ORIGINAL ARTICLE

Food Microbiology, Safety and Toxicology

Screening of Five *Lactobacillus* Bacteria with Probiotic Properties from Indigenous *Klila* Cheese

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ABSTRACT

Background: The *Lactobacillus* genus is the most widespread lactic acid bacteria (LAB) species globally. These bacteria are known for their probiotic properties, which benefit human health.

Aims: This study aims to identify and screen the principal probiotic selection criteria of five *Lactobacillus* strains isolated from *Klila*, a traditionally fermented cheese product from Algeria, *in vitro*.

Materials and Methods: The main probiotic selection criteria were screened *in vitro* through biochemical and physiological tests, such as tolerance to low pH, bile salts, and phenol, their aggregation capacity, cell surface hydrophobicity, antibiotic sensitivity, and antimicrobial activity. Sequencing the 16S-rRNA gene identified the five isolates as *Lactobacillus plantarum* (LP1, LP2, LP3, and LP4) and *Lactobacillus fermentum* (LF1).

Results: The experimental results showed that all five isolates survived after exposure to low pH (2.2) for 3 hours. They also showed tolerance to bile salts ranging from 57.67 to 70.68% and 0.4% phenol, ranging from 39.22 to 61.01%. The auto-aggregation capacity varied between 31.35% and 57.38%, while co-aggregation varied respectively from 14.57% to 22.17% with *Escherichia coli*, from 13.04% to 23.62% with *Staphylococcus aureus*, and from 11.15% to 17.03% with *Candida albicans*. The hydrophobicity towards xylene ranged from 41.67 to 60.47%, and the biofilm formation ability ranged from 32.94 to 70.19%. Isolate LF1 presented the highest hydrophobicity and biofilm formation percentages, with 60.47 and 70.19%, respectively. All five isolates demonstrated significant antioxidant capacities, suggesting their potential to improve food preservation and health benefits. Exceptional antimicrobial activities were revealed against the tested food-borne pathogens, ranging from 12.6 to 45 mm. A safety profile was shown without hemolytic, gelatin liquefaction, or coagulase activity.

Conclusion: The *Lactobacillus* bacteria isolated from *Klila* presented physiological characteristics that make them potential probiotic candidates beneficial for health.

Keywords: *Lactobacillus*; Lactic Acid Bacteria; Probiotic Potential; Antimicrobial Activity; Traditional Algerian Cheese.

ARTICLE INFORMATION

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1 INTRODUCTION

Lactobacillus species possess remarkable adaptability to diverse environments and are endowed with specific enzymatic machinery and physiological mechanisms that enable them to metabolize a broad spectrum of carbohydrates and energy sources necessary for proliferation. These attributes render *Lactobacillus* the predominant genus among lactic acid bacteria (LAB) found in nature (Dempsey & Corr, 2022). Consistent with other LAB, the *Lactobacillus* genus comprises Gram-positive, short rod-shaped, non-motile, facultatively anaerobic, catalase-negative, oxidase-negative, and non-spore-forming (Ibrahim, 2016; Limsowtin *et al.*, 2002). The majority of *Lactobacillus* species can thrive in environments with elevated salt concentrations (approximately 6.5%) and tolerate diverse challenging conditions, such as high acidity and bile salts. Furthermore, their high adhesive and hydrophobic properties render the digestive tract a favorable environment for their proliferation (Menconi *et al.*, 2014).

Given their varied natural sources, encompassing a major component of the indigenous flora of fermented dairy and non-dairy products, and their capacity to colonize the digestive tract, *Lactobacillus* and LAB generally exhibit significant probiotic attributes and confer numerous health

benefits (Shi *et al.*, 2016). The International Scientific Association of Probiotics and Prebiotics (ISAPP) describes probiotics as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill *et al.*, 2014). To be classified as probiotics or functional foods, fermented products must contain a minimum concentration of one million colony-forming units per gram or milliliter (CFU/mL or CFU/g) of viable microorganisms, demonstrate the ability to survive passage through the human digestive tract, and offer verifiable health advantages to the host (Tavakoli *et al.*, 2017).

Klila is a traditional fermented cheese produced through the spontaneous fermentation of goat, sheep, or camel milk within a "*Chekoua*" (a pouch crafted from tanned goat skin). Following the separation and drainage of the whey, the cheese undergoes salting, drying, and occasional flavoring with regional seasonings. *Klila* is frequently utilized for culinary applications but can also be consumed fresh, prior to drying, establishing it as a prominent variety of traditional Algerian cheese (Benamara *et al.*, 2022; Leksir *et al.*, 2019). As a dry cheese, previous studies have yielded contradictory results regarding the diversity and richness of *Kilal*'s lactic bacterial flora. It has been considered a valuable source of LAB, particularly strains exhibiting slow growth rates and adaptation to challenging conditions of water activity (Aw) and osmotic pressure (Doukaki *et al.*, 2024; Hadef *et al.*, 2023). Conversely, it has also been considered a poor source due to its low water content (Benamara *et al.*, 2022).

The present study aimed to isolate and characterize the probiotic properties of specific *Lactobacillus* strains comprising the indigenous flora of traditionally produced *Klila* cheese. In addition, this research seeks to evaluate the relevance of *Klila* cheese as a potential source of functional and probiotic microorganisms, particularly considering its characteristics as a hard, dry, and relatively saline food, and to evaluate to what extent these attributes affect the properties of the isolated LAB.

2 Material and Methods

2.1 Isolation and Phenotypic Characterization of LAB

A total of 21 LAB isolates were obtained from three samples of *Klila*, a traditional Algerian fermented cheese sourced from the Wilaya of Mascara (western Algeria). Five isolates exhibiting superior probiotic attributes, including resistance to simulated gastric conditions, aggregation properties, antimicrobial activity, and a favorable safety profile, were selected and genetically identified. Homogenized samples (20 g) were serially diluted tenfold using 0.9% sterile physiological saline solution. Aliquots from the final dilutions were subsequently inoculated onto de Man, Rogosa, and Sharpe (MRS) agar medium (Liofilchem® s.r.l., Roseto degli Abruzzi, Italy), adjusted to pH 5.7 ± 0.1. Plates were incubated anaerobically at 37°C for 48 hours using incubation jars. The isolated colonies were consecutively purified using the streaking method on MRS agar. Subsequently, phenotypic characterization was conducted using Gram staining, catalase test, oxidase test, motility test, and identification of cell morphology. Isolates were cryopreserved at -20°C in MRS broth supplemented with 25% glycerol (Soda *et al.*, 2003).

