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Posted Date: 6 March 2024

doi: 10.20944/preprints202403.0364.v1

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Micaela Ivana Núñez [1], María Cecilia Verni [1], Fernando Eloy Argañaraz Martinez [2], Jaime Daniel Babot [3], Victoria Terán [1], Mariana Elizabeth Danilovich [1,2], Elena Cartagena [1,2,*], María Rosa Alberto [1,2,†] and Mario Eduardo Arena [1,2,†]

[1] Instituto de Biotecnología Farmacéutica y Alimentaria (INBIOFAL) CONICET–UNT, Avenida N Kirchner 1900, CP 4000, Tucumán, Argentina; mario.arena@fbqf.unt.edu.ar (M.E.A.); maria.alberto@fbqf.unt.edu.ar (M.R.A.); ivanamicaela28@gmail.com (I.M.N.), mariaceciliaverni@gmail.com (M.C.V.); victoriateran@hotmail.com (V.T.); mariana.danilovich@fbqf.unt.edu.ar (M.E.D.)

[2] Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán (UNT), Ayacucho 471, CP 4000, Tucumán, Argentina; eloy.arganarazmartinez@fbqf.unt.edu.ar (F.E.A.M.)

[3] Reference Centre for Lactobacilli (CERELA-CONICET), Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina jbabot@cerela.org.ar

* Correspondence: elena.cartagena@fbqf.unt.edu.ar

† Alberto and Arena contributed equally to this work.

Abstract: This study provides a comprehensive investigation of lactic acid bacteria (LAB) isolated from Argentinean *Capsicum annum* L. The research covers important aspects, including genotypic characterization, bacterial stress tolerance, adhesion ability, safety evaluation, and functional and technological properties. The predominant isolates were identified as *Lactilactobacillus curvatus* and *Lactiplantibacillus plantarum*. Rep-PCR analysis grouped the isolates into 11 clonal groups. *Lp. plantarum* LVP 40 and LV 46; *Levilactobacillus brevis* LVP 41, *Pediococcus pentosaseus* LV P43, and *Lt. curvatus* LVP44, displayed both safety and resilience against adverse conditions such a slow pH, bile, and simulated gastric and intestinal juices. Moreover, the LAB strains exhibited high hydrophobicity and auto-aggregation percentages, NaCl tolerance, and substantial acidifying capacity. LAB supernatants demonstrated promising surfactant and emulsifying properties. Likewise, they differentially inhibited *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms, showcasing their potential as antipathogenic agents. Noteworthy some strains displayed considerable co-aggregation with these pathogens, and several isolates showed an effective antimutagenic and detoxifying power, further emphasizing their multifaceted capabilities. Five pepper bacterial strains showcased probiotic properties, suggesting their potential for gut health enhancement. In summary, these LAB strains hold promise as vegetable fermentation starters, contributing to food safety and versatile applications in food science.

Keywords: red and green peppers; lactic acid bacteria; probiotics; antimutagenic activity; antipathogenic activity; biosurfactants

1. Introduction

Among vegetables, peppers contribute significantly to the human diet due to their high content of antioxidants, vitamins (A, C, and E, mainly), minerals, polyphenols, and pigments. However, peppers have a comparatively short storage and shelf life than other vegetables, such as root vegetables. Therefore, developing methods to preserve the nutritional content and extend the shelf life of peppers could provide significant benefits for both the food industry and consumers. Multiple ancient civilizations have used pickling as one of the oldest preservation methods for various food products, including vegetables and fruits [1]. Although traditionally fermented pickles were homemade products obtained by spontaneous fermentation, they are now evolving as a select entrée to improve quality and safety [2]. It is well known that using a preselected mixed starter culture for lactic acid (LA) fermentation leads to superior quality in different fermented vegetables or fruits [3].

 doi:10.20944/preprints202403.0364.v1

Functional foods are defined as foods or ingredients with additional physiological benefits and nutritional value. In recent years, there has been an increase in interest in probiotics, and a significant body of research now recognizes probiotic products and fermented foods as potential functional foods that may contribute to improved gut health, prevention, and treatment of diseases.

Lactic acid bacteria (LAB) have traditionally been the main probiotics used in food processing as starter cultures, pharmaceuticals, and biological control agents. Currently, more than 62 different genera of LAB are widely used in commercial products as safe fermentation culture. However, it is necessary to address tools of different complexity for the isolation, molecular characterization, identification, and evaluation of probiotic properties of LAB, before they can be considered [4].

Since probiotics are expected to provide beneficial health effects to the host, they must show resistance to the acidic conditions of the stomach and the presence of bile salts and pancreatin in the small intestine. It is also essential that probiotic strains have good patterns of susceptibility to antibiotics, and cell surface properties, such as, auto-aggregation, cell surface hydrophobicity, autoaggregation, and coaggregation to facilitate the LAB colonization in the intestinal wall. In addition, functional attributes like antimicrobial and anti-mutagenic activities are desirable for probiotic strains to be effective [5].

The search for new probiotic strains with unique properties continues to be an area of great interest despite the availability of numerous well-characterized probiotic strains worldwide. In this sense, there is a growing interest in exploring the probiotic and biotechnological potential of microorganisms already adapted to the food matrices in which they are found naturally. This is especially relevant in the case of bacteria isolated from red and green peppers (*Capsicum annum* L.), which have the advantage of being already adapted to this specific microbiome. By evaluating these pepper isolates' probiotic and technological capabilities, we can identify new strains with unique properties useful in various applications, including the food and pharmaceutical industries.

The primary focus of this study was to examine the probiotic properties of lactic acid bacteria extracted from peppers in the northern region of Argentina. Given this vegetable's crucial role in the local economy, exploring ways to increase its market value is imperative. Our main goal was to isolate and identify strains of lactic acid bacteria that can potentially promote human health by conducting extensive investigations of their probiotic properties and searching for the development of innovative and sustainable agricultural practices in the region.

2. Materials and Methods

2.1. Isolation and LAB Identification

2.1.1. Source

Lactic acid bacteria were isolated from fresh *Capsicum annum* L. (green and red peppers) obtained from three different sources in Tucumán, Argentine: home garden peppers that did not receive fungicides and peppers purchased at the local market.

2.1.2. Isolation of Peppers' Bacterial Strains

Each sample aseptically collected was washed with 1 mL of sterile saline solution (0.85%). Serial dilutions were performed, of which 100 µL aliquots were plated out onto Man Rogosa and Sharpe (MRS) agar (MRS-agar) supplemented with 0.02% cycloheximide and 0.1 g/L sodium azide for the lactobacilli isolation. Cultures were carried out in triplicate using the pour plate method. Plates were incubated at 37 °C for 48 h under microaerophilic and anaerobic conditions generated by Mitsubishi Gas Chemical envelopes, Japan. Then, the isolates were examined microscopically for Gram reaction and catalase production, and their morphology was also analysed under an optical microscope. All isolates were kept at –20 °C and –80 °C in MRS glycerol (20%). Likewise, each isolate was lyophilized for its adequate conservation.

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2.1.3. Identification of LAB Isolates

For genotypic characterization, the total genomic DNA of isolates was extracted with a commercial kit (PrestoTM Mini gDNA Bacteria Kit Quick Protocol, Geneaid) according to the manufacturer's instructions. Amplification of the 16s rDNA was carried out using a pair of primers 27F (5'-GTGCTGCAGAGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CACGGATCCTACGGGTACCTTGTTACGACTT-3') [6]. The polymerase chain reaction mixture consisted of 1.5 mmol/L MgCl 2.5 µL 10x reaction buffer, 100 µmol/L dNTPs, 0.5 µmol/L of each primer, 4 mL bacterium DNA, and 1.5 U Taq polymerase (Invitrogen). Amplification consisted of initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 m, hybridization at 52 °C for 2 ms, and extension at 72 °C for 2 ms with final extension step at 72°C during 7 min. The polymerase chain reaction was conducted in a thermocycler My Cycler (Bio-Rad Laboratories). Amplification products were separated by electrophoresis (80 v) on a 0.8% (w/v) agarose gel stained with SYBR Gel DNA Safe Stain (Invitrogen) in 1x TAE buffer (40 mmol/L Tris-acetate, 1 mmol/L EDTA, pH 8). PCR products were purified with PCR Purification AccuPrep Kit (Bioneer, USA) and then sequenced using 3730 XL Sequencer (Applied Biosystems, USA) by MACROGEN Inc. (Korea). The 16S rRNA gene sequences obtained were edited with Chromas Pro software (1.5 version Technelysium Pty. Ltd. 2003-2009) and analysed with DNAMAN software (2.6 version Lynnon-Biosoft). Sequence homologies were examined by comparing the obtained sequences with those of the GenBank/EMBL/DDBJ database using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and identified according to the closest relative. The 16S ribosomal DNA sequences of the isolated strains were deposited in the GenBank.

2.1.4. Bacterial Strain Identification

Subsequently, the isolates were differenced at strain-level by repetitive sequence-based (rep-PCR) fingerprinting using the primer (GTG)5 (5'-GTGGTGGTGGTGGTG-3') described by Gevers et al. (2001) [7]. The PCR reactions were carried out as follows: 5 min of denaturation at 94 °C, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 40 ºC for 1 min, and extension at 65 ºC for 8 min, with a final extension step at 65 °C for 16 min [7]. The mastermix (20 µL) contained 4 µL of 5 × buffer (Inbio Highway, Buenos Aires, Argentina), 4 µL of 2.5 mM dNTPs (Inbio Highway, Buenos Aires, Argentina), 2 U of Taq polymerase (Inbio Highway, Buenos Aires, Argentina), 1 µL of DNA template (50 ng), 2 µL of 10 µM (GTG)5 primer (Genbiotech, Argentina), 4 µL of 25 mM MgCl2, and 4.8 µL of deionized water. PCR reactions were performed in a MyCycler device (Bio-Rad Laboratories, Hercules, USA), and the amplification products were separated by electrophoresis at 40 V for 200 minutes on 1.5 % (w/v) agarose stained with GelRedTM (Biotium, Hayward, USA) in 1 × TAE Buffer (40 mM Tris-acetate, 1 mM EDTA). Rep-PCR fingerprints were analysed as indicated by Versalovic et al. (1994) [8], using GelJ software v.2.0 [9] using the Dice correlation coefficient, and dendrograms were constructed employing the UPGMA clustering method according to Ishii and Sadowsky (2009) [10].

