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Selection of Probiotic Isolated from Marine Species

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Abstract

The objective of this study is to investigate the probiotic potential of marine lactic acid bacteria (LAB) by isolating them from a range of fish samples, identifying the different strains, and analysing their probiotic characteristics. Using 16S rDNA sequencing, three LAB strains were identified and evaluated for their probiotic potential, including their enzymatic activities (such as hemolytic and DNase activities, and API ZYM profile) and their resilience to gastrointestinal conditions. The study also examined their antimicrobial properties, adhesion abilities (including biofilm formation capacity), auto-aggregation, co-aggregation with pathogenic bacteria, and surface hydrophobicity. Their antibiotic susceptibility was also evaluated.
The LAB strains; Saf1 (*Lacticaseibacillus paracasei*), Saf2 (*Pediococcus pentosaceus*), and Saf3 (*Pediococcus acidilactici*) demonstrated significant probiotic potential, tolerating harsh gastric and intestinal conditions (low pH, pepsin, and trypsin resistance) without exhibiting detectable DNase or hemolytic activity. Furthermore, all strains displayed strong auto and co aggregation capacities, and high hydrophobicity. The tested isolates effectively inhibited various fish pathogens isolated from seabass and seabream, including *Vibrio alginolyticus*, *Vibrio vulnificus, Vibrio parahaemolyticus*, *Aeromonas salmonicida and Aeromonas hydrophila*. In addition, all isolates were sensitive to veterinary antibiotics and produced phosphatase enzymes, which support growth, boost immunity, and ensure proper mineral absorption, making probiotics an attractive alternative to traditional antibiotics in aquaculture systems. Overall, the findings of this current investigation indicate that *Lacticaseibacillus paracasei* (Saf1) is the most promising probiotic strain, which could be exploited for functional use in aquaculture.

Introduction

For several countries, achieving food self-sufficiency is a major factor in food security. The continuous increase as well as the diversification of the food needs of individuals have forced the public authorities to develop the national natural food resources, both terrestrial and marine (Iheanacho et al., 2025). Aquaculture has long been recognized as a vital sector for global food security, offering a sustainable source of high-quality protein and supporting economic development in coastal and rural regions (Gadhiya et al., 2025).

In recent decades, aquaculture has experienced remarkable expansion, establishing itself as one of the most rapidly developing sectors in animal production.

Marine aquaculture, in particular, is largely oriented toward high-market-value fish species. In the Mediterranean region, production is dominated by the European seabass (*Dicentrarchus labrax*) and the gilthead seabream (*Sparus aurata*) (FAO, 2024).

However, the emergence of infectious diseases, particularly in intensive farming conditions, poses a significant challenge to the expansion of aquaculture. Infectious diseases cause the most significant losses in aquaculture and can lead to severe economic and social consequences. Bacterial infections, in particular, represent the most prevalent and impactful diseases affecting farmed aquatic organisms (Muniesa et al., 2020; Sanchéz et al., 2022).

Given the increased risks of mortality and infection transmission, it is vital to implement effective disinfection and antimicrobial strategies to control and reduce diseases and pathology in aquaculture. Overuse of antibiotics can promote the development of antibiotic-resistant bacteria, lead to the accumulation of antibiotic residues in fish, disrupt beneficial gut microbes, and alter the microbiota (impacting non-target organisms) within the aquatic ecosystem (Monzón-Atienza et al., 2021; Parashuramappa, 2024). These issues pose significant threats to both environmental and human health (FAO/WHO, 2001; Nomoto, 2005; WHO, 2006).

In recent years, the application of probiotics in aquaculture has attracted significant attention as an alternative to antibiotics. Probiotics, which are live microorganisms that benefit the host, have been increasingly used to enhance the health and disease resistance of farmed aquatic species. This global shift toward reducing antibiotic use, especially in aquaculture, has been intensified and motivated by the widespread use of antibiotics in this sector.

The routine use of these drugs in fish farming has accelerated the emergence of resistant bacterial strains, which pose serious risks to public health as they spread through water systems and potentially transfer resistance genes to human pathogens (Gomez-Gil et al., 2000; Wang, 2007; Ringo et al., 2005). Probiotics have demonstrated benefits in improving fish gut health, boosting immune function, and supporting enhanced growth.

For instance, lactic acid bacteria (LAB) are commonly used as probiotics in aquaculture because they can inhibit pathogenic bacteria by producing bacteriocins and organic acids, thereby reducing the need for antibiotics (Medina et al., 2020, Arun et al., 2018).