2.2 Genotypic Identification of LAB

The different DNAs were extracted from pure, young cultures using the GF-1 Nucleic Acid Extraction Kit (Vivantis Technologies Sdn Bhd, Selangor, DE, Malaysia), following the protocol described by O'Sullivan and Klaenhammer (1993) with minor modifications. Polymerase chain reaction (PCR) amplification was conducted using a thermocycler (iCycler Bio-Rad, USA) with specific primers (27F: 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492R: 5'-CCG TCA ATT CCT TTG AGT TT-3') (Edwards *et al.*, 1989). Consensus sequences were analyzed by comparison with the GenBank database.

2.3 Assessment of the Probiotic Capabilities of LAB

Acid Tolerance

The acid tolerance of LAB strains under simulated human digestion conditions was evaluated using the method delineated by Botta *et al.* (2014). Briefly, 0.1 mL aliquots of young bacterial cultures, grown in MRS liquid medium to a concentration of 10^8 CFU.mL^{-1}, were centrifuged at 6000 rpm for 15 minutes. The resulting pellet was resuspended in MRS liquid medium adjusted to pH 2.2. Subsequently, these suspensions were incubated at 37°C for three hours (Li *et al.*, 2020). Absorbance was measured at 600 nm. Tolerance percentage was determined using the following formula:

$$Tolerance\ (\%) = \frac{(Absorbance\ of\ control\ broth - Absorbance\ of\ acidic\ broth)}{Absorbance\ of\ control\ broth} \times 100$$

Bile Salt Tolerance

Briefly, 1 mL aliquots of young bacterial isolates grown in MRS liquid medium to a concentration of 108 CFU.mL^{-1} were centrifuged at 6000 rpm for 15 minutes. The resultant pellet was suspended in 1 mL MRS liquid medium containing 0.3% (w/v) bile salts (Loba Chemie, Mumbai, India). For control purposes, 1 mL of MRS liquid medium without bile salts was added to sterile Eppendorf tubes containing 1 mL of the same bacterial pellets. All suspensions were incubated at

37°C for 4 hours (Nami *et al.*, 2019). Viability percentage was determined by measuring the absorbance at 600 nm, using the following formula:

$$Tolerance\ (\%) = \frac{(Absorbance\ in\ control\ broth - Absorbance\ in\ 0.3\%\ bile\ broth)}{Absorbance\ in\ control\ broth} \times 100$$

Auto-Aggregation and Co-Aggregation Assessments

The aggregation capability of LAB was assessed utilizing the methodology outlined by Collado *et al.* (2008). Briefly, after centrifugation at 6000 rpm for 15 minutes, overnight cultured LAB cell pellets were harvested, washed twice with Phosphate-Buffered Saline (PBS), and resuspended in the same buffer. These suspensions were vortexed, and the initial absorbance (A_i) of each cell suspension measured at 620 nm. The suspensions were then incubated at 37°C for four hours, and the final absorbance (A_f) was measured. The percentage of auto-aggregation was calculated using the following formula:

$$Autoaggregation\ (\%) = 1 - \left(\frac{A_f}{A_i}\right) \times 100$$

To determine the percentage of co-aggregation between LAB and *C. albicans* ATCC 10231, *E. coli* ATCC 25922, and *S. aureus* ATCC 25923, equal volumes of LAB isolates and pathogens were combined and incubated at 37°C for four hours without shaking. The co-aggregation percentage was determined using the aforementioned formula (Jena *et al.*, 2013).

Cell Surface Hydrophobicity

The cell surface hydrophobicity of *Lactobacillus* isolates was assessed using the method outlined by Krausova *et al.* (2019). Overnight cultures of lactic acid bacteria were centrifuged at 3000 rpm for 15 minutes at 4°C. The resulting cell pellets were collected and resuspended in PBS buffer adjusted to pH 7.0 ± 0.1. The initial optical density (OD) was measured at 600 nm. A 1.5 mL volume of xylene was combined with 1.5 mL of bacterial cell suspension, and the mixture was vigorously vortexed for 120 seconds. The mixtures were then allowed to stand at room temperature for two hours to facilitate phase separation. The absorbance of the aqueous phase was measured at 600 nm. Cell surface hydrophobicity percentage was determined using the formula previously used for auto-aggregation.

Biofilm Formation

The biofilm formation capability of the isolated bacteria was assessed using the crystal violet staining method (Shaaban *et al.*, 2020). A volume of 100 µL of 24-hour young bacterial cultures were inoculated into a 96-well microplate, each well pre-filled with 100 µL of MRS liquid medium. The microplate was incubated at 30°C for 24 hours. Following incubation, wells were rinsed thrice with PBS buffer. A volume of 100 µL of 0.1% crystal violet was added to each well and allowed to stand for 30 minutes. Subsequently, a five-fold successive rinse with PBS buffer was performed to remove excess crystal violet. Thereafter, the plate was air-dried for 30 minutes, and absorbance was measured at 640 nm using a microplate reader (Thermo Fisher Scientific, Multiskan SkyHigh, USA). Wells containing only MRS liquid medium served as a negative control. Results were expressed as percentages, calculated by determining the difference between the absorbance of the negative control and that of each inoculation well.

Phenol Tolerance (0.4% v/v)

Bacterial isolates were inoculated into MRS liquid medium tubes supplemented with 0.4% (v/v) phenol (Merck 00206, Darmstadt, Germany), following the method of Xanthopoulos *et al.* (2000). The tubes were incubated at 30°C for 24 hours. Viability was assessed using the pour plate method on MRS agar, and calculated using the following formula:

$$Viability\ (\%)\ \frac{Ni}{Nx} \times 100$$

Where Ni represents the $\log^{10}$CFU.mL^{-1} after 24 hours, and Nx represents the $\log^{10}$CFU.mL^{-1} before incubation.

