±0.03	0.19±0.04	0.19±0.03	0.26±0.02	0.16±0.02	0.18±0.05	0.30±0.26
	4% NaCl	Lag phase (h)	8.61±0.50	6.90±0.51	16.53±1.45	4.94±0.47	6.91±1.06	5.30±1.05	4.94±0.76
		OD (560nm)	1.38±0.04	0.96±0.02	1.50±0.02	1.34±0.04	1.11±0.05	1.37±0.06	1.03±0.05
		$\mu_{máx.}$	0.17±0.04	0.26±0.05	0.17±0.04	0.19±0.02	0.09±0.01	0.16±0.03	0.16±0.02
	6% NaCl	Lag phase (h)	NG	8.75±0.87	NG	22.40±2.46	NG	4.46±1.16	5.85±1.47
		OD (560nm)	0.11±0.03	0.89±0.05	0.02±0.02	1.05±0.07	0.17±0.02	1.48±0.05	0.83±0.06
		$\mu_{máx.}$	0.00±0.00	0.14±0.05	0.08±0.10	0,21±0,12	0.01±0.00	0.09±0.01	0.11±0.02
	8% NaCl	Lag phase (h)	NG	22.55±0.36	NG	NG	NG	9.48±1.00	17.83±2.72
		OD (560nm)	0.12±0.01	0.62±0.08	0.02±0.02	0.09±0.00	0.06±0.03	0.46±0.07	0.71±0.11
		$\mu_{máx.}$	0.01±0.00	0.09±0.03	0.02±0.00	0.00±0.00	0.01±0.00	0.03±0.03	0.06±0.14

Table 3. Effect of high salt concentration on the viability of selected strains.

Culture condition / Strain	Survival rate (CFUA/CFUB)[a]	
	MRS	MRS + 4% NaCl
L. gasseri CRL 1509	0.98±0.01	0.91±0.00
L. rhamnosus CRL 1332	1.05±0.03	0.91±0.01
L. reuteri CRL 1327	1.04±0.01	0.97±0.02

[a]Survival rate, where CFUB and CFUA are the CFU/ml before and after the stress.

Table 4. Compatibility between vaginal lactobacilli strains.

Metabolic group[d]	VL producers[a]	OHo						FHe		OHe	
		L. gasseri CRL 1290	*L. gasseri* CRL1314	*L. gasseri* CRL1322	*L. jensenii* CRL1349	*L. johnsonii* CRL1292	*L. salivarius* CRL1296	*L. paracasei* CRL1512	*L. rhamnosus* CRL1511	*L. reuteri* CRL1327	*L. mucosae* CRL1508
	VL Indicator[b]	Inhibition of growth[c]									
OHo	CRL1290[A]	0	0	4	0	0	0	0	4	0	0
	CRL1322[A]	4	0	0	0	0	0	0	3	4	0
	CRL1322[B]	0	0	0	0	0	0	0	3	4	0
	CRL1313[A]	0	11	0	0	0	0	0	0	0	0
	CRL1313[B]	5	10	0	0	0	0	0	0	3	0
	CRL1317[A]	7	0	0	0	0	0	0	0	0	0
	CRL1317[B]	4	6	0	0	5	0	0	3	4	3
	CRL1333[A]	8	0	0	0	10	3	0	6	0	4
	CRL1333[B]	5	3	0	3	0	0	3	4	0	0
	CRL1349[A]	5	0	0	0	10	0	0	4	0	0
	CRL1292[A]	0	0	0	0	0	0	0	0	3	0
	CRL1292[B]	0	0	0	0	0	0	0	0	3	0
OHe	CRL1287[A]	4	0	0	0	0	0	0	0	0	0

[a]Vaginal lactobacilli (VL) used as producers of inhibitory substances against lactobacilli.

[b]Vaginal lactobacilli (VL) used as indicator (sensitive) strains of inhibitory substances. Cellular concentrations of VL indicators, A: 1x106 UFC/ml; B: 1x107 UFC/ml.

[c]The inhibition of the growth of VL indicator strains by the effect of supernatants of VL producers was evidenced by the presence of a zone of inhibition around the well and expressed in millimeters of the inhibition halo (grey colour).