2.2. Stress Tolerance

2.2.1. pH Resistance

Bacterial cells were incubated at 37 °C for 18 h (5% initial culture overnight). Cells were harvested by centrifugation (3500 rpm, 15 min at 4 °C, 2193 x g) and washed once with phosphate-buffered saline (PBS, pH 7.2). The resistance of the isolates was evaluated as survival in PBS buffer at different pH (3, 4, and 7) for 2 h with an initial inoculum adjusted to an optical density (OD) of 0.35 at 600 nm (10^7–10^8 CFU/mL) (Spectrophotometer Genesys 50, Thermo Scientist). The number of CFU/mL was determined at 0, 1, and 2 h of incubation at 37 °C at the different pH values in microaerophilic conditions. A decrease less than three logarithmic cycles in 2 h was considered resistant.

 doi:10.20944/preprints202403.0364.v1

2.2.2. Bile Tolerance

In order to assess how the strains respond to the presence of bile, 0.3% (w/v) bile (oxgall, Sigma) was added to the MRS medium. The active cultures of each strain were subjected to centrifugation (3500 rpm, 15 min at 4 °C, 2193 x g) , and the resulting pellets were resuspended in an appropriate volume of medium to achieve an initial inoculum of OD 600 nm = 1 (Spectrophotometer Genesys 50, Thermo Scientist). The bacterial suspensions were used to inoculate (5%) MRS medium with or without oxgall (10^7 CFU/mL initial inoculum), and the cultures were then incubated at 37 °C under microaerophilic conditions. The growth of the cultures was monitored by observing changes in absorbance over 24 h (microplate reader, Multiskan Go, Thermo). To identify bacteria that are resistant to bile, the Gilliland growth retardation criterion was utilized. This criterion determines the delay time to reach an OD of 0.3 in the presence of bile relative to a control without bile [11]. Bacteria are classified as resistant if the delay time falls between 0 and 15 min, tolerant if the delay time is between 15 and 40 min, slightly tolerant if the delay time is between 40 and 60 min, and non-tolerant if the delay time exceeds 60 min.

Also, the percentage of bacterial survival was determined in the different growth phases (Lag, logarithmic, and stationary) by the following equation:

Survival (%) = Absorbance in the presence of bile/Absorbance in the absence of bile x 100.

The LAB strains were classified as resistant above 68%, tolerant between 34.0 –867.9%, and sensitive below 33.9%, according to Vera-Mejía et al. (2018) [12].

2.2.3. Resistance to Sequential Exposition of Simulated Gastric and Intestinal Juices

To determine the bacterial resistance to gastrointestinal tract conditions [13], 100 µL of each bacterial cell suspension containing 10^9 CFU/mL were transferred to 900 µL of sterile peptone water, and the cell count was performed by plate counting method on the agar culture medium suitable for each strain. Cells were washed twice with sterile saline water, harvested by centrifugation, resuspended in 1 ml of simulated gastric juice at pH 3 and 4, and then incubated 1 h at 37 °C in the shaker. Subsequently, cells were washed twice, resuspended in 5 mL of simulated intestinal juice, and incubated for 90 minutes at 37 °C in a shaker. The cell count was performed before and after the gastric and intestinal treatments by plating onto MRS agar. Plates were incubated at 37 °C for 48 h under microaerophilic conditions. The simulated gastric juice composition was 125 mM NaCl, 7 mM KCl, 45 mM NaHCO3, and 3 g/L pepsin. The pH was adjusted to 3 and 4 with 100 mM HCl. The composition of simulated intestinal juice was 0.3% (w/v) oxgall (dehydrated fresh bile, Sigma, MO, USA) and 0.1% (w/v) pancreatin (ICN Biomedicals, OH, USA).The pH adjusted to 8 with a solution of 5 N NaOH.

2.3. Adhesion Capacity

2.3.1. Surface Hydrophobicity

Bacterial cells were incubated at 37 °C for 18 h (5% initial culture overnight). Cells were harvested by centrifugation (3500 rpm, 15 min at 4 °C, 2193 x g), washed once with PBS, and resuspended in PBS. Cell density was adjusted to an OD of 0.8 (10^8 CFU/mL) at 600 nm (A_0). A 3 mL volume of the cell suspension was added to 1 mL of an organic solvent (xylene, chloroform and ethyl acetate) and stirred for 2 min. The phases were allowed to separate for 15 min at room temperature, and the OD at 600 nm of the aqueous phase was measured to determine the decrease in its absorbance, which was taken as an indicator of cell surface hydrophobicity (A_1) [14]. The percentage of bacterial adhesion to the solvent is determined by the following equation:

Hydrophobicity (%) = ($A_0 – A_1$ / A_0) x 100, where A_0 is the absorbance at zero time and A_1 is the absorbance at 15 min.

The strains were classified as strongly hydrophobic above 50%, moderately hydrophobic between 20 –50%, and hydrophilic below 20%, according to Tyfa et al. (2015) [15].

 doi:10.20944/preprints202403.0364.v1

2.3.2. Auto-Aggregation

The auto-aggregation assay based on the Hojjati et al. (2020) method [14] was conducted with slight modifications. In brief, formerly *Lactobacillus* strains were cultured in MRS broth at 37 °C for 18 h. The bacterial cells were collected by centrifugation (3500 rpm, 15 min at 4 °C, 2193 x g), washed twice, and re-suspended in PBS to attain an optical density of 0.8 at 600 nm. The suspended solution was left to incubate at room temperature for 24 h, after which the optical absorption of the top portion (Abs final) was measured. A microplate reader (Multiskan Go, Thermo) monitored the absorbance at 600 nm of the cell suspensions in different time intervals. The results were expressed as a percentage using the following formula:

Auto-aggregation (%) = $(A_0 - A_t / A_0) \times 100$, where A_t represents the absorbance at time t = 2, 4, and 24 h, where A0 is the absorbance at zero time.

2.4. Safety Assessment

2.4.1. Hemolytic Activity

The bacterial cells were subjected to an overnight culture at 37 °C with an initial inoculum of 5%. After incubation, they were harvested by centrifugation (3500 rpm, 15 min at 4 °C, 2193 x g), washed once with PBS, and resuspended to obtain an optical density of 0.8 (equivalent to 10^8 CFU/mL) at 600 nm. BHI agar-blood 5% medium, which comprised 100 mL of 2.5% w/v agar medium and 5 mL of complete human blood, was used for the experiment. The medium was vortexed and poured into small Petri dishes (10 mL), and allowed to solidify for 10 minutes, and a single study bacterium was inoculated per plate using a sterile loop for streaking (10 µL). The plates were then incubated for 48 h at 37 °C. The presence of a halo around the bacterial inoculum, which indicating lysis of erythrocytes was used as a criterion to determine hemolytic activity. The strains were then classified according to their hemolytic ability as α-hemolysis (green), β-hemolysis (clear), and γ-hemolysis (no hemolysis).

2.4.2. Antibiotic Sensitivity Test

The antibiotic susceptibility test was performed using the disk diffusion method, employing ten commercially available antibiotic disks (Brizuela-Lab.). These discs were placed on the surface of an MRS agar culture medium (2.5%, w/v), previously covered with soft MRS agar (5 mL) inoculated with 250 µL of a standardized suspension of the indicator strain (10^8 CFU/mL). Incubation was carried out for 48 h at 37 °C under microaerophilic conditions. After incubation, zones of inhibition surrounding the disks were observed.

2.5. Technological Properties

2.5.1. Compatibility of Strains

The agar diffusion method was used to check the compatibility between the selected probiotic strains [16]. MRS agar plates (2.5%, w/v) were covered on the surface with soft MRS agar (15 mL) inoculated with 750 µL of the indicator strain (10^8 CFU/mL). The dishes were allowed to solidify for 1 h at 25 °C, and wells were made in the top agar layer of the plates. Then, 30 µL of the culture cell-free supernatant of each strain was added to study their compatibility with the grass strain in question (indicator). The plates were incubated at 37 °C for 48 h, and the presence or absence of inhibition halos around the wells was observed.

2.5.2. NaCl Resistance

An inoculation of 5% of a cell culture adjusted to 10^8 CFU/mL was carried out in MRS broth culture media with and without NaCl (2.5%, 5%, 7.5%, and 10%, w/v). All variants were incubated in microaerophilic conditions at 37 °C, and cell growth (OD 600 nm) was measured using a microplate reader (Multiskan Go, Thermo) after 24 h of incubation at 37 °C.

 doi:10.20944/preprints202403.0364.v1

2.5.3. Acidification Capacity

The strains were cultivated in MRS broth (inoculated at 5% from a culture 10^8 CFU/mL) for 24 h at 37 °C under microaerophilic conditions. Subsequently, the pH of the supernatants of the different cultures obtained by centrifugation (3500 rpm, 15 min at 4 °C, 2193 x g) was determined.

2.6. Biosurfactant Production

2.6.1. Surfactant Capacity

The oil dispersion assay is a rapid and susceptible method for detecting surfactants. Therefore, it is an excellent tool for exploring the air-liquid surface activities of LAB supernatants. After 24 h of incubation, the whole bacterial cells were removed by centrifugation (3500 rpm, 15 min at 4 °C, 2193 x g), and the supernatants were filtered through a 0.22 µm pore size filter to obtain cell-free supernatants. For the bioassay, 20 µL of mineral oil was placed in a crystallizer of 250 mm in diameter, containing deionized water (100 mL), over millimetre paper according to a protocol described by Cartagena et al. (2020) [17]. Then, 10 µL of each cell-free supernatant was gently placed in the centre of the oil film. If biosurfactant is present, in the supernatant, the oil is displaced, and a clearing zone is formed. The diameter of this clearing zone on the surface of the oil correlates with the biosurfactant production and its activity [18,19]. The diameters of clear halos (mm) visualized under visible light were measured fivefold concerning the control supernatant. Tween 80 (polysorbate 80, Merck, Darmstadt, Germany) was used as a reference standard (positive control) [17]. The MRS medium (without bacteria) showed activity due to the presence of the sorbitan monooleate emulsifier in its composition, so this activity was discounted from each experiment.