Antioxidant Capacity

The total antioxidant capacity of *Lactobacillus* bacteria was assessed using the potassium permanganate ($KMnO_4$) agar method (Hanchi *et al.*, 2022; Zhou *et al.*, 2015). Petri dishes (90 mm diameter) were filled with 25 mL of 1.5% agar containing $KMnO_4$ at a concentration of 0.5 mmol/L. Following solidification, wells were drilled with a 0.6 mm cork borer. Then, 80 µL of Cell-Free Supernatant (CFS) of each bacterial isolate was inoculated into the wells. The Petri dishes were subsequently stored at 4°C in the dark. Measurements of decolorized zones were performed at time intervals of 10, 30 minutes, 1, 4, and 24 hours. Wells inoculated with MRS liquid medium served as the control. Antioxidant activity was determined by measuring the diameter difference between the discolored zones in the control sample and those in the samples treated with CFS from each isolate.

Antagonistic Activity

The antagonistic activity of *Lactobacillus* isolates against indicator strains, obtained from the Pasteur Institute of Algeria (IPA), was assessed using the agar spot method (Akman *et al.*, 2021). The indicator strains included: *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 14990, *Salmonella* spp., *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Bacillus cereus* ATCC 10987, *Enterococcus* spp., and *Candida albicans* ATCC 10231. Each bacterial isolate was inoculated as a spot on MRS agar (four spots per 90 mm Petri dish). Following incubation at 37°C for 24 hours, each spot was covered with 12 mL of 0.7% MRS Muller-Hinton agar tempered at 45°C, containing 100 µL of an overnight culture of each indicator strain. After gentle agitation, the contents of these tubes were poured onto the Petri dishes containing the *Lactobacillus* spots. Following incubation at 37°C for 24 hours, the diameters of the inhibition zones were measured.

2.4 Health and Safety Assessment

Antibiotic Sensitivity

The antibiotic susceptibility of *Lactobacillus* isolates was evaluated using the Kirby-Bauer disk diffusion method (Hudzicki, 2009). Bacterial suspensions adjusted to 0.5 McFarland turbidity were prepared from young (18–24 hours) cultures in a 0.9% sterile saline solution. Each suspension was uniformly swabbed onto Mueller-Hinton agar plates. Antibiotic disks (Liofilchem® s.r.l., Roseto degli Abruzzi, Italy) — including Penicillin (P, 10 µg), Augmentin (AUG, 30 µg), Ampicillin (AMP, 30 µg), Cefepime (FEP, 30 µg), Cefoxitin (FOX, 30 µg), Ofloxacin (OFX, 5 µg), Nalidixic acid (NA, 30 µg), Chloramphenicol (C, 30 µg), Erythromycin (E, 15 µg), and Tetracycline (TE, 30 µg) — were aseptically placed onto the inoculated Mueller-Hinton agar surfaces. Following incubation at 37°C for 24 hours, the diameters of the inhibition zones surrounding the antibiotic disks were measured. Sensitivity results were categorized as resistant (R, zone diameter ≤ 4 mm), moderately susceptible (I, zone diameter between 14 and 20 mm), or susceptible (S, zone diameter >20 mm), in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2020).

Hemolytic Activity

The hemolytic activity of *Lactobacillus* isolates was assessed using the method described by Pieniz *et al.* (2014). Young cultures of bacterial isolates were plated on blood agar that contained 7% (w/v) sheep blood. After incubation for 48 hours at 37°C, the blood agar plates were examined for β-hemolysis, α-hemolysis, and non-hemolytic (γ-hemolysis) activities.

Gelatin Liquefaction Test

Gelatinase activity in *Lactobacillus* isolates was assessed following the protocol described by Dela Cruz and Torres (2012). Glass tubes containing gelatin nutrient medium, pre-refrigerated at 4°C, were inoculated with LAB isolates. Following incubation at 25°C for 7 days, the tubes were submerged in an ice bath for 15 to 30 minutes. Tubes retaining a liquid state after chilling were considered positive for gelatinase activity. *Pseudomonas. aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 served as positive and negative controls, respectively.

Coagulase Test

Free coagulase activity in *Lactobacillus* isolates was determined according to the protocol described by De Almeida Júnior *et al.* (2015). A volume of 0.3 mL of a bacterial suspension from each isolate was transferred into a sterile tube containing 0.3 mL of rabbit plasma. Following incubation at 37°C for 6 hours, the formation of a large clot or complete coagulation was indicative of a positive result. *Staphylococcus aureus* ATCC 25923 was employed as a positive control.

2.5 Statistical Study

All experiments were conducted in triplicate. Results are expressed as mean ± standard deviation (SD). Statistical analysis and comparison of the obtained data were performed using GraphPad Prism 9.5.1.733 software, employing two-way ANOVA, one-way ANOVA, and the Tukey post-hoc test. Statistical significance was defined as a p-value less than 0.05 ($p < 0.05$).

3 Results

3.1 Phenotypic Characterization

The five *Lactobacillus* isolates were identified based on their macroscopic and microscopic attributes. The macroscopic appearance of each colony cultivated on MRS agar was examined to ascertain morphological characteristics such as shape, size, contour, color, and opacity (Table 1). Gram staining revealed all five isolates as Gram-positive and short, rod-shaped bacilli. In addition, motility was assessed on semi-solid agar (Table 1).

3.2 Genotypic Identification of LAB

Genetic analysis identified the five isolates, designated LP1, LF1, LP2, LP3, and LP4 as follows: LF1 exhibited 99.36% homology to *Lactobacillus fermentum*; furthermore, LP1, LP2, LP3, and LP4 demonstrated 99.82%, 100%, 99.83 and 90.69% homology to *Lactobacillus* plantarum, respectively (Table 2).