Probiotics, typically composed of beneficial bacterial strains such as *bacillus* function by outcompeting pathogenic bacteria in the gastrointestinal tract of fish and shellfish (Felix et al., 2019). They achieve this by creating antimicrobial substances that inhibit the growth of dangerous germs, such as bacteriocins and organic acids (Felix et al., 2019; Ringo, 2020). Moreover, probiotics have been shown to enhance the immune responses of fish, improving their resistance to diseases (Nayak, 2010). Lactic acid bacteria (LAB) are particularly notable for their fermentative ability and nutritional benefits, exhibiting good antimicrobial activity towards a wide range of pathogenic microorganisms (Chauhan and Singh, 2018; Ringo et al., 2020; Balcazar et al., 2006; Maitreya et al., 2024).

Despite these promising attributes, there remains a research gap in identifying and characterizing LAB strains derived from marine environments, particularly regarding their potential dual application in aquaculture and the human food sector (Hai, 2015; Newaj-Fyzul & Austin, 2014). Furthermore, comprehensive in vivo studies are crucial to validate their efficacy and safety, thereby confirming their potential as functional probiotics for both fish health management and human nutrition (Kesarcodi-Watson et al., 2008; Hoseinifar et al., 2018; Vázquez-Euan et al*.,* 2022; Almeida et al., 2025).

Given these valuable attributes, this study focused on isolating lactic acid bacteria (LAB) from various marine species samples, identifying these LAB strains, and exploring their probiotic potential as a preliminary step for their application in the food industry and/or the aquaculture sector.

Material and Methods

Sample Collection

Twenty samples were collected, including fish, algae, and shrimp. The algae samples were obtained from the Tunisian coast, while the fish and shrimp samples were sourced from either Tunisian aquaculture farms or local markets (Table 1).

These samples were stored on ice for 2 hours until they reached the laboratory. Fish specimens weighing 100-200 g were sacrificed in ice water, after which their intestines were excised, and the intestinal contents were extracted through dissection. For each sample, 5 g was added to 10 mL of MRS (Man, Rogosa, and Sharpe) broth (Biomérieux, Marcy-L'Etoile, France) and incubated anaerobically at 37°C for 48 hours. After this period, 100 µL of each sample was cultured on MRS agar (Biomérieux, Marcy-L'Etoile, France) and incubated at 37°C for 24 hours, after which clear colonies were selected for analysis.

Only Gram-positive, catalase-negative cells were selected for additional research after Gram staining, catalase activity, and cell morphology evaluations were completed.

Twenty LAB strains were initially separated from the samples. Because of their strong resistance to low pH, gastric juice, and digestive enzymes (pepsin and trypsin), three of these strains were recognized as possible probiotics. These selected strains were preserved in MRS broth with 20% glycerol at -20°C for future analysis.

Molecular Identification and DNA Sequencing

DNA Extraction and PCR Amplification

Following the manufacturer's instructions, the Wizard Genomic Purification Kit (Promega, Lyon, France) was used to extract the genomic DNA from three isolated strains. Two universal primers, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), were used to amplify a 16S rDNA gene fragment using the purified DNA as a template (Relman, 1993).

Polymerase chain reaction (PCR) was carried out in a final volume of 50 µL containing 5 µL of 10× PCR buffer (Mg-free), 5 µL of dNTP mix (2 mM), 1.5 µL of $MgCl_2$ (10×), 1 µL of each primer (30 $pM\mu L^{-1}$), 34 µL of sterile deionized water, 0.5 µL of Taq DNA polymerase (Thermo Fisher Scientific, Wilmington, USA), and 2 µL of template DNA (50–100 ng). The amplification profile consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 1 min, and extension at 72 °C for 2.5 min. A final extension step was performed at 72 °C for 10 min before cooling to 4 °C.

DNA Sequencing Analysis

Amplifications were performed in a Veriti thermal cycler (Applied Biosystems, Foster City, CA, USA). At Ran Bio Links Sarl (Tunis, Tunisia), the resultant amplicons were purified using the ATPTM Gel PCR Fragment Purification Kit (ATP Biotech Inc., Taipei, Taiwan) and sequenced using the 27F primer. BLAST (Basic Local Alignment Search Tool) was used to compare the acquired sequences to the GenBank database at the National Center for Biotechnology Information (NCBI) and the sequences were submitted to the GenBank 16S rRNA database under accession numbers. Bacteria with 99–100% sequence similarity in GenBank were identified as the same species, while 97–99% similarity indicated the same genus.

A phylogenetic tree was constructed using the Maximum Likelihood (ML) method implemented in the online software IQ-TREE (Trifinopoulos et al., 2016), applying the best-fit evolutionary model TIM2+F+I+G4. Complementary distance-based phylogenetic analyses were performed in MEGA version 6 (Tamura et al., 2013) using the Neighbor-Joining method (Saitou and Nei, 1987), with statistical support evaluated through 1000 bootstrap replicates. Evolutionary distances, expressed as the number of base substitutions per site between sequences, were estimated with the Kimura 2-parameter model (Kimura, 1980), incorporating rate variation among sites modelled with a gamma distribution (shape parameter = 1). The analysis involved 22 nucleotide sequences, with all positions containing gaps or missing data removed, resulting in a final dataset of 870 positions.