2.6.2. Emulsifying Capacity

The emulsifying properties of the cell-free supernatants were evaluated using the emulsification activity (E24) test. This test was conducted in quintuplicate using a micro-method described by Verni et al. (2022) [20] which employs mineral oil as the oil phase, and tween 80 as a reference standard. Briefly, a volume of the oil phase was added to an equal volume of cell-free supernatant, and the resulting mixture was vigorously vortexed for 2 min and left to stand for 24 h. Then, the height of the emulsion layer and total height of both phases were measured. The liquid-liquid interfacial activity was calculated using the following formula:

Emulsification activity (%) = height of the emulsion layer (mm) / total height (mm) x 100.

2.7. Antipathogenic Activity

2.7.1. Inhibition of Bacterial Pathogenic Biofilm Adhesion

The non-stick activity test was conducted to evaluate the effects of probiotic bacteria metabolites on the biofilm formation of pathogenic bacteria, using polystyrene microplates. In each well was added 160 µL of Mueller Hinton broth (for *Staphylococcus aureus* ATCC 6538P and HT1) or Luria Bertani broth (for *Pseudomonas aeruginosa* ATCC 27853 and PAO1) and 20 µL of supernatants obtained after 24 h of incubation of the tested LAB, and 20 µL of each culture of pathogenic bacterium with biofilm phenotype (final concentration of 10^8 CFU/mL). The microcultures were incubated at 37 °C for only 1 h. Biofilm quantification was performed as previously described [20] using a crystal violet solution. Positive controls for the inhibition of bacterial adhesion were included, which comprised azithromycin (5 µg/mL), a known quorum-sensing inhibitor in *P. aeruginosa* PAO1 [21], ciprofloxacin (5 µg/mL), an antibiofilm antibiotic [22], and the non-ionic surfactant tween 80. Wells without LAB supernatants were also included as negative control (100% biofilm formation). Results were expressed as inhibition percentage concerning to the negative control.

 doi:10.20944/preprints202403.0364.v1

2.7.2. Co-aggregation with Pathogens

Non-pathogenic and pathogenic bacteria were mixed in equal amounts and vortexed for 30 seconds. The mixture was left at room temperature for 24 h, and the absorbance was measured at 600 nm. The absorbance of each suspension was also determined individually. Co-aggregation was quantified by calculating the reduction percentage of the absorbance in the mixed suspension in comparison to the individual suspensions [14], using the following formula:

% Co-aggregation = $[(OD_X + OD_Y / 2) - OD_{X+Y}) / (OD_X + OD_Y / 2)] \times 100$, where OD_X = Absorbance of the non-pathogenic bacterial suspension at zero incubation time, OD_Y = Absorbance of the pathogenic bacterial suspension at zero incubation time, OD_{X+Y} = Absorbance of the mixed bacterial suspension at 1, 4 or 24 h of incubation at 600 nm.

2.8. Antimutagenic Activity

The probiotics' mutagen-binding ability was assessed by measuring the inhibition of the *Salmonella typhimurium* TA100 mutation by sodium azide, as previously described by Díaz et al. (2022) [23]. One hundred µL of the potential probiotic bacterial suspensions (adjusted to 0.1, 0.4, and 0.9 at 600 nm) were mixed with 100 µL of the mutagenic solution. A positive control (100% revertants) was prepared with sodium azide only (without probiotic bacteria). Each suspension was incubated at 37 °C for 2 h and then centrifuged at 5000 rpm at 4 °C. The supernatants containing the mutagenic substance no bound to lactic acid bacteria (residual mutagen) were then incubated with *S. typhimurium* TA 100 (10^9 CFU/mL). LAB with the ability to bind mutagens have anti-mutagenic activity and detoxify power of important mutagens.

The anti-mutagenic activity was expressed as the percentage of inhibition of *S. typhimurium* mutation, as Maron and Ames (1983) reported [24].

Inhibition (%) = [(A–B) / (A–C)] x 100%, where A = Number of His+ revertants induced by the mutagen (positive control), B = Number of His+ revertants with bacteria and mutagen, and C = Number of spontaneous His+ revertants (negative control) determined in PBS containing the LAB culture without mutagens.

2.9. Statistical Analysis

Data are presented as mean ± SD from at least three independent experiments. Tukey's test evaluated the statistical significance of differences between mean values. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Isolation and Strains Identification

The comparative analysis of the sequences obtained from the isolated strains was conducted using different databases (BLAST, NCBI, and RDP). This study identified nine species with high identity scores ranging from 97.38% to 100% (Table 1). These sequences were deposited in GenBank, making them publicly available for further research.

Table 1. Identification of the isolates using the 16S rRNA gene sequence.

Bacteria	Access number GenBank	BLAST	PPeppers	Origin
Lactilactobacillus curvatus LVP 32	SUB5345111 LVP32 MK659876	100%	Red/Green	DG
Lactiplantibacillus plantarum LVP 33	SUB5659046 LVP33 MK965193	100%	Green	M
Lactilactobacillus curvatus LVP 34	SUB5345224 LVP34 MK659883	99.86%	Leaf	DG
Enterococcus casseliflavus LVP 35	SUB5345348 LVP35 MK659877	99.93%	Green	M
Pediococcus acidilactici LVP 36	SUB5659133 LVP36 MK965101	99.93%	Red	M

 doi:10.20944/preprints202403.0364.v1

Leuconostoc mesenteroides LVP 37	SUB5345695 LVP37 MK659879	97.38%	Red	DG
Lactilactobacillus curvatus LVP 38	SUB5345760 LVP38 MK659880	99.93%	Green	DG
Lactilactobacillus curvatus LVP 39	SUB5349021 LVP39 MK676004	100%	Green	DG
Lactiplantibacillus plantarum LVP 40	SUB5349099 LVP40 MK676008	100%	Green	M
Levilactobacillus brevis LVP 41	SUB5349336 LVP41 MK676009	99.16%	Green	M
Weisella cibaria LVP 42	SUB5515270 LVP42 MK825577	100%	Green	M
Pediococcus pentosaceus LVP 43	SUB5349351 LVP43 MK676007	100%	Red	DG
Lactilactobacillus curvatus LVP 44	SUB5349355 LVP44 MK676006	99.79%	Green	DG
Lactilactobacillus sakei LVP 45	SUB5349726 LVP45 MK676005	99.93%	Green	DG
Lactiplantibacillus plantarum LVP 46	SUB5515733 LVP46 MK825575	100%	Green	M

Domestic garden (DG). Market (M).

The isolated strains were lyophilized and incorporated into the LIVAPRA strain collection with the LVP nomenclature followed by a number. The table shows these microorganisms' origin and the pepper colour from which they were isolated. Interestingly, the number of isolates obtained from market and orchard peppers was almost proportional. However, the colour of the pepper from which the strains were obtained varied significantly, with the majority being derived from green peppers.

Most isolated strains were Lactilactobacillus curvatus (Lt. curvatus, 33%), and Lactiplantibacillus plantarum (Lp. plantarum, 20%), as also from the genus Pediococci (13%), particularly: Pediococcus acidilactici and Pediococcus pentosaceus, and others bacteria identified were: Levilactobacillus brevis (Lv. brevis) and Lactilactobacillus sakei (Lt. sakei), Enterococcus casseliflavus (En. casseliflavus), Leuconostoc mesenteroides (Ln. mesenteroides), and Weisella cibaria (W. cibaria).

Overall, this comprehensive analysis provides essential insights into the microbial diversity of peppers and highlights the importance of understanding the microbial composition of food products to ensure their safety and quality.

Based on the 16S rRNA gene sequences a phylogenetic relationship was established between the isolates to indicate the species and distribution of selected isolates.

The genetic relatedness of the isolated strains was assessed through Rep-PCR, and a dendrogram was constructed based on the calculated percentage of similarity (Figure 1). The power of discrimination denotes the ability of the typing method to differentiate unrelated strains was 97%, indicating that isolates with a similarity percentage of less than 97% were considered distinct.

Conversely, the strains with similarly percentage greater than 97% were regarded as identical strains with indistinguishable profiles. Based on these criteria, a similarity coefficient of 0.97 derived from the dendrogram indicates that LVP 33 and 40 are the same strain, and our analysis revealed 100% genetic similarity. Likewise, LVP 32, 34, 39, and 44 were identified as the same strain, with a 100% similarity score. The remaining strains resulted genetically distinct. Overall, the Rep-PCR analysis grouped the isolates into 11 clonal groups.

 doi:10.20944/preprints202403.0364.v1

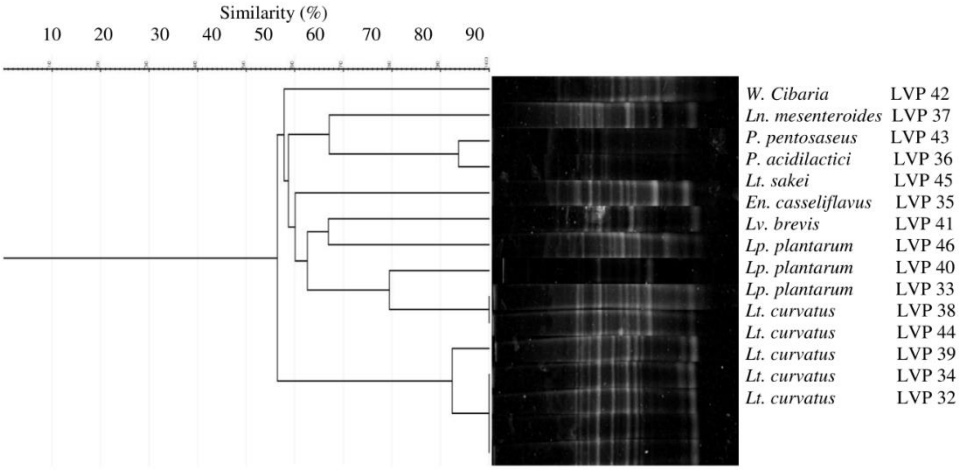

Figure 1. Dendrogram generated from rep-PCR fingerprints using UPGMA analysis and Dice's correlation coefficient.