Table 1. Macroscopic Appearance, Microscopic Appearance, and Motility of *Lactobacillus* strains isolated from *Klila*

Isolates	Macroscopic appearance					Microscopic appearance		
	Shape	Size (mm)	Margin	Chromogenesis	Opacity	Gram-Stain	Form	Motility
LP1	Circular	01	Entire	White	Opaque	+	Short rods	Nonmotile
LF1	Circular	01	Entire	White	Opaque	+	Short rods	Nonmotile
LP2	Circular	01	Entire	White	Opaque	+	Short rods	Nonmotile
LP3	Circular	01	Entire	Yellowish white	Opaque	+	Short rods	Nonmotile
LP4	Circular	01	Entire	Yellowish white	Opaque	+	Short rods	Nonmotile

Table 2. Genotypic Identification of *Lactobacillus* Strains Isolated from *Klila*

Isolates	Molecular identification			
	Species	Strain	Similarity (%)	Accession number
LP1	*Lactobacillus plantarum*	Strain 2546	99.82	MT611578
LF1	*Lactobacillus fermentum*	Strain KLAB15	99.36	KM485578
LP2	*Lactobacillus plantarum*	Strain B_16LAB	100.00	MF405177
LP3	*Lactobacillus plantarum*	Strain B_16LAB	99.83	MF405177
LP4	*Lactobacillus plantarum*	Strain 1583	90.69	MT597488

3.3 Assessment of the probiotic capabilities of lactic acid bacteria

Acid tolerance

The evaluation of *Lactobacillus* isolates' ability to tolerate acidic conditions was carried out under a singular pH condition of 2.2. This evaluation is critical, as tolerance to stomach acid is one of the crucial criteria for the selection of probiotic strains. The results are expressed as a percentage of acid tolerance. The five *Lactobacillus* isolates exhibited varying tolerance percentages (Table 3). All bacterial isolates demonstrated significant resistance to acidity at pH 2.2 for up to 3 hours, with isolate LF1 showing over 40% survival and isolate LP2 exhibiting over 90% survival. The mean tolerance or survival percentages of bacterial isolates were compared. A statistically significant difference was observed between the means of the compared groups as determined by one-way ANOVA ($p < 0.0001$). A Tukey post-hoc test revealed that statistical significance across all pairwise comparisons of isolates.

Bile salt tolerance

The capacity of the *Lactobacillus* isolates to tolerance bile salts was expressed as a tolerance percentage. All five bacteria isolates survived exposure to a 0.3% concentration of bile salts, demonstrating survival rates ranging from approximately 57.61 to 70.68%. Isolates LP3 exhibited the highest tolerance level, with a survival percentage of 70.68%, while LP4 isolate displayed the lowest tolerance level (Table 3). Similar to acid tolerance at pH 2.2, a statistically

Table 3. Percentage of acid tolerance, bile salts tolerance, auto-aggregation, and co-aggregation of *Lactobacillus* strain isolates

Isolates	Tolerance percentage (%)		Aggregation percentage (%)			
				Co-aggregation		
	Acid pH (2.2)	Bile salts (0.3%)	Auto-aggregation	*E. coli*	*S. aureus*	*C. albicans*
LP1	80.80 ± 1.09	62.40 ± 0.85	35.00 ± 0.90	20.40 ± 0.17	23.62 ± 1.09	11.15 ± 0.59
LF1	40.29 ± 1.22	60.04 ± 1.55	57.39 ± 5.28	14.57 ± 0.56	13.04 ± 0.88	17.03 ± 0.20
LP2	91.75 ± 1.60	66.03 ± 0.87	31.36 ± 1.63	19.62 ± 0.43	16.75 ± 0.38	12.23 ± 0.90
LP3	71.38 ± 0.67	70.69 ± 1.27	41.39 ± 0.70	17.35 ± 0.05	19.50 ± 1.23	13.47 ± 1.07
LP4	55.97 ± 0.45	57.61 ± 1.74	36.64 ± 0.47	22.17 ± 1.05	13.53 ± 0.24	14.21 ± 0.22

significant difference was observed among the means of the compared groups using one-way ANOVA ($p < 0.0001$). A Tukey post hoc test revealed that only the comparisons between isolates LF1 and LP1 ($p = 0.2497$) and LF1 and LP4 ($p = 0.2301$) did not yield statistical significance.

Auto-aggregation and Co-aggregation assessments

Lactobacillus isolates demonstrated auto-aggregation potential capacity with rates ranging from 31.36% to 57.39% (Table 3). The co-aggregation test of these isolates with selected indicator strains, *Candida albicans* ATCC 10231, *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923, yielded rates ranging from 14.57 to 22.17% for *E. coli*, 13.04 to 23.62% for *S. aureus*, and 11.15 to 17.03% for *C. albicans* (Table 3). One-way ANOVA revealed significant differences in the comparisons between groups for both auto-aggregation and co-aggregation ($p < 0.0001$).

Cell surface hydrophobicity

The hydrophobicity of the cell surfaces of the five Lactobacillus isolates was investigated through their interaction with xylene, a hydrocarbon, to stimulate their adhesion to intestinal epithelium cells. The hydrophobicity percentages ranged from 41.68 to 60.47% (Figure 1). The Isolate LF1 exhibited the highest affinity for xylene, with a hydrophobicity percentage of 60.47%, while isolate LP4 displayed the lowest affinity at 41.68%. One-way ANOVA test revealed statistical significance ($p < 0.0001$). Post-hoc statistical comparisons are detailed in Figure 1 (C).

Biofilm formation

The crystal violet staining assay was employed as an indirect method to assess the capacity of isolated LAB to form biofilms (Djordjevic *et al.*, 2002). All five *Lactobacillus* isolates demonstrated biofilm formation capability with varying percentages ranging from 32.94% for isolate LP1 to 70.10% for isolate LF1 (Figure 1). One-way ANOVA test revealed statistical significance ($p < 0.0001$). Post-hoc statistical comparisons are presented in Figure 1 (A).

Resistance to 0.4% (v/v) phenol

The evaluation of phenol tolerance at concentration of 0.4% of the five bacterial isolates revealed varying levels of sensitivity (Figure 1). Isolate LP4 represented the highest viability percentage (61.01%), while isolate LP2 represented the lowest viability percentage (39.22%). One-way ANOVA test demonstrated statistical significance ($p < 0.0001$). Post-hoc statistical comparisons are referenced in Figure 1 (B).

Antioxidant capacity

The investigation of the antioxidant capacity of *Lactobacillus* isolates is significant due to their potential to produce antioxidant enzymes and organic acids, which may confer protection against free radical-induced damage, a process implicated in the pathogenesis of numerous chronic diseases (Zehiroglu & Ozturk Sarikaya, 2019). The employed methodology has recently been adapted for LAB to estimate the overall antioxidant capability of their native CFS (Atanasov *et al.*, 2023).