Safety Aspects

Hemolytic Activity

The hemolysis test is used to determine whether the selected bacteria can digest host hemoglobins. Hemolysis was assessed on TSA agar supplemented with 5% fresh sheep blood. The sheep blood used was sterile and free of bacterial contamination.

After incubating for 24 to 48 hours at 37°C, three types of hemolysis can be observed based on the halo around the colony: complete hemolysis, classified as β-type; partial hemolysis, classified as α-type and no hemolysis, indicating a γ-type strain (Dbeibia et al., 2023).

A clear zone on blood agar plates was interpreted as a positive result.

Staphylococcus aureus ATCC 25923 served as the positive control.

DNase Activity

The DNase assay was used to determine the presence of nuclease activity (nucleic acid degradation) in bacteria. For this test, the medium DNase agar (Bio Rad, Hercules, CA, USA) was used and the bacteria were streaked as a single horizontal line on a Petri plate and incubated for 24 hours at 37°C. If the bacteria exhibit positive DNase activity, clear zones (around the colonies) will be observed. If there was no DNase activity, no clear zones will be present.

Enzymatic Activity

The investigation of enzymatic activity in the selected strains of lactic acid bacteria was carried out using Api ZYM strips (ref 25200, Biomérieux, Marcy-

Table 1. List of samples and their origin

Nature	Species	Origin
Algae	*Ulva lactuca*	Monastir Coastal
Wild fish	*Sardina pilchardus*	Monastir Coastal
	Pagellu serythrunus	Monastir Coastal
	Mullus surmuletus	Monastir Coastal
	Diplodus annularis	Monastir Coastal
Aquaculture fish	*Dicentrarchus labrax*	Offshore Fish farms
	Sparus aurata	Offshore Fish farms
	Oreochromis niloticus	Land -based fish farms
Aquaculture shrimp	*Penaeus vannamei*	Land -based fish farms

L'Etoile, France), according to the study described by Papamaloni et al. (2002).

In each well, 65 µL of the bacterial suspension at 0.5 McFarland to be evaluated was added. The strips were incubated at 37°C in the dark for 4 hours. A drop of ZYM A (ref 70,494, Biomérieux, Marcy-L'Etoile, France) and ZYM B (ref 70,493, Biomérieux, Marcy-L'Etoile, France) reagents was then included. After 10 minutes of incubation under white light, a colorimetric reaction was developed (purple, orange, blue or brown coloration).

Based on the intensity of the colour reaction, results are expressed on a scale from 0 to 5, corresponding to a semi-quantitative measure of the amount of substrate hydrolyzed: (0): no enzymatic activity, (1): 5 nmol, (2): 10 nmol, (3): 20 nmol, (4): 30 nmol, and (5): 40 nmol.

Antibiotic Susceptibility

Antibiotic susceptibility was assessed on Mueller-Hinton plates (Biokar, Beauvais, France) using the agar diffusion disc method according to European Food Safety Authority (EFSA, 2018). Antimicrobial discs were obtained from BioRad, and the following antimicrobial agents were tested: Oxytetracycline (OT), Tetracycline (T), Ciprofloxacin (CIP), Chloramphenicol (C), Ampicillin (AMP), Enrofloxacin (ENF), Amoxicillin-clavulanic acid (AUG), Trimethoprim (TM), Neomycin (NEO), Florfenicol (FFC). A Pure culture was selected and subcultured in liquid MRS medium, followed by incubation for 18 hours. The cell density of overnight bacterial suspensions was estimated to be around 10^8 CFU/mL, and then spread onto MRS agar using a swab. The plates were allowed to dry for 15 minutes before antibiotic discs were applied using sterile forceps.

After being incubated at 37°C for 24 hours, the inhibition zones surrounding each disc were measured to assess bacterial growth suppression. Based on these zones, each bacterial strain was classified as 'susceptible,' 'intermediate,' or 'resistant' in accordance with French national guidelines (CASFM, 2023).

Characteristics of Strains Associated with Probiotic Activity

Assay of Low pH

The acid tolerance of the three isolated bacteria was assessed following the method outlined by Dbeibia et al. (2023).

Each strain was inoculated into MRS broths (10%, w:v) adjusted to pH levels of 2, 3, and 4 from bacterial seed cultures. After three hours of incubation at 37°C, the absorbance at 600 nm was used to measure the growth of the bacteria. The control was an unmodified PBS.

The following formula was used to get the survival rates:

Growth capacity= (OD 600 control group/OD 600 experimental group) x 100 (1)

Assay of NaCl Resistance

The salt stress tolerance of the isolated strains was evaluated according to the method outlined by Badis et al. (2004). Fresh cultures of each isolate were introduced into MRS broth medium with progressively higher NaCl concentrations (4%, 8%, and 15%). The cultures were incubated at 37°C for 48 to 72 hours. For comparison, a control was set up for each strain in MRS broth medium without added salt. Subsequently, bacterial growth was measured spectrophotometrically using the aforementioned equation (Equation 1).