[d]OHo: obligate homofermentative group. FHe: facultative heterofermentative group. OHe: obligate heterofermentative group.

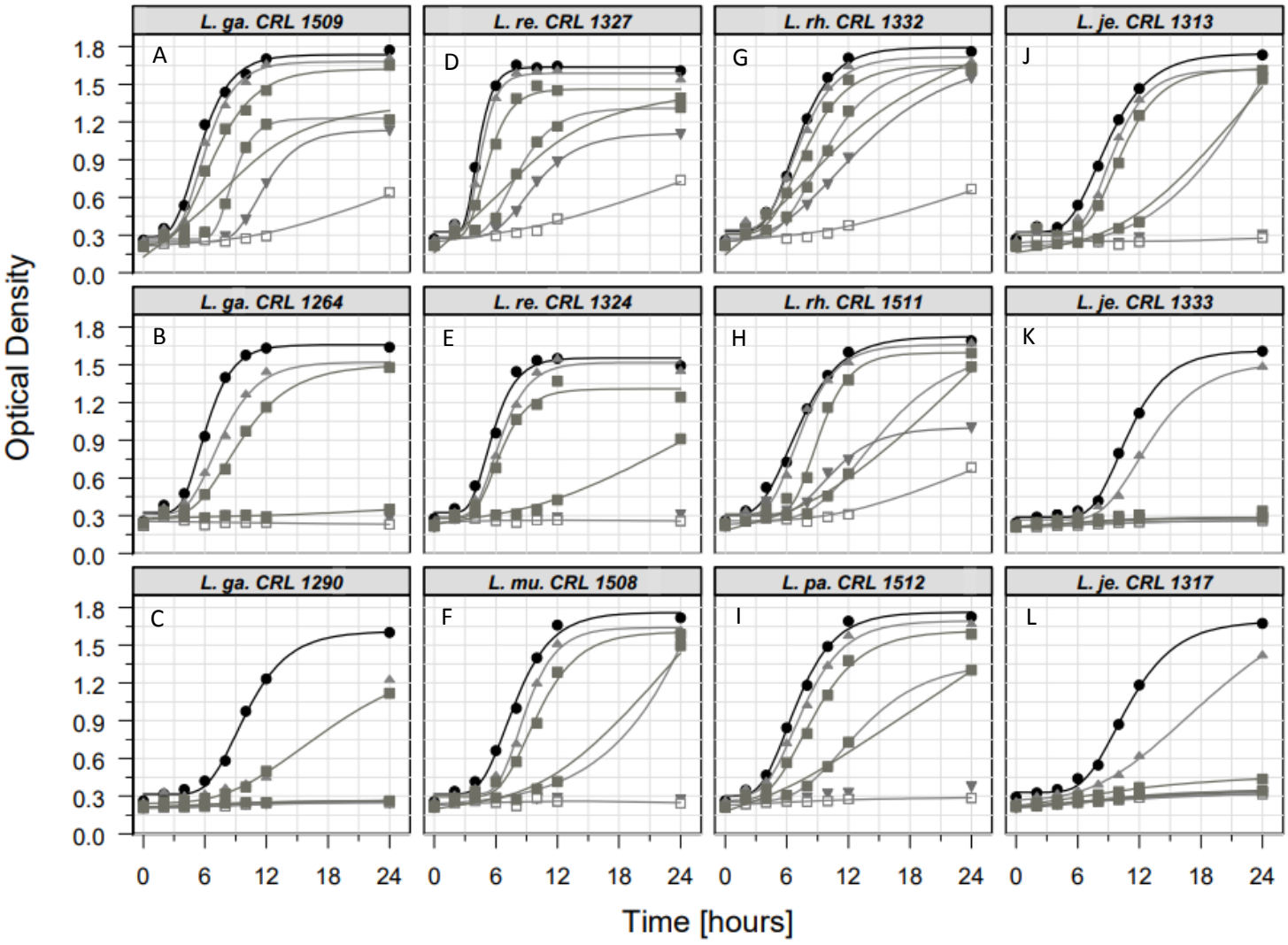

Fig. 1. Effect of increasing concentrations of salt on the growth of lactobacilli strains. *L. gasseri* CRL 1509 (A); *L. gasseri* CRL 1264 (B); *L. gasseri* CRL 1290 (C); *L. reuteri* CRL 1327 (D); *L. reuterii* CRL 1324 (E); *L. mucosae* CRL 1508 (F); *L. rhamnosus* CRL 1332 (G); *L. rhamosus* CRL 1511 (H); *L. paracasei* CRL 1512 (I); *L. jensenii* CRL 1313 (J); *L. jensenii* CRL 1333 (K) and *L. jensenii* CRL 1317 (L). Method is described in the text. MRS broth was used as control. Line represents: —●— MRS medium (as control); —▲— MRS 1% NaCl; —■— MRS 2% NaCl; —○— MRS 4% NaCl; —▼— MRS 6% NaCl and —□— MRS 8% NaCl.

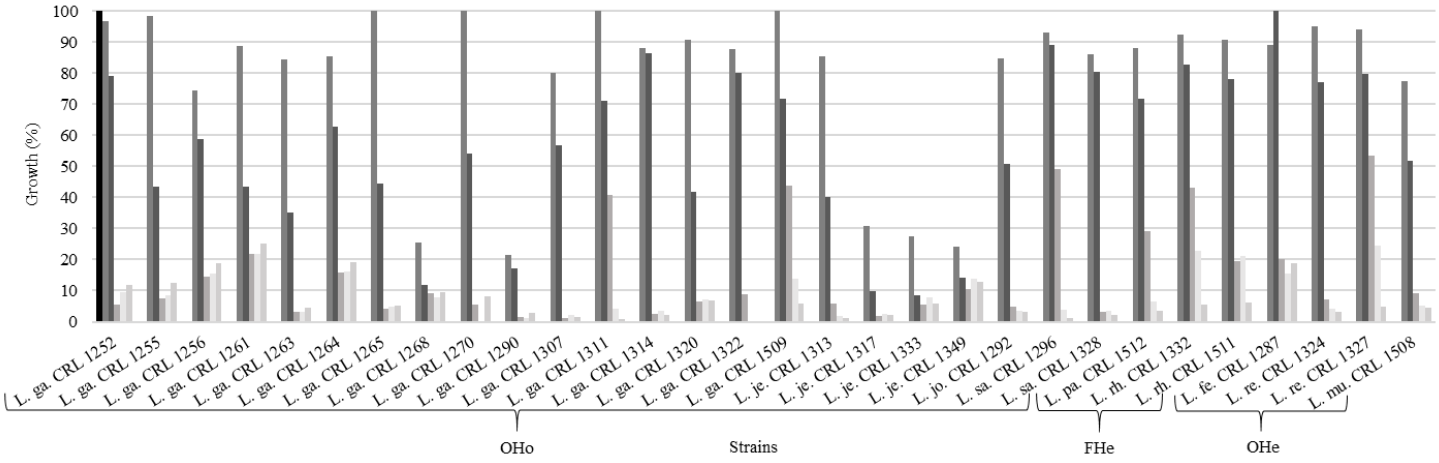

Fig. 2. Growth of lactobacilli strains in culture medium with different salt concentration 1%; 2%; 4%; 6%; 8% NaCl (w/v). The growth in salted media was calculated as percentage of the growth in control media (MRS pH 6.5, no salt added). Metabolic group of Lactobacillus strains (OHo: Obligate homofermentative group. FHe: Facultative heterofermentative group. OHe: Obligate heterofermentative group). Colour represents: MRS medium (as control); MRS 1% NaCl; MRS 2% NaCl; MRS 4% NaCl; MRS 6% NaCl and MRS 8% NaCl.