3.2. Bacterial Stress Tolerance

3.2.1. pH Resistance

All strains survived under the different pH conditions tested, with variable survival rates, as shown in Table 2. Most isolated microorganisms exhibited good tolerance to pH 7.0 and pH 4.0. However, a notable sensitivity at pH 3 was observed in En. casseliflavus LVP 35, Ln. mesenteroides LVP 37, W. cibaria LVP 42, and Lt. curvatus LVP 38. In acidic conditions, these strains experienced a decrease in cell viability and growth, suggesting a higher susceptibility to acidic environments than other microorganisms.

In contrast, the study highlights the resilience of P. acidilactici LVP 36, Lp. plantarum LVP 40, LVP 44, and LVP 46; Lv. brevis LVP 41, P. pentosaceus LVP 43, and Lt. sakei LVP 45 at pH 3.0. Given their ability to tolerate low pH, these strains were selected for additional parameter evaluation.

Table 2. Survival of LAB isolated from peppers during 2 h of exposure to different pH.

Bacteria	**pH 3**	**pH 4**	**pH 7**
Enterococcus casseliflavus LVP 35	3.20	0.07	0.18
Pediococcus acidilactici LVP 36	2.97	0.14	0.04
Leuconostoc mesenteroides LVP 37	3.85	0.58	0.007
Lactilactobacillus curvatus LVP 38	4.11	0.13	0.11
Lactiplantibacillus plantarum LVP 40	2.33	0	0.52
Levilactobacillus brevis LVP 41	1.46	0.26	0.07
Weisella cibaria LVP 42	7.08	1.23	0.30
Pediococcus pentosaceus LVP 43	1.57	0.11	0.11
Lactilactobacillus curvatus LVP 44	0.78	0.003	0
Lactilactobacillus sakei LVP 45	2.74	0.39	0.25
Lactiplantibacillus plantarum LVP 46	2.67	0	0.41

Results of log CFU/mL reduction after 2 h exposure to different pH.

3.2.2. Bile Tolerance

According to Gilliland's growth retardation criteria [11], the isolated strains of *Lp. plantarum*, *Lt. curvatus*, and *P. pentosaceus* demonstrated tolerance to bile. However, *Lv. brevis* LVP 41 exhibited

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resistance to bile. Conversely, *P. acidilactici* LVP 36 and *Lt. sakei* LVP 45 strains were unable to tolerate bile (Table 3).

Table 3. Growth retardation due to the presence of bile.

Bacteria	Time (h) to reach DO=0.3		Δ time (h)	Δ time (min)	Growth retardation criteria
	Without Bile (Control)	With Bile (Treated)			
P. acidilactici LVP 36	4.6	6.2	1.4	81	Not tolerant
Lp. plantarum LVP 40	3.6	4	0.4	24	Tolerant
Lv. brevis LVP 41	3.2	3.3	0.1	6	Resistant
P. pentosaceus LVP 43	4.4	4.9	0.5	30	Tolerant
Lt. curvatus LVP 44	2.1	2.6	0.5	30	Tolerant
Lt. sakei LVP 45	2.8	4.4	1.6	96	Not tolerant
Lp. plantarum LVP 46	2.6	3.2	0.6	36	Tolerant

Resistant: delay between 0–15 min; Tolerant: delay between 15–40 min; Little Tolerant between 40–60 min; Not tolerant greater than 60 min.

This study found that the three strains of *Lp. plantarum* exhibited tolerance to bile salts in different growth phases: Lag (3 h), logarithmic (7 h) and stationary (24 h) (Figure 2). However, the LVP 40 strain displayed the highest survival rate throughout all growth phases, as depicted in Figure 2. Notably, strains LVP 40, LVP 41, LVP 43, and LVP 44 consistently maintained a survival rate of 70–100% when exposed to bile salts during the lag, exponential and stationary growth phases. This remarkable resilience and robustness in the face of gastrointestinal conditions highlight their potential. Based on these promising results, further investigations have been planned to explore the resistance of these four strains to the harsh environment of the gastrointestinal tract.

The results obtained regarding tolerance to bile and growth retardation in the presence of bile are in agreement, confirming the resistance of lactic acid bacteria to bile. This consistency between the two methodologies validates their use in assessing bile tolerance. In both cases, it was observed that the strains *P. acidilactici* LVP 36 and *Lt. sakei* LVP 45 did not exhibit tolerance to bile.

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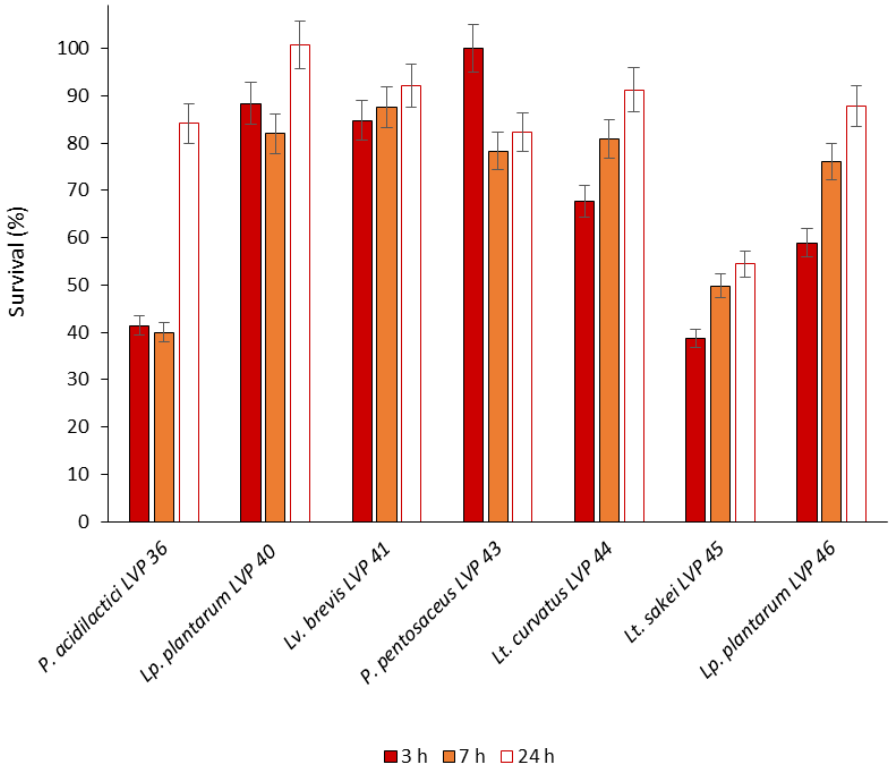

Figure 2. Bile tolerance of lactic acid bacteria isolated from peppers.

3.2.3. Resistance to Sequential Exposition to Stimulated Gastric and Intestinal Juice

The ability of lactic acid bacteria to survive the harsh conditions of the gastrointestinal tract is a crucial factor in determining their potential probiotic efficacy. In this study, lactic acid bacteria were exposed to artificial juices that simulated the gastric and intestinal juice. The results indicated that all the tested bacteria strains exhibited reasonable survival rates under these conditions (Table 4). The maximum reduction in viability was observed for *Lt. curvatus* LVP 44, with a decrease of 2.78 log CFU/mL, which is an acceptable value for a probiotic strain. The strain that showed the best adaptation to the digestion process was *Lp. plantarum* LVP 40, with decreases of 2.16 and 1.58 log CFU/mL to pH levels of 3 and 4, respectively. These findings suggest that the selected isolates have the potential to be used as probiotics as they can survive and adapt to harsh digestive system conditions.

Table 4. Resistance of lactic acid bacteria to the gastrointestinal tract.

Bacteria		Log CFU/mL		
		Initial	After juice exposition:	
			Gastric	Intestinal
Lp. plantarum LVP 40	pH 3	9.21	8.23	7.06
	pH 4	9.40	8.29	7.83
Lv. brevis LVP 41	pH 3	9.80	7.88	7.09
	pH 4	9.81	7.99	7.38
P. pentosaceus LVP 43	pH 3	9.75	7.79	7.14
	pH 4	9.88	7.92	7.17

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Lt. curvatus LVP 44	pH 3	9.96	8.04	7.18
	pH 4	9.89	8.10	7.30

Bacteria were incubated for 1 h in a simulated gastric juice at pH 3 or 4 and then, incubated in a simulated intestinal juice at pH 8.

3.3. Adhesion Capacity

3.3.1. Surface Hydrophobicity

The isolates evaluated showed varying levels in the hydrophobicity across all solvents, with lower values than the reference strain, except for *Lv. brevis*. The most effective solvent for increasing the hydrophobicity of all strains was ethylene acetate. It is important to note that hydrophobicity is a strain-depending property. Thus, the differential behaviour observed between the two strains of *Lp. plantarum* can be attributed to this surface property. *Lv. brevis* resulted strongly hydrophobic, with the highest adherence observed in all three solvents tested (84.45 in xylene, 98.92 in chloroform, and 96.92% in ethyl acetate). These hydrophobicity values were similar to or even higher than those obtained with the ATCC reference strain (Table 5).

Table 5. Hydrophobicity ability of the LAB strains.

Bacteria	**Strains**	**Hydrophobicity (%)**		
		Xylene	**Chloroform**	**Ethyl acetate**
Lp. plantarum	LVP 40	3.88 ± 0.02	9.50 ± 0.00	11.61 ± 0.09
Lv. brevis	LVP 41	84.45 ± 0.19	98.92 ± 0.13	96.92 ± 0.15
P. pentosaceus	LVP 43	–	9.21 ± 0.09	17.16 ± 0.12
Lt. curvatus	LVP 44	–	5.61 ± 0.08	24.55 ± 0.24
Lp. plantarum	LVP 46	11.35 ± 0.09	1.78 ± 0.02	18.06 ± 0.05
Lp. plantarum	ATCC 10241	87.26 ± 0.45	71.70 ± 0.77	74.57 ± 0.73

Hydrophobicity in different organic solvents expressed in %.