Survival Under a Simulated Fish GI Tract Condition

The isolated bacteria's tolerance to pepsin and trypsin was assessed using the methodology previously detailed by Dbeibia et al. (2023). Fresh bacterial cultures were centrifuged at 10,000 g for 5 minutes at 4°C to collect the cells. The cell pellets were washed twice with phosphate-buffered saline (PBS on pH 7.4 was composed with 10 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl, 1.76 mM KH_2PO_4) and then re-suspended in either an acidic PBS solution (pH 2 or 3) with pepsin (3 mg/mL) or an alkaline PBS solution (pH 8) with trypsin (1 mg/mL).

Plate counts on MRS agar medium were used to measure the density of LAB cells at 0 hours after inoculation (in all cases), 3 and 4 hours after incubation at 37°C for pepsin and trypsin, respectively. The developed colonies were counted following an incubation time of 48 hours at 37°C. To express survival rates, the following formula was used:

Survival rate %= (Number of cells (logCFU/ml) after incubation/Number of cells (logCFU/ml) after inoculation) x 100 (2).

Antibacterial Activity

The antibacterial activity of probiotic bacteria isolated from aquaculture species was evaluated using the well diffusion agar method. The test was conducted against five marine pathogenic strains: *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio vulnificus*, *Aeromonas hydrophila*, and *Photobacterium damselae*, all isolated from infected farmed marine fish, seabass (*Dicentrarchus labrax*) and seabream (*Sparus aurata*), from coastal areas of Monastir, Tunisia. To further assess their antimicrobial potential, the probiotic isolates were also tested against reference strains of marine pathogens, including *Aeromonas hydrophila* ATCC 7966, *V. alginolyticus* ATCC 17749, and *V. parahaemolyticus* ATCC 17802.

Pathogenic strains were cultured in Mueller–Hinton (MH) broth (Biokar, Beauvais, France) with 10% NaCl at 37°C for 24 hours, and suspensions were

adjusted to a 0.5 McFarland standard turbidity. A 100 µL aliquot of each prepared suspension was spread onto MH agar plates with 10% NaCl. Sterile wells, 5 mm in diameter, were created in the MH agar plates inoculated with the pathogenic indicator strain. Each well was then filled with 100 µL of the lactic acid bacteria culture being tested. Plates were subsequently incubated overnight at 37°C. After incubation, the diameter of the inhibition zones round the wells was measured. Each test was performed in triplicate. The antibacterial test of each isolate was assessed using the spot-on-lawn method (Fleming et al., 1975).

Biofilm Formation and Adhesion

The adhesion protein "Slime" production capacity of the LAB is evaluated on the agar medium of Congo Red (CR) according to the method described by Badi et al. (2020). After sterilizing the MRS agar, 100 microliters of overnight liquid culture were streaked on it with CR (0.01%, w/v) added. Following a 48-hour incubation period at 37°C, plates were analyzed, and the bright red colonies were identified as CR-bound cells.

To evaluate the biofilm-forming potential of the three isolated strains, the crystal violet assay was conducted following the method outlined by Taheur et al. (2016). Strains were incubated in MRS broth with 2% glucose at 37°C for 24 hours in 96-well plates, with a sterile broth as a negative control. After incubation, non-adherent cells were removed by rinsing with PBS. The attached bacteria were fixed with 95% ethanol for 30 minutes, then stained with 1% crystal violet for 5 minutes.

Excess dye was removed, and the wells were rinsed three times with PBS, then drained and air-dried. The optical density (OD) at 570 nm was measured for each well using an ELISA reader. Based on the OD570 values, biofilm formation by the strains was classified into three categories: “strong biofilm formers” (OD570>1), “weak biofilm formers” (0.1<OD570<1), and “non-biofilm formers” (OD570<0.1) (Leveau et al., 1991).

Cell Surface Hydrophobicity Assay

This test was performed by measuring the cellular affinity for organic solvents. Three organic solvents were used: chloroform, n-hexadecane and ethyl acetate. The degree of hydrophobicity of isolated LAB was determined by estimating cellular adhesion to hydrocarbons according to Dbeibia et al. (2023). Bacterial strains (MRS, 24 h, 37°C) were spuned in a centrifuge at 6000 rpm for 5 minutes. Then, the supernatant was discarded and the pellets were collected, washed twice with Ringer’s buffer (0.1 g/L $CaCl_2$, 60 g/L NaCl, 0.0075 g/L KCl and 0.1 g/L $NaHCO_3$), and re-suspended in the same solution.