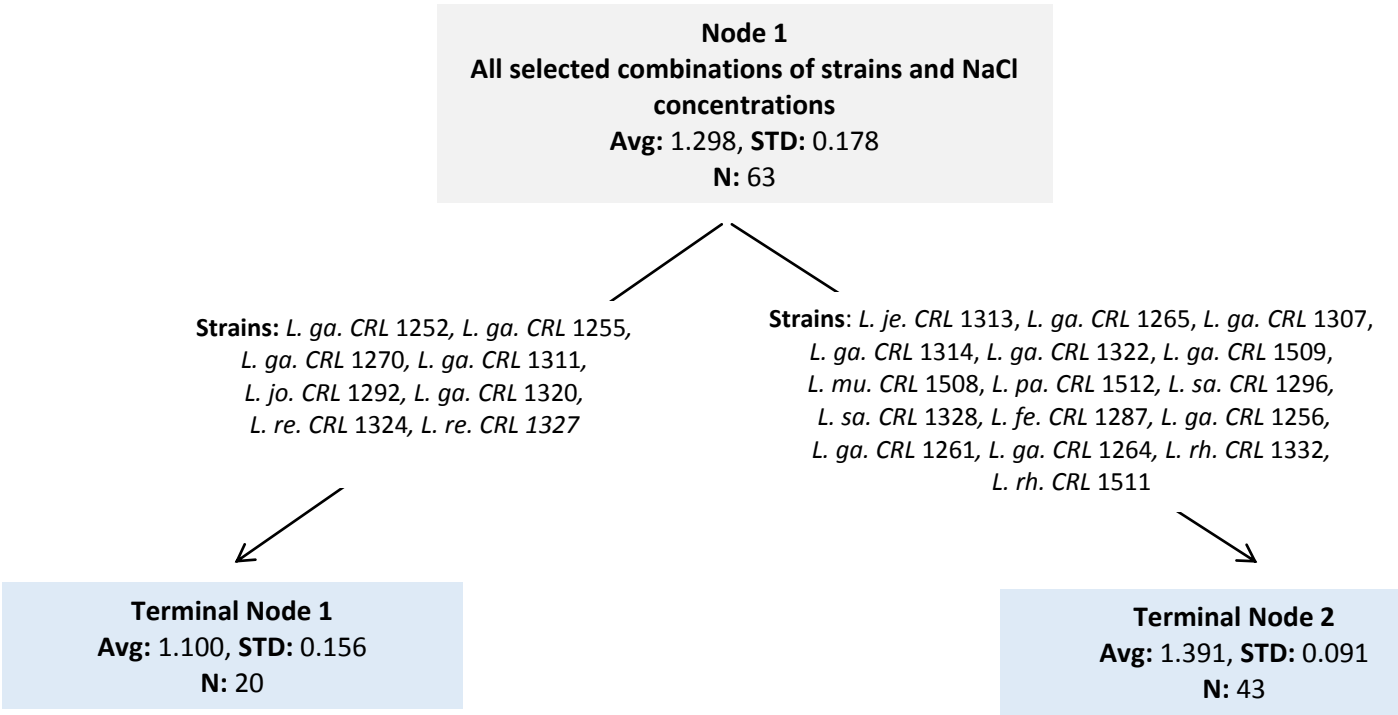

Fig. 3. Resistance to stressed conditions by CART analysis.