3.3.2. Auto-Aggregation

All the tested strains displayed a remarkable capability to undergo self-aggregation when they were incubated at 37 °C for 24 h in PBS. Notably, the strains *Lp. plantarum* LVP 40, *P. pentosaceus* LVP 43, *Lt. curvatus* LVP 44, and *Lp. plantarum* LVP 46 exhibited the highest degree of auto-aggregation. Following the 24-h incubation period, all the strains demonstrated auto-aggregation percentages surpassing 70%, except for *Lv. brevis* LVP 41, which exhibited a lower auto-aggregation rate of about 31% (Table 6).

Table 6. Auto-aggregation ability of the LAB strains.

Bacteria	**Strains**	**Auto-aggregation (%)**				
		1h	**2h**	**3h**	**4h**	**24h**
Lp. plantarum	LVP 40	11.09 ± 1.50	14.30 ± 0.64	15.28 ± 1.16	18.61 ± 0.04	72.84 ± 7.54
Lv. brevis	LVP 41	4.85 ± 1.54	6.29 ± 0.91	10.61 ± 0.07	10.46 ± 0.61	30.83 ± 2.06
P. pentosaceus	LVP 43	14.57 ± 0.27	21.78 ± 0.53	28.98 ± 1.59	33.67 ± 1.10	72.36 ± 1.59
Lt. curvatus	LVP 44	14.11 ± 1.90	14.09 ± 2.19	23.45 ± 2.00	30.57 ± 4.67	74.27 ± 0.47
Lp. plantarum	LVP 46	10.71 ± 0.93	12.01 ± 0.33	18.09 ± 0.84	19.83 ± 1.64	75.55 ± 1.59
Lp. plantarum	ATCC 10241	4.35 ± 1.92	8.68 ± 0.46	15.27 ± 2.05	16.17 ± 0.35	72.61 ± 3.91

Determination of bacterial auto-aggregation percentages after 1, 2, 3, 4 and 24 h of incubation in phosphate-buffered saline.

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3.4. Safety Assessment

3.4.1. Hemolytic Activity

Among the strains subjected to testing, none produced the formation of hemolysis halos on the blood agar medium used in the experiments (data not shown). The absence of clear/green halos indicated that the selected strains cannot cause red blood cell hemolysis.

3.4.2. Antibiotic Sensitivity Test

The pattern of antibiotic resistance exhibited by the chosen strains was similar to the *Lp. plantarum* ATCC 10241 reference strain. Specifically, these strains demonstrated sensitivity to ampicillin, cephalothin, chloramphenicol, clindamycin, erythromycin, gentamicin, and rifampicin. On the contrary, they resisted the antibiotics vancomycin, ciprofloxacin, and norfloxacin. These findings provide a guarantee of safety in the use of these strains since their antibiotic resistance profiles conform to established standards (Table 7).

Table 7. Antimicrobial susceptibility.

Groups	**ATB**	**Antimicrobial susceptibility**					
		***Lp. plantarum* LVP 40**	***Lv. brevis* LVP 41**	***P. pentosaceus* LVP 43**	***Lt. curvatus* LVP 44**	***Lp. plantarum* LVP 46**	***Lp. plantarum* ATCC 10241**
Group 1 Inhibitors of cell wall synthesis	Ampicillin	S	S	S	S	S	S
	Cephalothin	S	S	S	S	S	S
	Vancomycin	R	R	R	R	R	R
Group 2 Inhibitors of protein synthesis	Chloramphenicol	S	S	S	S	S	S
	Clindamycin	S	S	S	S	S	S
	Erithromycin	S	S	S	S	S	S
	Gentamicin	S	S	S	S	S	S
Group 3 Inhibitors of nucleic acid synthesis	Ciprofloxacin	R	R	R	R	R	R
	Norfloxacin	R	R	R	R	R	R
	Rifampicin	S	S	S	S	S	S

Concentration in the disk (μg): Ampicillin 10; Cephalothin 30. Vancomycin 30. Chloramphenicol 30. Clindamycin 2. Erythromycin 15. Gentamicin 10. Ciprofloxacin 5. Norfloxacin 10. Rifampicin 5. R: resistant, S: sensitive.

3.5. Technological Properties

3.5.1. Compatibility of Strains

The compatibility test on the five chosen strains revealed the absence of inhibition halos in all experiments. That is, no supernatant from the selected bacteria prevents the growth of other bacteria (data not shown). Consequently, the creation of mixtures of these lactic acid bacteria can be used effectively in food fermentation.

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3.5.2. NaCl Resistant

The experimental evaluation of tolerance to high concentrations of NaCl showed that the LVP 40, LVP 43, LVP 46, and LVP 44 strains had a high tolerance level (2.5 and 5% NaCl). Conversely, the LVP 41 strain demonstrated a high tolerance to a 2.5% NaCl solution but a lower tolerance to a 5% solution.

It is worth highlighting that none of the strains exhibited tolerance to NaCl concentrations of 7% and 10%, as detailed in the supplementary material. In all cases, the tolerance was more significant than the collection strain *Lp. plantarum* ATCC 10241, isolated from sauerkraut, which tolerates only 2.5% salt (Figure S1, Supplementary Material). The osmotic resistance of potential probiotic bacteria makes them suitable for fermenting vegetables in which concentrations close to 2% NaCl are generally used.

3.5.3. Acidification Capacity

The isolated microorganisms were cultured at 37 °C in MRS broth with an initial pH of 6.5 for 24 h. The pH was measured at the end of this period. Regarding acidifying capacity, all strains, except LVP 41, demonstrated the ability to lower the pH of the culture medium by more than two units after 24 h of incubation. The final pHs were 3.56, 3.72, 3.73, and 3.57 for *Lp. plantarum* LV P40, *P. pentosaceus* LVP 43, *Lt. curvatus* LVP 44, and *Lp. plantarum* LVP 46, respectively. In the reference strain *Lp. plantarum* ATCC 1024, isolated from fermented food, the final pH was 4.24. This characteristic is particularly advantageous from a technological perspective since it implies a quicker process to reach acidity levels that protect the product from contaminating microorganisms. The higher final pH in the culture with *Lv. brevis* LVP 41 (final pH 6.04) may be attributed to its obligate heterofermentative nature, producing both lactic and acetic acid. Acetic acid is less acidic than lactic acid, contributing to the observed difference in acidity.

3.6. Surfactant and Emulsifying Properties

All LAB cell-free supernatants (CFS) showed oil dispersing activities similar to those obtained with the commercial surfactant tween 80 (Figure 3). *Lp. plantarum* LVP 46, LVP 44, and LVP 40 CFS had the highest activity. Nevertheless, there were no significant differences between them. However, the reference surfactant had a lower oil dispersion halo than the *Lactobacillus* strains.

On the other hand, the emulsification activity of the bacterial supernatants was generally greater than 30% but lower than that of tween 80 (50%). Only *Lp. plantarum* LVP 40 supernatant exhibited an emulsification activity of 67%.

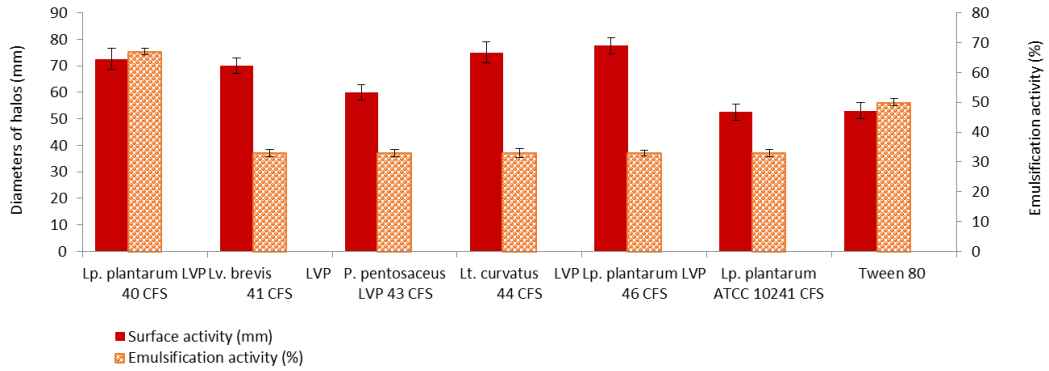

Figure 3. Surface and interfacial activities of LAB cell-free supernatants.

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3.7. Antiphatogenic Activity

3.7.1. Inhibition of Bacterial Pathogenic Biofilm Adhesion

All LAB supernatants inhibited the formation of bacterial biofilms at 1 h of treatment (Figure 4). The biofilm of both S. aureus strains decreased in the presence of all CFS. For the ATCC 6538P strain, the most significant effect was observed with the supernatant of the *Lp. plantarum* LVP 46 strain (68%). Concerning the HT1 strain, the highest inhibition was noticed for the supernatant of *Lv. brevis* LVP 41 (67%).