The optical density was measured at 600 nm (absorbance i). Afterwards, equal volumes of each bacterial suspension were transferred and mixed to 1.5 mL of organic solvent: chloroform, ethyl acetate and n-hexadecane and vortexed for 1.5 minutes. After 30 minutes of incubation at room temperature, 1 mL of the water-based layer was taken, and the absorbance was measured again (absorbance f). The percentage of hydrophobicity was calculated using the following formula:

$$\text{Hydrophobicity (\%)} = [(Ai - Af) / Af] \times 100 \quad (3)$$

Using (Ai) and (Af) as the initial and final absorbances, respectively.

Auto and Co-aggregation Capacities

With minor adjustments, the auto-aggregation ability of the isolated strains was assessed in accordance with Dbeibia et al. (2023).

The three isolated strains were cultured in MRS broth at 37°C for 18 hours. Afterward, they were centrifuged at 5000 rpm for 15 minutes, washed twice, and resuspended in phosphate-buffered saline (PBS, pH 7.4) to reach an absorbance of 0.5±0.005, corresponding to approximately 10^9 CFU/mL. A 0.1 mL aliquot of this suspension was mixed with 3.9 mL of PBS, and the optical density at 600 nm (OD_{600}) was measured immediately. The mixture was then incubated at 37 °C for 5 hours, after which the OD_{600} was measured again. The percentage of auto-aggregation was calculated using the following equation:

$$\text{The percentage of auto-aggregation} = [1-(\text{Absorbance at 0 h} / \text{Absorbance after 5 h})] \times 100$$

The co-aggregation test consists of determining the ability of bacteria from heterogeneous groups to attach to each other via specific molecules.

The lactic acid bacteria strains were tested against eight pathogenic strains: *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Photobacterium damselae*, *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Vibrio alginolyticus* ATCC 17749, *Vibrio parahaemolyticus* ATCC 17802, and *Aeromonas hydrophila* ATCC 7966 was determined by following the protocol of Dbeibia et al. (2023).

Bacterial suspensions for co-aggregation were prepared similarly to those for auto-aggregation, by mixing equal volumes (2 mL) of suspensions from different strains, including three probiotics and common aquaculture bacteria. The mixed suspensions were vortexed for 10 seconds to ensure proper mixing. After a 5-hour incubation at room temperature, samples were collected to calculate co-aggregation percentages according to the following equation:

$$\text{Co-aggregation (\%)} = [(A\ path + Apro) - 2 \times (A\ pro.path) / (A\ path + A\ pro)] \times 100 \quad (4)$$

Where, Apro: absorbance of the probiotic bacteria alone, Apath: absorbance of the pathogen bacteria alone, Apro.path: absorbance of the probiotic and pathogen mixture after 5 hours of incubation.

Statistical Analysis

In this study, results were presented as the mean±standard deviation (SD) and analysed using a one-way ANOVA. For each experiment, three replicates (n=3) were performed per strain tested. Significant differences between isolated strains were then evaluated through Tukey's test. A p-value of less than 0.05 was considered statistically significant, and analyses were conducted using GraphPad Prism software (version 7.04 for Windows, GraphPad Software, CA, USA).

Results

Molecular Identification of Lactic Acid Bacteria (LAB)

From the twenty samples collected, three LAB strains with significant antimicrobial activity were isolated from the gut of marine fish and shrimp, while those obtained from algae showed no probiotic potential. The selected strains were identified as Saf1 from seabream (*Sparus aurata*), Saf2 from shrimp (*Penaeus vannamei*), and Saf3 from seabass (*Dicentrarchus labrax*). Genomic DNA was extracted from each isolate, and a portion of the 16S rRNA gene was amplified and subsequently sequenced. PCR amplification of the 16S rRNA gene from the three strains produced clear single bands of approximately 1500 bp on agarose gel, consistent with the expected size of the 16S rRNA gene fragment. This confirms the successful amplification of the target gene for all isolates presented in Figure 1.

Molecular identification based on 16S rRNA gene sequencing revealed that Saf1 shares 100% sequence identity with *Lacticaseibacillus paracasei*, Saf2 with *Pediococcus pentosaceus*, and Saf3 shows 99.51% identity with *Pediococcus acidilactici*. The 16S rRNA gene sequences of Saf1, Saf2, and Saf3 have been submitted to GenBank at the NCBI under the accession numbers PP196113, PP196116, and PP196117, respectively (Table2).