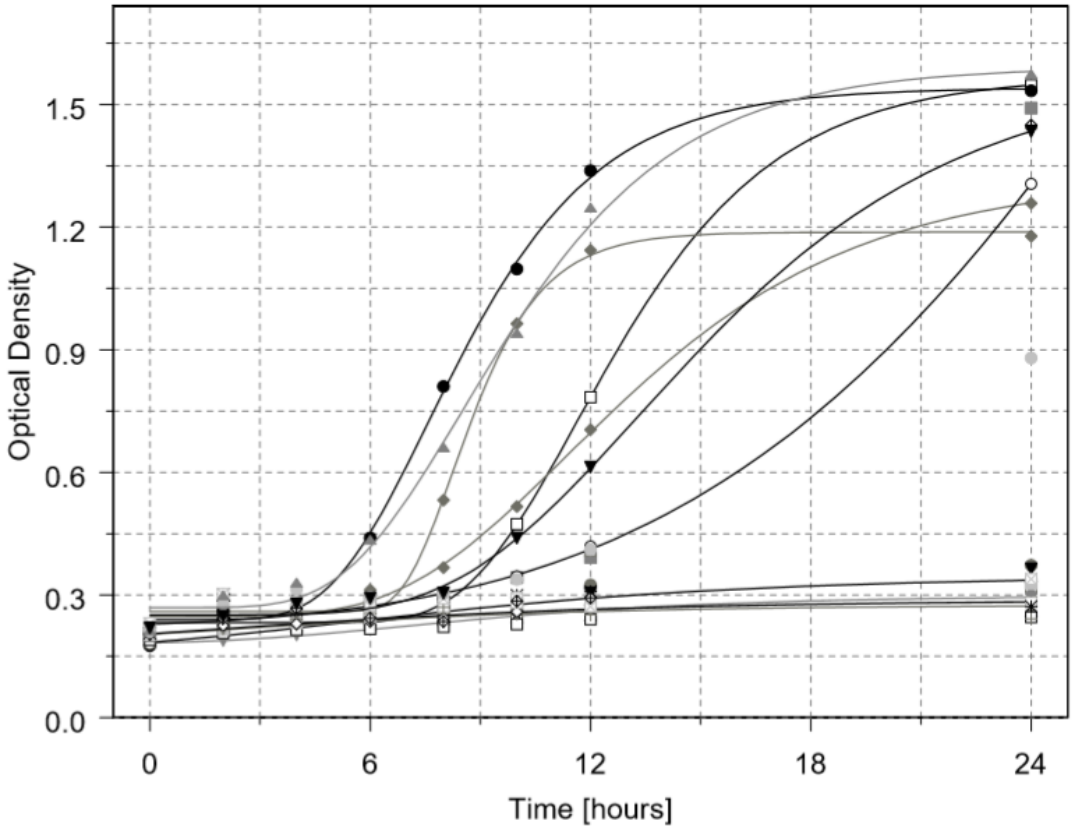

Fig. 4. Different growth kinetics of lactobacilli strains in MRS-4% NaCl. Line represents: *L. je.* CRL 1313, *L. ga.* CRL 1252, *L. ga.* CRL 1255, *L. ga.* CRL 1263, *L. ga.* CRL 1265, *L. ga.* CRL 1268, *L. ga.* CRL 1270, *L. ga.* CRL 1290, *L. ga.*

CRL 1307, *L. ga.* CRL 1311, *L. ga.* CRL 1314, *L. ga.* CRL 1322, *L. ga.* CRL 1509, *L. je.* CRL 1317, *L. je.* CRL 1333, *L. je.* CRL 1349, *L. jo.* CRL 1292, *L. mu.* CRL 1508, *L. pa.* CRL 1512, *L. sa.* CRL 1296, *L. sa.* CRL 1328, *L. fe.* CRL 1287, *L. ga.* CRL 1256, *L. ga.* CRL 1261, *L. ga.* CRL 1264, *L. ga.* CRL 1320, *L. re.* CRL 1324, *L. re.* CRL 1327, *L. rh.* CRL 1332 and *L. rh.* CRL 1511.