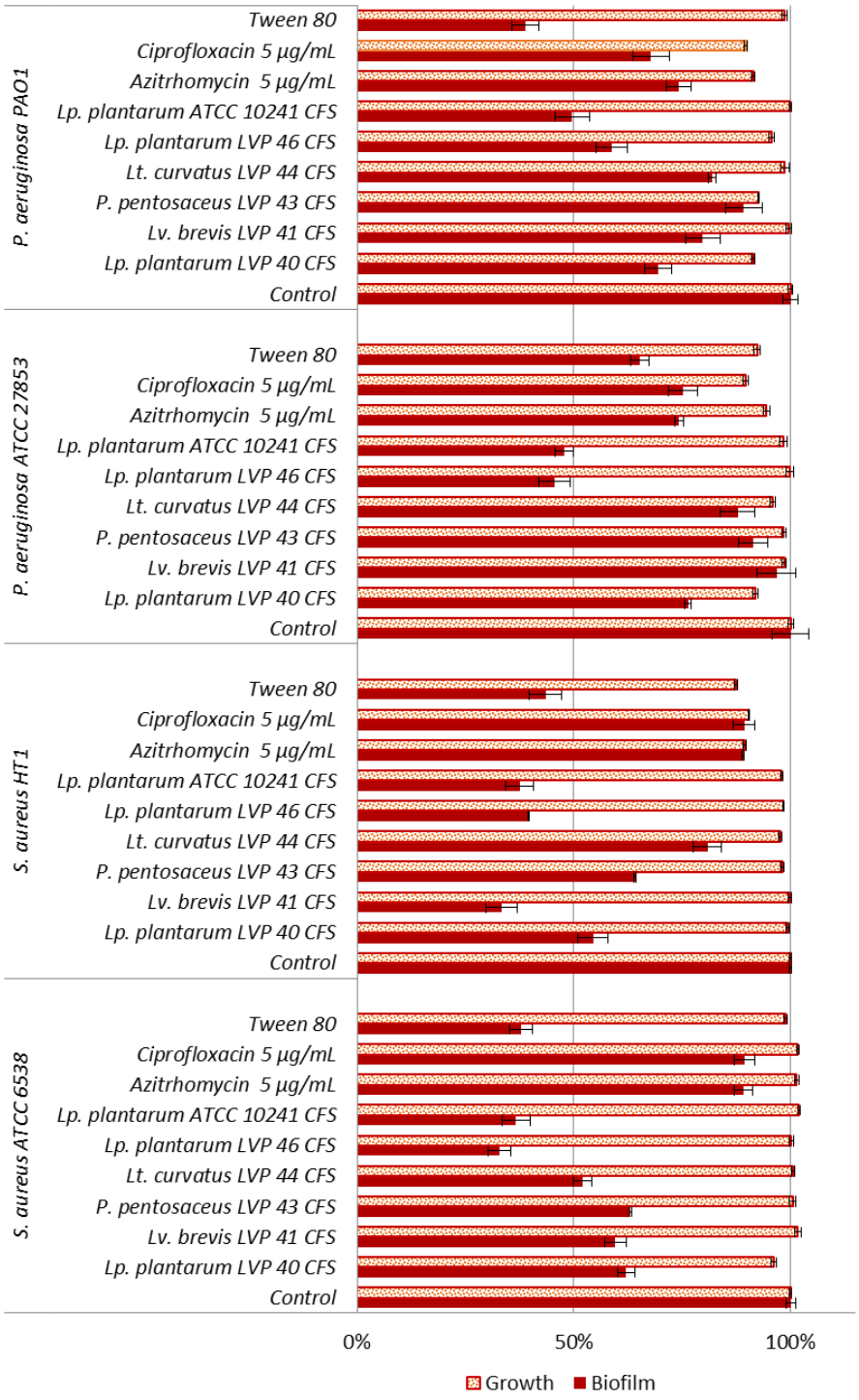

Figure 4. Effects of LAB supernatants on growth and biofilm formation of pathogenic bacteria.

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For the *P aeruginosa* strains, all CFS inhibited biofilm formation in both strains assayed but with lower values than those presented for *S. aureus*. Among the strains, the most potent supernatant was derived from *Lp. plantarum* LVP46, showcasing 55% and 42% inhibition against *P. aeruginosa* ATCC 27853 and PAO1, respectively.

It is noteworthy that the inhibitory effects on biofilm adhesion surpassed those of commonly used antibiotics, ciprofloxacin and azithromycin, in all instances. Additionally, the observed inhibitions were comparable or even higher than those achieved with tween 80.

In contrast to what was observed in the biofilm formation by pathogenic bacteria, the supernatants of the potential probiotics did not significantly affect the growth of the pathogens under the conditions studied.

3.7.2. Co-Aggregation with Pathogens

Table 8 shows the results of co-aggregation between isolated and selected strains and four pathogenic bacteria after 1, 4, and 24 h incubation. All strains tested exhibited some degree of co-aggregation with pathogens. The highest effect was observed in the bacterial strains *Lv. brevis* LVP 41 and *Lp. plantarum* LVP 40 and ATCC 10241 with both strains of *P. aeruginosa* (100 and 50%, respectively). The greatest co-aggregation with S. aureus was observed with *P. pentosaceus* LVP 43 and Lt. curvatus LVP 44, although the values varied depending on the strain.

Table 8. Co-aggregation ability of the lactic acid bacteria.

Lactic acid bacteria	**Pathogenic bacteria**		**% Coaggregation**		
			1h	**4h**	**24h**
Lp. plantarum LVP40	*P. aeruginosa*	ATCC 27853	7.83 ± 1.59	25.06 ± 2.83	51.20 ± 3.33
		PAO1	9.84 ± 1.64	24.97 ± 1.04	48.14 ± 2.70
	S. aureus	ATCC 6538	4.31 ± 3.04	4.31 ± 3.04	4.31 ± 3.04
		HT1	5.11 ± 0.74	18.07 ± 2.12	18.07 ± 2.12
Lv. brevis LVP41	*P. aeruginosa*	ATCC 27853	19.43 ± 4.60	67.46 ± 4.83	100 ± 0.12
		PAO1	21.46 ± 1.69	89.16 ± 0.74	100 ± 1.14
	S. aureus	ATCC 6538	1.04 ± 0.74	1.04 ± 0.74	1.04 ± 0.74
		HT1	0.72 ± 0.51	3.73 ± 1.49	29.37 ± 6.67
P. pentosaceus LVP43	*P. aeruginosa*	ATCC 27853	6.33 ± 0.11	13.29 ± 3.05	13.29 ± 3.05
		PAO1	7.93 ± 1.84	19.08 ± 4.78	20.47 ± 0.99
	S. aureus	ATCC 6538	9.00 ± 6.36	15.42 ± 6.36	36.99 ± 0.07
		HT1	1.50 ± 0.70	8.03 ± 2.56	37.75 ±18.66
Lt. curvatus LVP44	*P. aeruginosa*	ATCC 27853	11.18 ± 4.66	26.35 ± 1.74	38.94 ± 0.46
		PAO1	4.37 ± 0.27	20.20 ± 1.00	24.35 ± 2.94
	S. aureus	ATCC 6538	7.47 ± 6.67	68.29 ± 0.11	76.89 ± 0.86
		HT1	-	11.39 ± 1.51	36.29 ± 7.61
Lp. plantarum LVP46	*P. aeruginosa*	ATCC 27853	7.64 ± 0.15	19.52 ± 0.40	34.32 ± 10.47
		PAO1	12.39 ± 0.57	23.55 ± 3.13	30.22 ± 2.69
	S. aureus	ATCC 6538	-	-	34.32 ± 10.47
		HT1	2.46 ± 0.50	7.43 ± 1.64	18.79 ± 14.39
Lp. plantarum ATCC10241	*P. aeruginosa*	ATCC 27853	3.65 ± 0.86	21.84 ± 5.55	40.84 ± 24.42
		PAO1	0.35 ± 0.24	10.00 ± 0.00	48.29 ± 5.03
	S. aureus	ATCC 6538	14.26 ± 7.95	14.26 ± 0.00	14.26 ± 0.00
		HT1	7.46 ± 3.79	27.87 ± 6.45	44.82 ± 5.42

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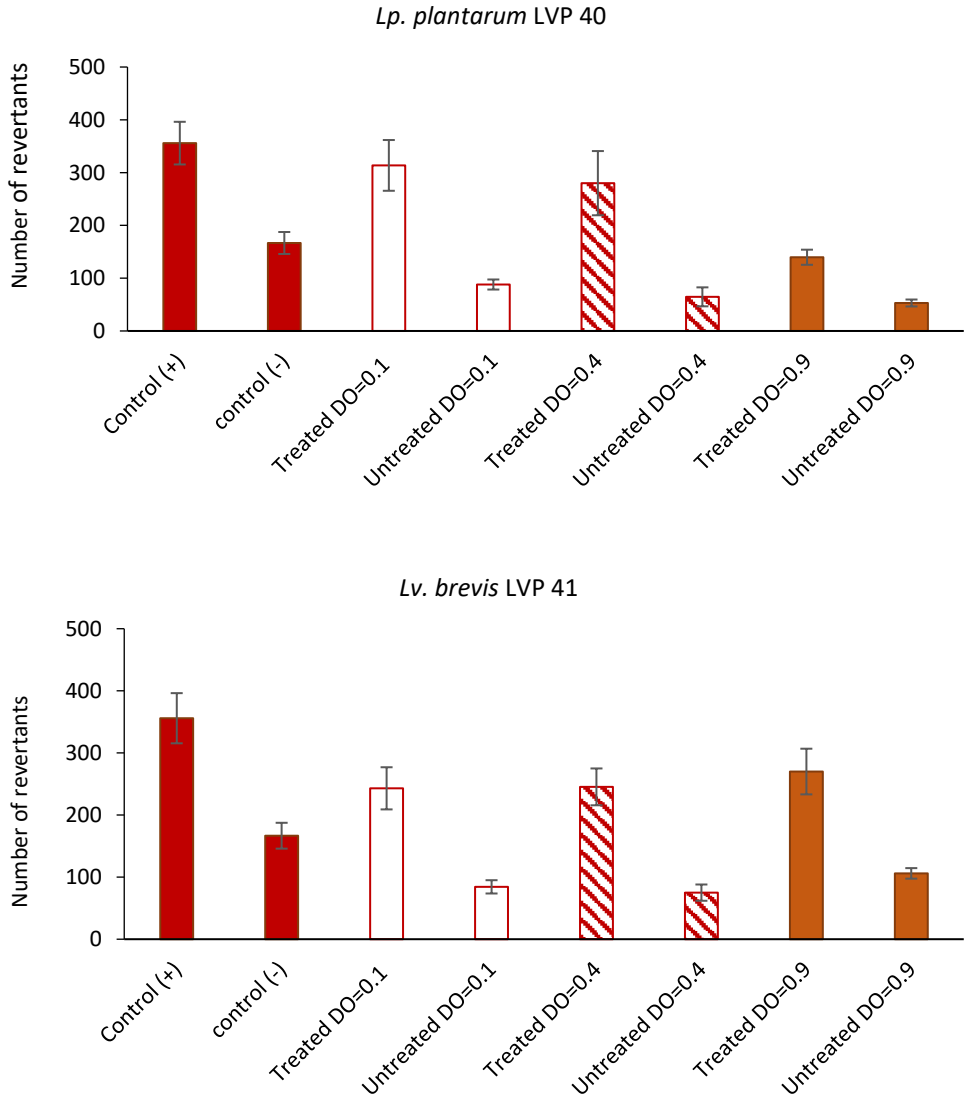
Co-aggregation capacity of LAB strains with strains of P. aeruginosa (ATCC 27853 and PAO1) and Staphylococcus aureus (ATCC 6538 and HT1) after 4 h of incubation in phosphate-buffered saline.