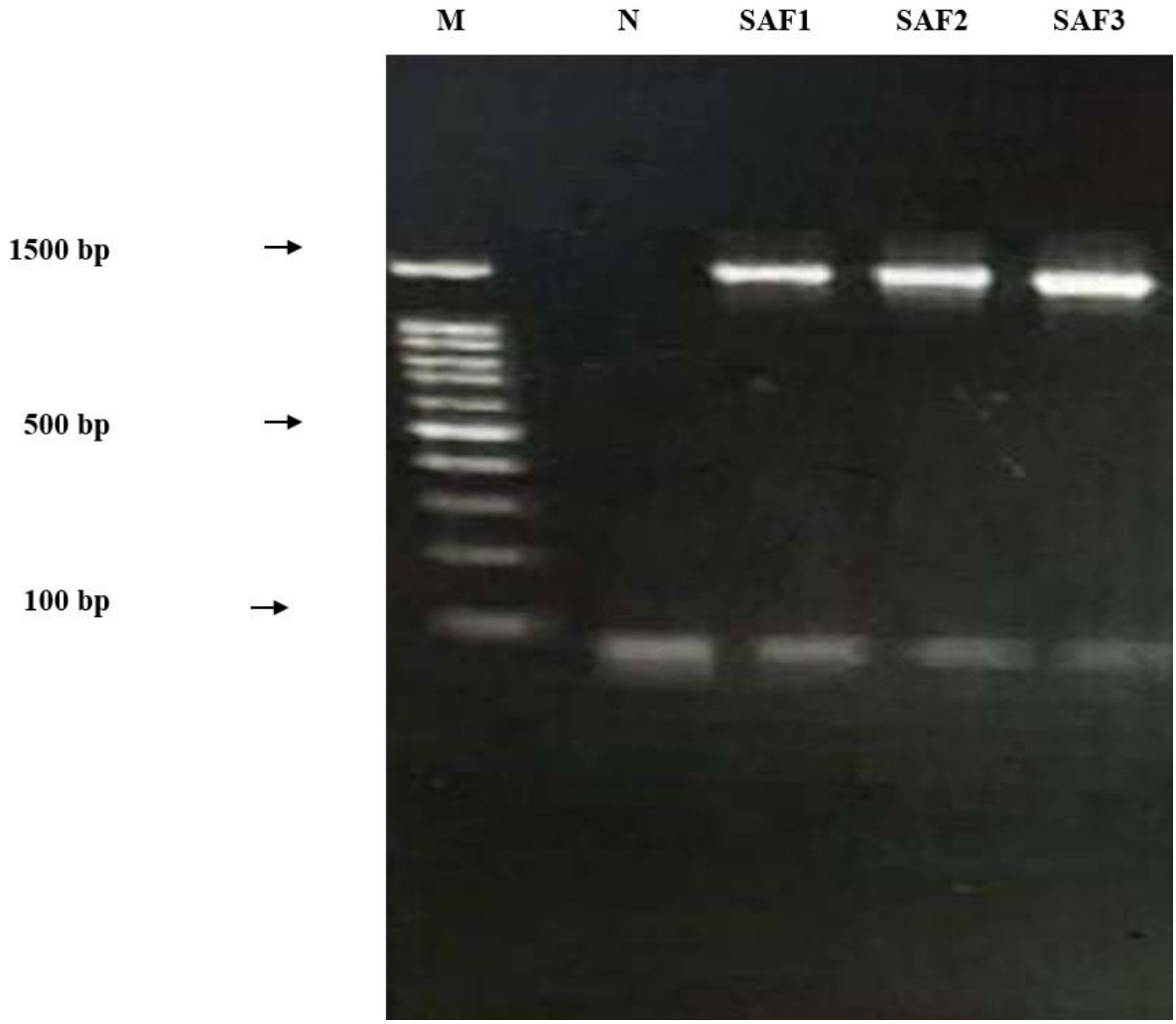

Figure 1. Migration on agarose gel (1%) of PCR products after DNA amplification of Lactic Acid Bacteria LAB isolates, Lanes: M (Molecular marker)–N (Negative control)–SAF1 (*L. paracasei*)–SAF2 (*P. pentosaceus*)–SAF3 (*P. acidilactici*).

The presence of single, distinct bands confirmed the successful amplification of the target gene; however, species-level identification required sequencing followed by phylogenetic analysis. The optimal phylogenetic tree obtained is shown in Figure 2. The tree, rooted with sequences from *Enterococcus faecium*, revealed two well-supported clusters, each with high bootstrap values (BS). The first cluster (BS = 100%) contained a single species of the genus *Lacticaseibacillus* (*Lacticaseibacillus paracasei*), with isolate Saf1 clearly grouping within this clade, thereby confirming its affiliation to *L. paracasei*. The second cluster included nine species of the genus *Pediococcus*. Within this group, isolate Saf2 clustered with *Pediococcus pentosaceus*, while isolate Saf3 was positioned in the *Pediococcus acidilactici* clade. These phylogenetic placements are consistent with their molecular identification.

Genetic distance analysis (Figure 3) further supported these results. The maximum evolutionary distance was observed between Saf1 (*L. paracasei)* and the isolates Saf2 (*P. pentosaceus*) –Saf3 (*P. acidilactici)*, whereas the minimum relationship was detected between Saf2 and Saf3. The percentage of divergence between Saf1 and both Saf2 and Saf3 was 8.4%, while Saf2 and Saf3 diverged by 5.2%. These values indicate that the three isolates represent distinct species within the lactic acid bacteria group.