All five bacterial isolates exhibited antioxidant capacity during the experiment (Figure 2). Clear and distinct zones were observed following the reaction of organic antioxidant compounds with $KMnO_4$. Reduced halo zones were already visible after 10 minutes. Halo zone boundaries became distinct after 30 minutes, 1, and 4 hours. CFS from all bacterial isolates demonstrated well-expressed antioxidant capacities. Among the strains tested, the LP1 isolate exhibited the highest antioxidant capacity. A steady increase in the diameter of the halo zone was observed throughout the experiment, except for LP2 and LP4 isolates, where a stabilization of the halo zone diameter was noted between 4 and 24 hours (Figure 2). Statistical analysis (two-way ANOVA) revealed significant differences between all groups (*Lactobacillus* isolates) with $p < 0.001$.

Antagonistic activity

Evaluation of the antimicrobial activity of the five isolated *Lactobacillus* bacteria strains demonstrated significant inhibition of pathogen growth (Figure 3). *S. aureus* exhibited the highest resistance among the various bacterial isolates, while *E. coli* was the most susceptible pathogen. More or less, all five *Lactobacillus* bacteria isolates showed satisfactory antagonistic effects against all the tested indicator bacteria, ranging from 12.66 to 45 mm. *E. coli*, followed by *P. aeruginosa*, showed the highest susceptibility against the five bacterial isolates. Regarding the overall averages, the LP3 isolate demonstrated the highest antibacterial activity against Gram-positive pathogens, while the LP3 and LP4 isolates exhibited the most potent antibacterial activity against Gram-negative pathogens. For antifungal activity, the isolate LP3 consistently demonstrated the highest activity.

3.4 Health Safety Assessment

Antibiotic sensitivity

The five isolates of *Lactobacillus* bacteria were screened for antibiotic resistance and susceptibility. The isolates, therefore, showed different degrees of susceptibility to ten of the antibiotics that were examined (five classes of antibiotics). All the isolates tested showed resistance to cefepime and nalidixic acid and were susceptible to Augmentin, Amoxicillin, and Chloramphenicol. The susceptibility of the five isolates to the rest of the antibiotics varied between susceptible, moderate, and resistant (Table 4).

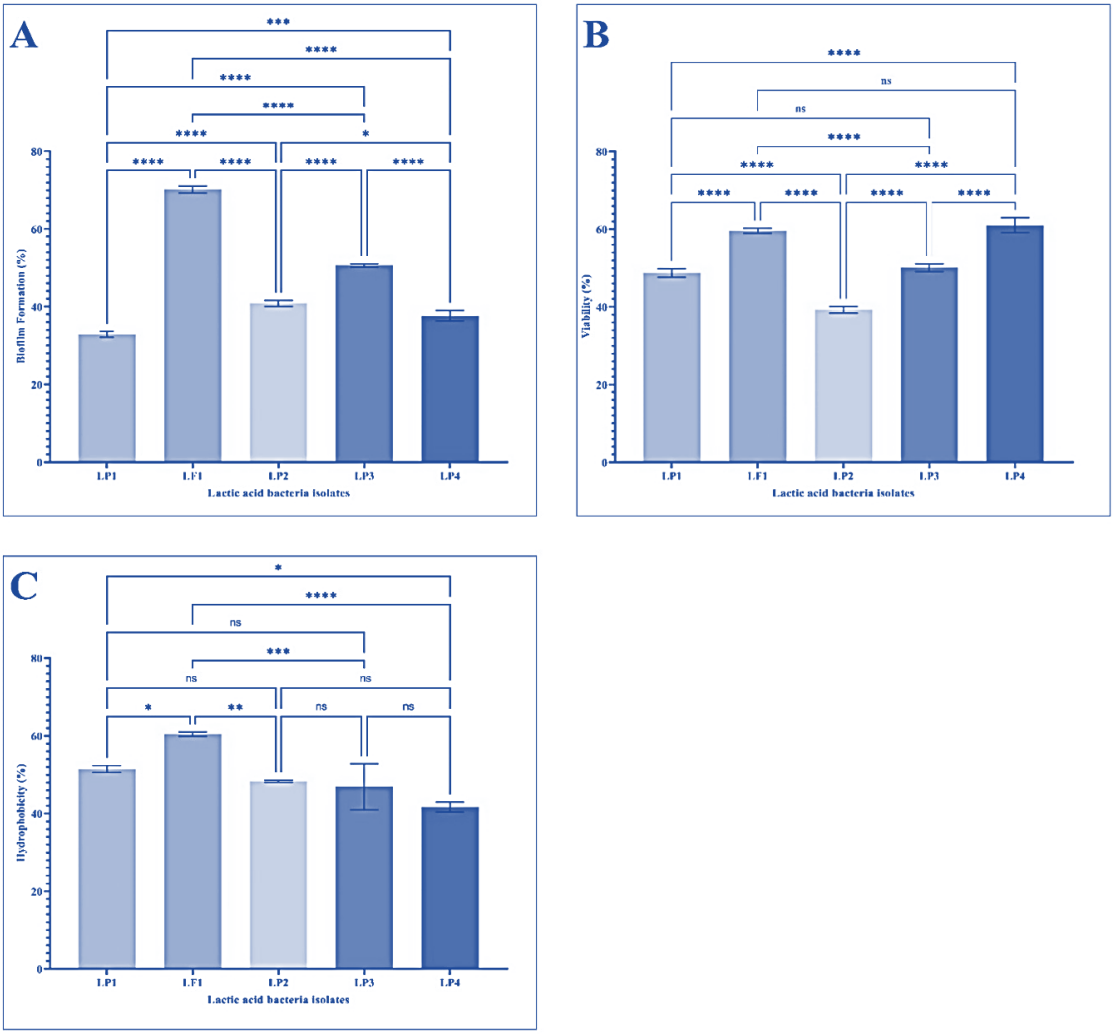

Figure 1. Biofilm Formation Percentage **(A)**, Resistance to 0.4% phenol percentage **(B)**, Cell surface hydrophobicity percentage of *Lactobacillus* strains towards xylene **(C)**. Asterisks follow GP Prism 5.04/d and later *p*-value style at a confidence level of 95%, ns: non-significant