3.8. Antimutagenic Capacity of Lactic Acid Bacteria

The antimutagenic activity of the lactic acid bacteria against sodium azide is shown in Figure 5. *S. typhimurium* TA 100 was assessed against cell-free supernatants (CFS) derived from LAB cultures treated (by the addition of mutagenic substance) and untreated cultures (control). The LAB concentrations used were 4x 10^7 CFU/mL (OD_{600} nm= 0.1), 1.5x 10^8 CFU/mL (OD_{600} nm = 0.4), 4 x 10^8 CFU/mL (OD_{600} nm = 0.9). As seen in the following figure, the inhibition in the reversion of *S. typhimurium* by CFS was strain-dependent. The results revealed that the LAB strains *Lv. brevis* LVP 41, *P. pentosaceus* LVP 43, and *Lp. plantarum* LVP 46 have the ability to remove the mutagen, evidenced by the decrease in revertant colonies (34 –38%, 30 –52%, 30–44%, respectively). Notably, in the case of strain *Lp. plantarum* LVP 40, the removal capacity increased as the used bacterial biomass increased (15–71%). The antimutagenic activity of these strains surpassed that of the reference strain *Lp. plantarum* ATCC 10241 (7–14%). On the contrary, *Lt. curvatus* LVP 44 did not show antimutagenic effects.



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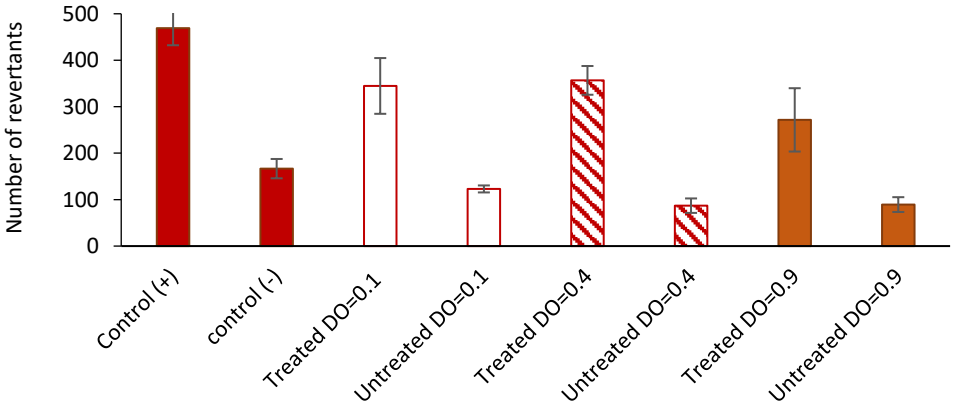
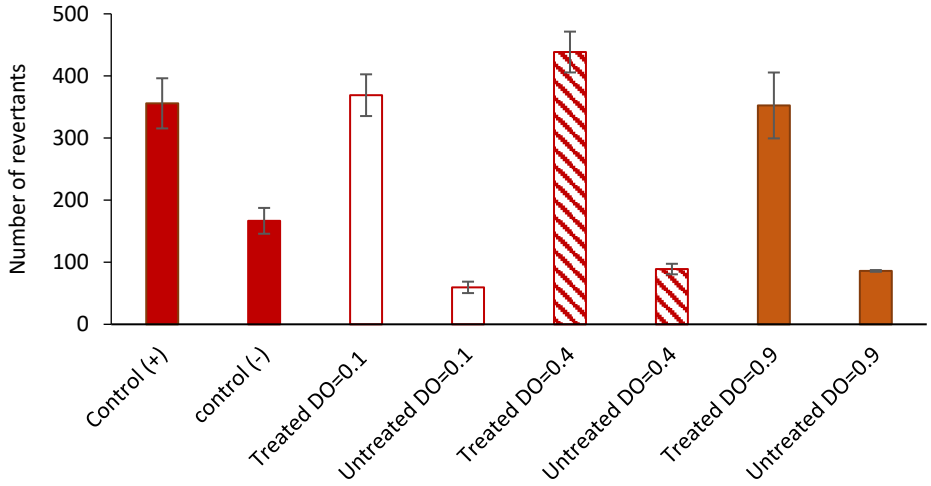
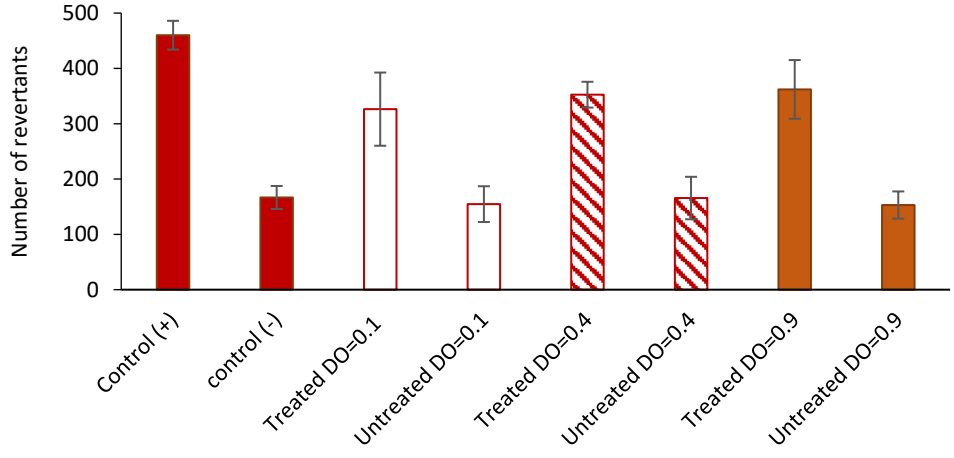
P. pentosaceus LVP43



Lt. curvatus LVP 44



Lp. plantarum LVP 46



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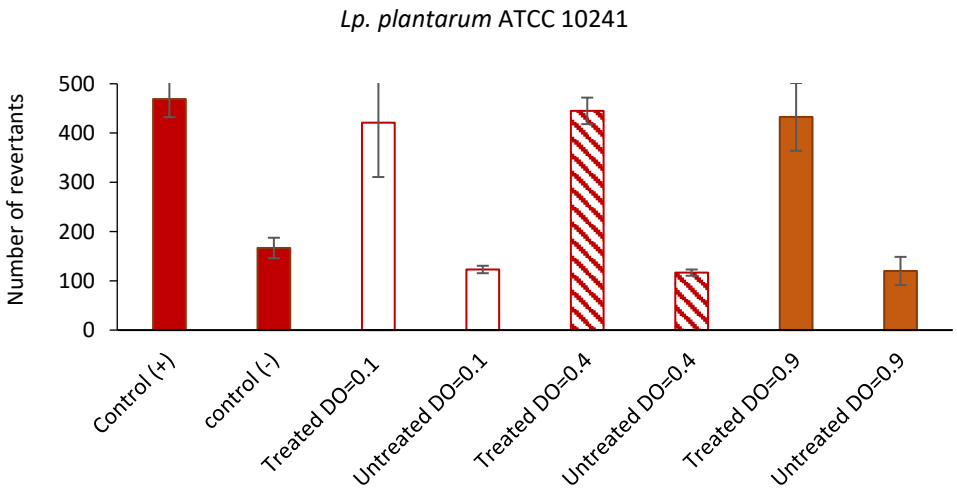

Figure 5. Revertants of *S. typhimurium* TA 100 versus CFS from different concentrations of treated (sodium azide) and untreated cultures. Control (+): The number of His+ revertants induced by the mutagen in the absence of CFS corresponds to a 100% reversion rate. Control (-): The number of spontaneous His+ revertants induced in the absence of both mutagen and CFS corresponds to the rate of spontaneous reversion. Treated: The number of His+ revertants induced by the mutagen in the presence of CFS. Untreated: The number of spontaneous His+ revertants induced in the absence of the mutagen but in the presence of CFS corresponds to the rate of spontaneous reversion.

4. Discussion

Ancestrally, pickling is one of the oldest preservation processes of several foodstuffs, such as vegetables, fruits, fish, and meat. Pickling imparts unique and desirable changes in flavour, texture and colour that take place over time in fermented pickles. In many cultures, pickles have been associated with many nutritional benefits over time. In particular, LAB microorganisms contributed to this process and are increasingly linked to consumer health benefits. In fact, *Lp. plantarum*, *Lv. Brevis*, *Lactobacillus acidophilus*, *Limosilactobacillus fermentum*; *Leuconostoc fallax*, and *Ln. mesenteroides* are the most studied [25].

Lactic acid bacteria dominate the fermented vegetable microbial community, providing several health-related properties. In concordance with our results, García-Burgos et al. (2020) stated lactobacilli are the most prevalent microorganisms in the fermentation of fruit and vegetable juices and species such as *Lp. plantarum*, *Lactobacillus bavaricus*, *Lactobacillus xylosus*, *Lactobacillus bifidus*, and *Lv. brevis* are frequently found [26]. In the same way, other authors reported that *Lp. plantarum*, *Lv. brevis*, *Ln. mesenteroides*, *P. pentosaceus*, *Limosilactobacillus fermentum*, and *Lactococcus lactis* were the main microorganisms isolated from vegetables [27,28].