Safety Aspects

Activities of Hemolysis and DNase

All tested strains exhibited a non-hemolytic phenotype. Additionally, subculturing the bacteria on DNase agar showed no halo formation around the streaks, indicating the absence of DNase enzyme secretion by the isolates.

In contrast, the positive control, *Staphylococcus aureus* ATCC 25923, displayed DNase activity and α-

Table 2. 16S rRNA gene sequencing-based identification of the three LAB strains

Strains	Species origin	Sequence size	Similarity rate	Strain species	Access number
Saf1	*Sparus aurata*	956	100%	*Lacticaseibacillusparacasei*	PP196113
Saf2	*Penaeus vannamei*	976	100%	*Pediococcus pentosaceus*	PP196116
Saf3	*Dicentrachus labrax*	881	99.51%	*Pediococcus acidilactici*	PP196117

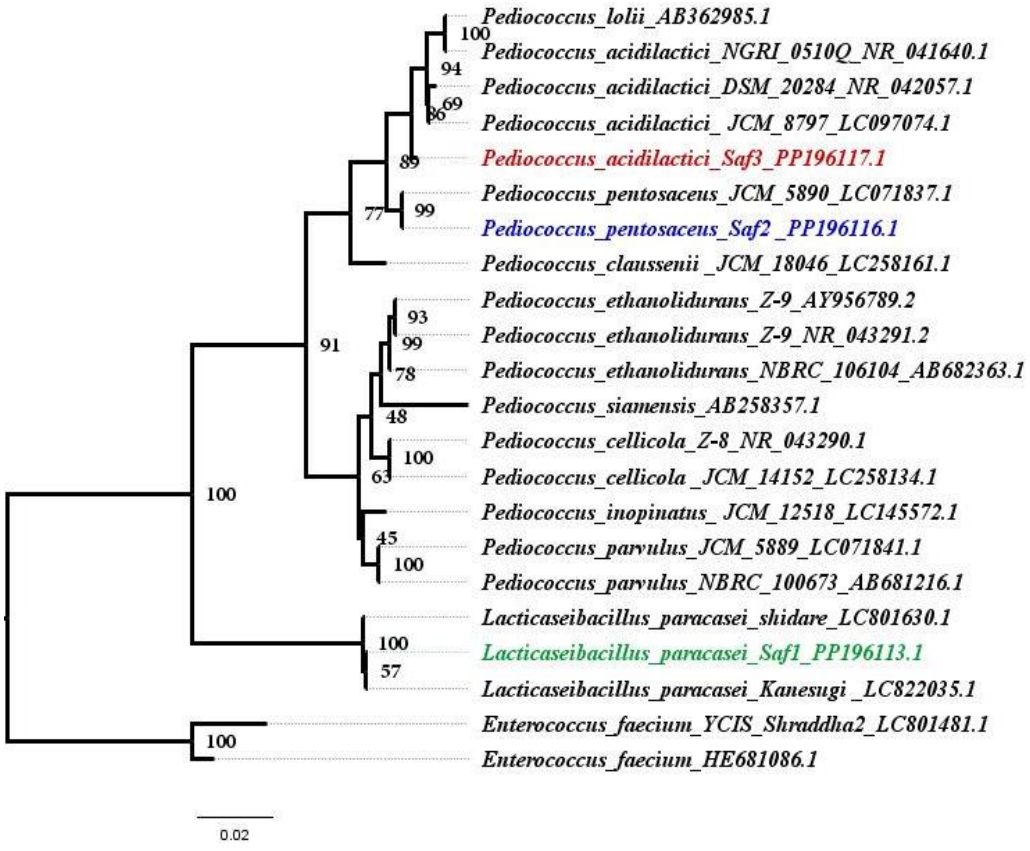

Figure 2. Molecular Phylogenetic tree according to the nucleotide sequences of the 16S rRNA fragments of the strains identified with their GenBank accession numbers. Lacticaseibacillus paracasei_SAF1 (PP196113), Pediococcus pentosaceus_SAF2 (PP196116), and Pediococcus acidilactici_SAF3 (PP196117) are known sequences in the NCBI database.

hemolysis (data not shown). Therefore, these LAB isolates are non-pathogenic and can be regarded as secure for use.

The lack of DNase enzyme production and hemolytic activity indicates that the isolates are non-virulent, making them suitable for use as probiotics, which is a critical criterion when assessing a strain's probiotic potential.