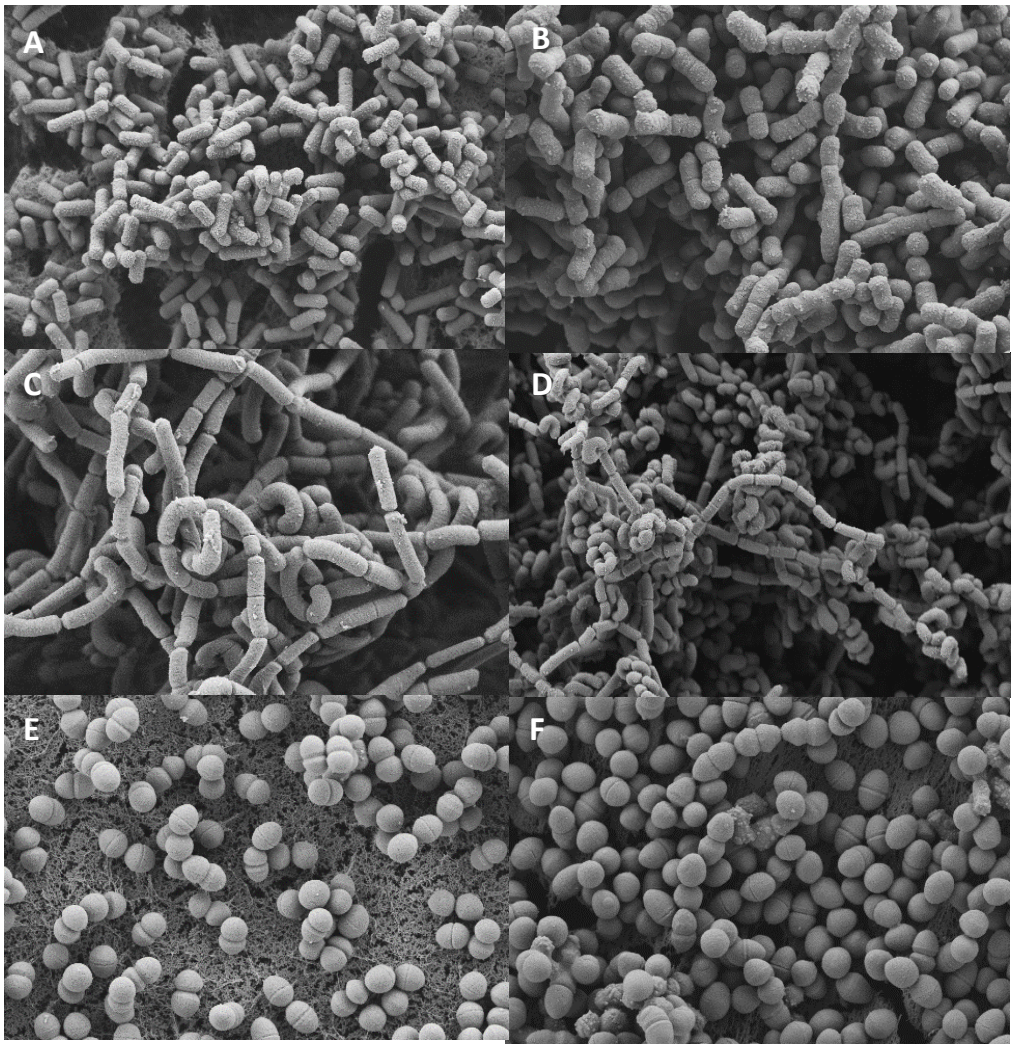

Fig. 5. Scanning electronic microscopy (SEM) of stressed and no stressed conditions of the most resistant strains. *L. gasseri* CRL 1509 in control (A) and at 4% NaCl (B); *L. rhamnosus* CRL 1332 in control (C) and at 4% NaCl (D); *L. reuteri* CRL 1327 in control (E) and at 4% NaCl (F).

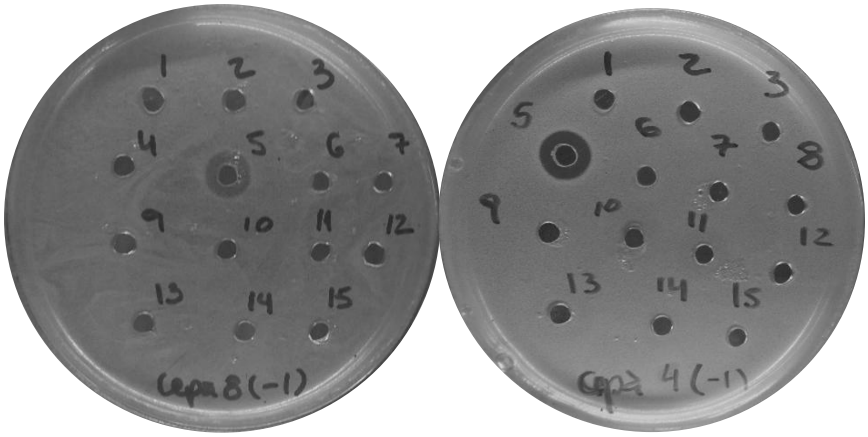

Fig. 6. Inhibition patterns showing non-compatible lactobacilli strains in agar plates.

Figure legends

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