Table 4. Antibiotic Susceptibility Profile of *Lactobacillus* Isolates, expressed as the Diameter (mm) of Inhibition Zone Based on CLSI

Isolates	ATB + inhibition zones (mm)									
	P (10µg)	AUG (30µg)	AM (10µg)	FEP (30µg)	FOX (30µg)	OFX (5µg)	NA (30µg)	C (30µg)	E (15µg)	TE (30µg)
LP1	R	S	S	R	R	R	R	S	I	R
LF1	S	S	S	R	I	I	R	S	S	S
LP2	S	S	S	R	S	R	R	S	S	S
LP3	S	S	S	R	I	R	R	S	R	I
LP4	I	S	S	R	I	R	R	S	I	S

Note: R, resistant (zone size <14 mm), I, intermediate (14 mm ≥ zone size ≤ 20 mm), S, sensitive (zone size > 20 mm) (CLSI)

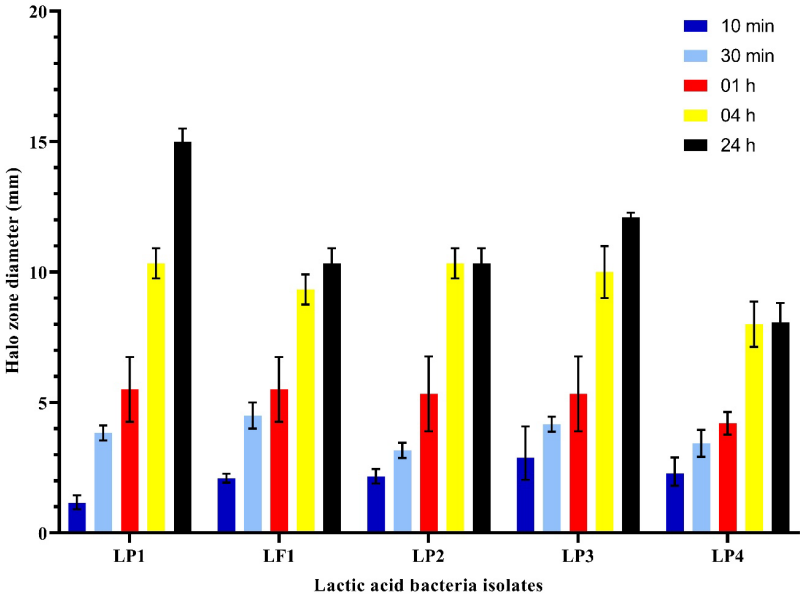

Figure 3. Total Antioxidant Capacity Represented in mm of the Zones of Decolorization of $KMnO_4$ of *Lactobacillus* strains Isolated from *Klila*

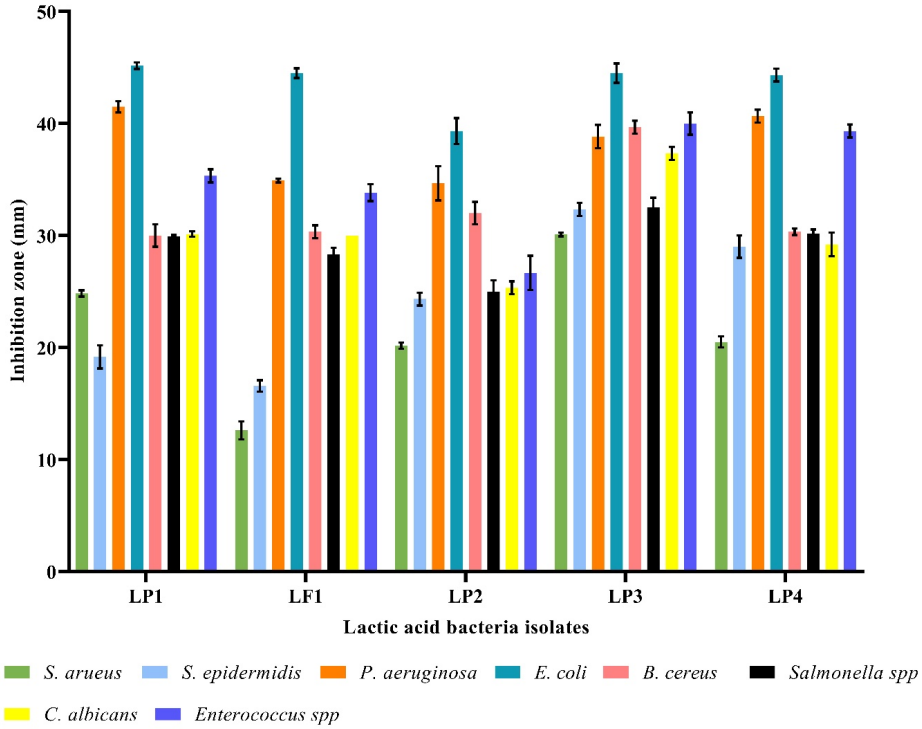

Figure 2. Antimicrobial Activity Represented in mm of the Zones of Inhibition of *Lactobacillus* Strains Isolated from *Klila* Against Selected Pathogenic Bacteria

Hemolytic activity

The hemolytic activity of five *Lactobacillus* bacterial isolates was evaluated on blood agar. The isolates consistently exhibited negative hemolysis, corresponding to a gamma (γ) hemolytic profile.

Gelatin liquefaction test

The presence of gelatinase enzyme in the five *Lactobacillus* bacteria isolates was assessed using the tube method. No gelatin liquefaction activity was revealed.

Coagulase test

All five *Lactobacillus* bacteria isolates demonstrated an absence of free coagulase enzyme.

4 Discussion

The present study investigates the probiotic potential of five *Lactobacillus* isolates, which are lactic acid bacteria, from *Klila*, a traditional Algerian fermented cheese. This investigation involved a detailed examination of physiological traits, including their capacity to endure harsh gastrointestinal conditions (such as acid and bile salts), their function as an antioxidant, and their ability to inhibit pathogenic bacteria.