It is important to note that genus previously called *Lactobacillus* is one of the most widely used bacterial genera as probiotics, and their use as microbial food supplements has obtained the status of *Generally Recognized as Safe* (GRAS). Lactobacilli are found in the gastrointestinal tract of humans and animals, in plant or animal-based fermented products, and most commercially available fermented foods. In this study, bacterial isolates of peppers were evaluated for their stress tolerance, safety, technology, and probiotic properties.

Resilience to the challenging conditions of the gastrointestinal tract stands as a primary factor restricting the application of microorganisms as live probiotic agents. The capability to endure acidity and bile salts is widely acknowledged as crucial for the survival of LAB in the gut. Specifically, the ability to survive at pH 3.0 is deemed as optimal acid tolerance for probiotic strains [29]. Consequently, this study assessed isolates for their capacity to withstand pH 3.0 and bile. Seven isolates were resistant to exposure to pH 3.0 and then were assessed for their bile tolerance. Only five strains supported the bile and were identified as lactobacilli and pediococci. This fact is in concordance with those found in *Lacticaseibacillus rhamnosus*, *Lactobacillus gasseri*, and *Limosilactobacillus*, which exhibited a bile resistance of 3.0 g/L [30,31].

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The adhesion capacity of LAB isolates that were able to survive the conditions of the digestive system was evaluated. The hydrophobicity property is strain-specific in concordance with an earlier report [32]. So, the high hydrophobicity found in *Lactobacillus* strains agrees with the observed in *Lactobacillus gasseri* and *Lacticaseibacillus rhamnosus*, and the mean values depended on the origins and genera [31]. De Souza et al. (2019) also confirmed this, by describing different strains of *Lacticaseibacillus casei* with hydrophobicity values ranging from 9.66 to 69.36%, and for *Lm. fermentum* strains from 0.30 to 68.81% [33], and they stated that bacteria with higher hydrophobicity can adhere better to epithelial cells and significantly influence the microbial composition in the intestine.

In addition, after 24 h of incubation, all LAB strains demonstrated self-aggregation percentages higher than 70%, except for *Lv. brevis*. In contrast, the auto-aggregation abilities of *Lactobacillus gasseri*, *Lacticaseibacillus rhamnosus*, and *Limosilactobacillus reuteri* were in a variable range between 5.8 and 28.5%, depending on incubation time and strain [31]. Hemolytic effects were not observed in the tested isolates, and our results agreed with those reported by Damaceno et al. (2021) [34].

On the other hand, evaluating the antibiotic resistance of potential probiotic microorganisms is vital to limit the antibiotic resistance gene transfer between the typical microbiota and pathogens [35]. In the present study, all the strains were resistant to vancomycin, ciprofloxacin and norfloxacin. In accordance with the present results, lactobacilli were previously reported as vancomycin and ciprofloxacin resistant [29,31]. In most *Lactobacillus* species, vancomycin resistance genes are chromosomally coded; therefore, there is no risk of transfer as in the case of plasmids [31]. Other authors noticed a variable resistance to gentamicin [36,37]. Nevertheless, in this work neither of the strains was resistance to gentamicin.

The new isolates also showed a high tolerance to 2.5% of NaCl and a low tolerance to 5% of NaCl; while other study informed that several probiotics could tolerate 6.5% NaCl [38]. The stability of pickles during the fermentation process and storage is due to their content of LAB, and its viability, and metabolic activity (acidifying activity). The suitability and good selectivity of LAB starter cultures for large-scale production are essential for keeping the fermenting microflora stable for a long time [39].

The bacterial surfactant property has also been investigated in the present work. Biosurfactants are predominantly synthesized by bacteria and fungi and can reduce interfacial and surface tension between two immiscible liquids. These compounds exhibit various properties such as detergency, wettability, and foaming, making them suitable for biomedical and industrial applications [40].

LAB biosurfactants have shown promise as anti-adhesive agents to prevent the adhesion of pathogens to the host epithelium and solid surfaces, including biomedical instruments [41,42]. Exploring bacterial supernatants' surface and interfacial activities, such as those produced by *Lactobacillus* strains, is essential for identifying new strategies to inhibit microbial adherence and control biofilm-forming pathogens [20,43]. Previous studies have identified glycoproteinaceous biosurfactants, such as surlactin, in various *Lactobacillus* species [17,44–46]. The findings found in this work are consistent with the results obtained by Verni et al. (2022) which revealed an emulsifying activity of the *Lactobacillus paracasei* subsp. *paracasei* CE75 supernatant similar to the *Lactobacillus crispatus* BC1' biosurfactant [20,47].

The biofilm formation is initiated by the adhesion of individual bacteria to a surface. Surface-sensing creates bacterial awareness of their adhering state on the surface. It is essential to initiate the phenotypic and genotypic changes that characterize the transition from initial bacterial adhesion to a biofilm. This first stage is controlled by complex combinations of the physicochemical interactions between the cell membrane and the material surface [48,49]. In fact, the observed effects on the biofilm biomass after only one hour of incubation did not depend on bacterial growth inhibition but rather on non-stick effects, and these results agree with previous works that demonstrated antibiofilm effects exclusively due to the *Lactobacillus* anti-adhesive property [20,43,50,51]. Indeed, Gudiña et al. (2010) observed an anti-adhesive activity of the crude biosurfactant isolated from *L. paracasei* subsp. *paracasei* A20 higher against *S. aureus* (76.80%) than against *P. aeruginosa* (21.20%) at 50 mg/mL after four hours of treatment [50]. While Verni et al. (2022) demonstrated a potent inhibition of *P. aeruginosa* HT5 biofilm (72.01%) at one hour linked to anti-adhesiveness effects of *L. paracasei* biosurfactant [20].

 doi:10.20944/preprints202403.0364.v1

Our results indicate that BAL supernatants are promising sources of biosurfactants with anti-adhesive properties. Specially, *Lp. plantarum* LVP46 supernatant exerts a surface activity that correlates with strong anti-biofilm effects against pathogenic bacteria Gram (+) and (-).

It is important to highlight that anti-biofilm and antipathogenic effects observed in LAB supernatants were consistent with previous studies [23,52–54]. These results are significant since biofilm formation, governed by Quorum sensing (QS), constitutes a significant problem for the safety of several food products. Probiotics have multiple properties, and although evidence is scarce, their involvement in the regulation of QS may bring new solutions in several areas, including food preservation. Several species of lactic acid bacteria (*Lp. plantarum*, *Lm. fermentum*, *Lactobacillus acidophilus*, *Ls. casei*, *Lv. brevis*, *Lactobacillus reuteri*, and *Lt. curvatus*) have already been reported at least once as quorum-quenching (QQ) agents [23,52,54,55]. Thus, a *Lp. plantarum* strain reduced the expression of some genes involved in the biofilm formation of *S. aureus* [56], *Lv. brevis* 3M004 inhibited biofilm formation of *P. aeruginosa* [57], and *Lp. plantarum* PA 100 inhibited the *N*-acyl-homoserine lactone (QS autoinducer) activity of *P. aeruginosa* by blocking their synthesis [53]. Recently, Diaz et al. demonstrated that *Ls. casei* probiotic strains inhibited biofilm formation and critical virulence factors of *S. aureus* and *P. aeruginosa* [23,54].

Co-aggregation between pathogens and probiotic strains, as well as auto-aggregation, is regarded as one of the critical indicators for using probiotics [58]. The co-aggregation againt *S. aureus* and *P. aeruginosa* was good and strain-dependent. A previous study found that *Enterococcus faecium* exhibited a significant co-aggregation effect with *Salmonella enterica* and *S. aureus* [32].

Finally, several studies suggest that fermented pickles containing probiotic strains may have a protective effect against colorectal cancer cells by potentially mitigating proliferative and mutagenic activity, suppressing the activity of enzymes involved in the production of mutagens, carcinogens, and tumour promoters [59]. According to the present results, previous articles have reported antimutagenic properties of lactobacilli, with this effect being strain-dependent.[5,23]. Nonetheless, additional research is imperative to validate the antimutagenic effects of fermented pickles that contain naturally occurring probiotics.

5. Conclusions

In summary, this study assessed the probiotic characteristics of 15 strains of lactic acid bacteria isolated from bell peppers, identifying them phenotypically and genotypically. The results highlight five strains that exhibit notable resistance to low pH, bile salts, and in vitro gastrointestinal conditions. These strains also display surface properties, such as auto-aggregation, hydrophobicity, coaggregation, and anti-adhesive capacity against pathogens. Furthermore, they demonstrate superior biosurfactant and antimutagenic properties compared to the reference strain, meeting safety requirements for probiotic classification.

Technological properties, including osmotic tolerance, acidifying capacity, and compatibility between these strains, suggest its potential to be used in mixed starter cultures for fermented food production. Overall, these characteristics indicate that the five strains have the potential to be promising probiotics with sufficient functional merits for application in the fermentation of bell peppers.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Growth curves of lactic acid bacteria in the absence and presence of different NaCl concentrations.

Author Contributions: : Núñez Ivana Micaela: Performed the experiments, analysed the data. Verni Maria Cecilia: Performed the experiments, analysed the data. Argañaraz Martinez Fernando Eloy: Performed the experiments, analyzed the data. Babot Jaime Daniel: Performed the experiments, analysed the data. Danilovich Mariana Elizabeth: Performed the experiments, analysed the data. Cartagena Elena: Conceived and designed the experiments, analysed the data, Wrote the paper. Alberto María Rosa: Conceived and designed the experiments, analysed the data, Wrote the paper. Mario Eduardo Arena: Conceived and designed the experiments, analysed the data, Wrote the paper.

 doi:10.20944/preprints202403.0364.v1

Funding: The authors acknowledge the financial support from the SCAIT-UNT (PIUNT 26D-702, 26D-708, and 26D-715), the Agencia Nacional de Promoción Científica y Técnica ANPCyT (PICT 2018-02071 and 02514, PICT 2021-0060, PICT-2021-I-A-00439 I), The Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET (PUE 0021).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data will be made available on request.

Acknowledgments: The authors gratefully acknowledge the financial support of the UNT, ANPCyT, and CONICET that made this work possible.

Conflicts of Interest: The authors declare no conflict of interest.

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