Since probiotic strains are typically administered orally, it is paramount that they are able to withstand transit through the gastrointestinal tract. Therefore, they are selected based on their capacity to survive the acidic conditions of gastric environment and the presence of bile salts in the small intestine (Angmo *et al.*, 2016; Meradji *et al.*, 2023).

All five isolates demonstrated high resistance to the acidity of pH 2.2. The survival rate varied between 40.29 and 91.75%, with significant differences in their capacity to survive under acidic conditions ($p < 0.05$). Isolates LP1, LP2, LP3, and LP4 all exceeded 50% tolerance to acidity, with isolate LP2 exhibiting the highest tolerance percentage (91.75%). Only isolate LF1 displayed a tolerance below 50% (40.29%). Sengun *et al.* (2024) found that some strains of *Lactobacillus plantarum* could survive at low pH levels of 2.5, 3.0, and 4.0. Similarly, in a study by Bao *et al.* (2010), certain *L. fermentum* strains showed low pH tolerance, ranging from 80.40 to 91.80%. According to Akman *et al.* (2021), the tolerance of the isolates of LAB to low pH may be attributed to variations in the growth phase of the microorganisms. The ability to survive in acidic conditions is also related to strain specificity (Sengun *et al.*, 2024). Resistance to high gastric acidity (pH 2.2) is an essential factor in selecting probiotic strains, as these strains must pass in adequate numbers through the acid digestion process to colonize the intestines (Meradji *et al.*, 2023). Beyond their functional and probiotic interests, LAB that are highly adaptable and resistant to high acidity also present technological applications, particularly in food fermentation and preservation processes (Razmi *et al.*, 2023).

In the human digestive tract, the concentration of bile is approximately 0.3%, with a residence time of 3–4 hours (Amenu & Bacha, 2023). All five isolates demonstrated high resistance to bile salts, indicating their capacity to survive in the small intestine. Their survival rates ranged from 57.61 and 70.68% with the LP3 isolate exhibited the highest viability percentage (70.68%). This is consistent with the findings of Shehata *et al.* (2024). In another study, high resistance to bile salts was observed in some probiotic strains of LAB isolated from fermented Gilaburu and Shalgam beverages (Akman *et al.*, 2021). The investigation conducted by Jin *et al.* (2021) also underlined that some strains of *L. plantarum* can resist bile salts at 0.3% concentration. This is further confirmed by Sengun *et al.* (2024), who reported that the survival rates of most strains studied in the presence of 0.3% bile salts ranged from 79.25 to 147.84%.

Probiotic stains possess other functional characteristics, such as the ability to auto-aggregate and co-aggregate with pathogens (Ruiz-Ramírez *et al.*, 2023). The adhesion of probiotic strains to the intestinal mucosa is influenced by their capacity to auto-aggregate and create a biofilm. This adhesion is critical for colonizing the human intestine, regulating immune functions, and promoting antimicrobial action against enteric pathogens (Ruiz-Ramírez *et al.*, 2023; Paul *et al.*, 2023). According to Bujnakova and Kmet (2002), probiotic strains co-aggregate with pathogens, thereby preventing their proliferation in the gastrointestinal tract. Ingesting probiotic strains that generate aggregation-promoting substances is crucial for host defense against infection. In the present study, all *Lactobacillus* isolates demonstrated auto-aggregation rates ranging from 31.36 to 57.39%, which are comparable to those reported by Divyashree *et al.* (2024), while being significantly higher than the values documented by Shehata *et al.* (2024). This difference may be attributed to strain-specific variations or distinct experimental conditions.

For all five isolates, the percentage of co-aggregation did not exceed 23.62% for the three tested pathogenic strains: *E. coli*, *S. aureus*, and *C. albicans*. The auto-aggregation results were also comparable to those reported by Divyashree *et al.* (2024). In another study, Atanasov *et al.* (2023) reported that co-aggregation percentages between some *Lactobacillus* strains with *C. albicans* ranged from 9.83 to 27.97%. The capacity of co-aggregation provides probiotics bacteria with a competitive advantage against enteric pathogens by reducing their colonization in the gastrointestinal tract and lowering the risk of infection (Aziz *et al.*, 2019). Limited co-aggregation may

negatively impact the presence and colonization of LAB strains in the gastrointestinal tract, potentially affecting their antagonistic capabilities against pathogens and thus their probiotic potency (Leska *et al.*, 2022).

The investigation of the hydrophobicity characteristics of probiotic isolates is essential. These studies enable us to analyze how probiotic bacteria colonize and adhere to the epithelial cells of the gastrointestinal tract, hence preventing colonization by pathogens (Abushelaibi *et al.*, 2017). The hydrophobicity percentages of the five isolates ranged from 41.67 to 60.47% after 4 hours of incubation. Isolates LF1 and LP1 exhibited the highest hydrophobicity, with rates of 60.47 and 51.41%, respectively. Divyashree *et al.* (2024) reported hydrophobicity percentages ranging from 46.60 to 69.40%. In an *in vitro* study, Vijayakumar *et al.* (2015) demonstrated that the isolated *L. plantarum* strain KCC-24 exhibited significant cell surface hydrophobicity in xylene hydrocarbon (41.13%). As one of the physicochemical properties of bacterial cell surfaces, hydrophobicity directly influences adhesive capabilities such as auto-aggregation or coaggregation, and thus the ability of bacteria to adhere to various biotic and abiotic surfaces (Guan *et al.*, 2020).

Among the critical mechanisms by which LAB exert their beneficial characteristics upon adhesion to mucosal tissues is their capacity to form biofilms. This mechanism also reinforces the antagonistic capacities of LAB against various pathogens that colonize the digestive tract (Mgomi *et al.*, 2023). The results of the biofilm formation test reveal significant differences between the different *Lactobacillus* isolates. Biofilm formation of the five isolates varied between 32.94 and 70.19%, where isolate LF1 (*L. fermentum*) displayed the highest biofilm forming capacity with a value of 70.19%. In a study by Atanasov *et al.* (2023), biofilm formation of the twelve strains of LAB tested varied between 20 and 96%. Gómez *et al.* (2016) reported significant differences between the different strains of LAB they studied; following 48 hours of incubation, strong biofilm formations were revealed. The ability to form biofilm may depend on the strain of LAB. It is also possible that it is influenced by environmental elements (Atanasov *et al.*, 2023). The ability of the five isolates to form biofilm was consistent with the rest of the adhesive properties tested, such as auto, co-aggregation, and hydrophobicity. In addition to their probiotic properties, the biofilm-forming ability of *Lactobacillus* isolates contributes to their protection against environmental factors such as antibiotics, food additives, phenolic conditions, and antagonistic activities (Balcázar *et al.*, 2015). Conversely, this feature poses a major challenge in the food industry, where the elimination of bacterial biofilms that can colonize industrial machinery is extremely difficult and threatens food safety and public health (Elafify *et al.*, 2024).

The viability of gut microbiota is influenced by phenolic conditions, which arise from the bacterial deamination of amino acids derived from dietary proteins (Huligere *et al.*, 2023). In accordance with the results of Divyashree *et al.* (2024), the five isolates exhibited resistance to 0.4% phenol with survival percentages ranging from 39.22 to 61.01%. The isolates LP4 and LF1 demonstrated the highest survival, with 61.01 and 59.58%, respectively. The study by Amenu and Bacha (2023) recorded survival percentages under 0.4% phenolic conditions ranging from 48.93 to 98.67%. Nandha and Shukla (2023) reported that a strain of *Lactobacillus lactis subsp* demonstrated resilience, with cell vitality increasing from 7.98 to 8.82 Log CFU.mL^{-1} when exposed to 0.4% phenol.

The metabolism of probiotic LAB produces several organic acids, such as acetic and lactic acid. Consequently, the CFS exhibits a low pH, increasing its antioxidant capacity. This observation is consistent with the findings of Atanasov *et al.* (2023) who noted that a lower CSF pH correlated with a greater antioxidant capacity. All *Lactobacillus* isolates exhibited a total antioxidant activity (Figure 2). The redox reaction between the CFS and $KMnO_4$ proceeds quantitatively, indicating that the size of each discolored zone is proportional to the quantity of antioxidants present. Hanchi *et al.* (2022) determined that the recently adapted $KMnO_4$ agar method for LAB effectively and reproducibly measures antioxidant capability. This evaluation showed that the procedure is linear and can be completed within 30 minutes to 4 hours (Hanchi *et al.*, 2022). The $KMnO_4$ agar method serves as a preliminary screening assay used to assess antioxidant capacity (Atanasov *et al.*, 2023).

LAB and probiotic microorganisms produce diverse metabolites, including organic acids, bacteriocins, diacetyl, enzymes, and hydrogen peroxide, which possess antibacterial properties against various foodborne pathogens (Lee *et al.*, 2021). All *Lactobacillus* isolates demonstrated significant antagonistic activities against different foodborne pathogens. *E. coli* was the most sensitive pathogen, with an inhibition zone of up to 45 mm, while *S. aureus* was the least sensitive pathogen. The antagonistic potential of *Lactobacillus* isolates against *E. coli* was higher than that of Fayemi *et al.* (2023). In a study conducted by Divyashree *et al.* (2024), the five *Lactobacillus* strains they studied exhibited antagonistic activity greater than 90% inhibition for some pathogens, such as *E. coli*, *S. aureus*, and *P. aeruginosa*. The significant antagonistic activity evidenced by large zones of inhibition, may be attributed not only to strain specificity but also to the nature of the confrontation assay employed. The substantial difference in the results of antimicrobial activity examinations in previous studies is attributed to the nature and objectives of the experimental

techniques, for instance, evaluating only the efficacy of bacteriocins or the total capacity of total metabolites to inhibit the growth of pathogens.

The excessive and irrational utilization of antibiotics to treat bacterial infections has made pathogen resistance a significant global challenge. Recently, attention has focused on the intrinsic or acquired antibiotic resistance capabilities of lactic acid bacteria (Yang & Yu, 2019). Since LAB can evolve genes against antibiotics, this ability has become an essential safety criterion in the selection of probiotic strains, according to the "Qualified Presumption of Safety" (QPS) concept developed by the European Food Safety Authority (EFSA) (Basbülbül *et al.*, 2015; Clementi & Aquilanti, 2011). The current study revealed that the five *Lactobacillus* isolates exhibited resistance to Ofloxacin and Nalidixic acid and more susceptible to Penicillin, Augmentin, Amoxicillin, and Chloramphenicol. Meanwhile, susceptibility was variable for the remaining antibiotics, as summarized in Table 4. In a study by Amenu and Bacha (2023), 11 isolates were susceptible to Tetracycline, while 9 out of 11 were susceptible to Ampicillin. Moreover, the study by Choi *et al.* (2018) reported that all 4 isolates studied were susceptible to Chloramphenicol. Meradji *et al.* (2023) reported that 14 LAB isolates were susceptible to Ampicillin and Chloramphenicol, while 13 out of 14 isolates were susceptible to Erythromycin. The same authors also noticed that all 14 isolates exhibited resistance to nalidixic acid. Susceptibility to other antibiotics, such as penicillin, was variable.

The absence of hemolytic and gelatinase activity is considered essential safety prerequisites for evaluating potential probiotic strains as non-pathogenic (Divyashree *et al.*, 2024). Gelatinase is considered a virulence factor due to its ability to hydrolyze collagen, potentially causing an inflammatory response (Leonardo & Pennypacker, 2009). The present study revealed that none of the five *Lactobacillus* isolates exhibited any hemolytic activity, gelatin liquefaction, or coagulase activity, identical to previous studies (Amenu & Bacha, 2023; Azevedo *et al.*, 2024; Nandha & Shukla, 2023; Sengun *et al.*, 2024). These findings indicate their suitability as safe probiotic candidates.

5 Conclusions

The current study involved the isolation of five strains of *Lactobacillus* bacteria from *Klila*, an Algerian fermented cheese product. Four isolates were identified as *L. plantarum*, while one was identified as *L. fermentum*. The isolates, in general, exhibited promising antioxidant capacity, aggregation properties, and significant antimicrobial activity against pathogens. These findings suggest that the isolates are promising probiotic candidates, with potential applications as natural preservatives or fermentation starters in the food industry. However, further experimental research is required to evaluate their probiotic potential *in vivo*, confirm their safety for human consumption by identifying the genes associated with virulence factors, and to exploring their potential use as therapeutic agents.

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