Enzymatic Activity

All the tested strains showed no activity forlipase, trypsin, α- and β-galactosidase, α- and β-glucosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, or α-fucosidase. However, the strains showed high activity for alkaline phosphatase, esterase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase, with enzyme release ranging between 30 to 40 nmol. For strains Saf1 and Saf3, no substrate hydrolysis was observed for valine arylamidase or cystine arylamidase, whereas strain Saf2 exhibited low hydrolysis activity (5 nmol). The production of other enzymes varied across strains. In addition, in all tested strains, no β-glucosidase and β-glucuronidase activities were detected (Table 3).

Antibiotic susceptibility

The results presented in Table 4 show that these strains exhibit sensitivity to all antibiotics tested and

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
1	Lacticaseibacillus_paracasei_Saf1_PP196113.1																					
2	Lacticaseibacillus_paracasei_Kanesugi__LC822035.1	0,000																				
3	Lacticaseibacillus_paracasei_shidare_LC801630.1	0,001	0,001																			
4	Pediococcus_lolii_AB362985.1	0,092	0,092	0,091																		
5	Pediococcus_acidilactici_NGRI_0510Q_NR_041640.1	0,092	0,092	0,091	0,000																	
6	Pediococcus_ethanolidurans_Z-9_AY956789.2	0,071	0,071	0,071	0,056	0,056																
7	Pediococcus_ethanolidurans_Z-9_NR_043291.2	0,071	0,071	0,071	0,056	0,056	0,000															
8	Pediococcus_cellicola_Z-8_NR_043290.1	0,067	0,067	0,068	0,052	0,052	0,014	0,014														
9	Pediococcus_acidilactici_DSM_20284_NR_042057.1	0,085	0,085	0,084	0,008	0,008	0,049	0,049	0,045													
10	Pediococcus_claussenii__JCM_18046_LC258161.1	0,079	0,079	0,079	0,034	0,034	0,040	0,040	0,034	0,028												
11	Pediococcus_acidilactici__JCM_8797_LC097074.1	0,084	0,084	0,082	0,007	0,007	0,048	0,048	0,044	0,001	0,026											
12	Pediococcus_inopinatus__JCM_12518_LC145572.1	0,069	0,069	0,069	0,053	0,053	0,020	0,020	0,018	0,047	0,034	0,045										
13	Pediococcus_cellicola__JCM_14152_LC258134.1	0,067	0,067	0,068	0,052	0,052	0,014	0,014	0,000	0,045	0,034	0,044	0,018									
14	Pediococcus_parvulus_JCM_5889_LC071841.1	0,072	0,072	0,072	0,049	0,049	0,013	0,013	0,015	0,043	0,031	0,041	0,009	0,015								
15	Pediococcus_pentosaceus_JCM_5890_LC071837.1	0,084	0,084	0,084	0,016	0,016	0,040	0,040	0,038	0,011	0,021	0,009	0,042	0,038	0,036							
16	Pediococcus_parvulus_NBRC_100673_AB681216.1	0,072	0,072	0,072	0,049	0,049	0,013	0,013	0,015	0,043	0,031	0,041	0,009	0,015	0,000	0,036						
17	Pediococcus_ethanolidurans_NBRC_106104_AB682363.1	0,069	0,069	0,069	0,054	0,054	0,001	0,001	0,013	0,048	0,039	0,047	0,019	0,013	0,012	0,039	0,012					
18	Pediococcus_siamensis_AB258357.1	0,079	0,079	0,081	0,067	0,067	0,019	0,019	0,020	0,060	0,049	0,059	0,026	0,020	0,024	0,052	0,024	0,018				
19	Pediococcus_acidilactici_Saf3_PP196117.1	0,084	0,084	0,082	0,012	0,012	0,045	0,045	0,042	0,006	0,026	0,005	0,045	0,042	0,041	0,007	0,041	0,044	0,056			
20	Pediococcus_pentosaceus_Saf2__PP196116.1	0,084	0,084	0,084	0,016	0,016	0,040	0,040	0,038	0,011	0,021	0,009	0,042	0,038	0,036	0,000	0,036	0,039	0,052	0,007		
21	Enterococcus_faecium_YCIS_Shraddha2_LC801481.1	0,121	0,121	0,120	0,110	0,110	0,103	0,103	0,113	0,102	0,104	0,101	0,112	0,113	0,109	0,107	0,109	0,102	0,116	0,104	0,107	
22	Enterococcus_faecium_HE681086.1	0,109	0,109	0,108	0,097	0,097	0,099	0,099	0,097	0,090	0,088	0,088	0,096	0,097	0,097	0,096	0,097	0,097	0,108	0,091	0,096	0,026

Figure 3. Estimates of Evolutionary Divergence between Sequences of the specimens under the K2P (Kumura 2 parameters) distance model.

Table 3. Enzymatic characteristics of LAB evaluated by Api ZYM gallery

	Enzymes	Saf1	Saf2	Saf3
01	Alkaline phosphatase	3	4	3
02	Esterase (C 4)	4	4	4
03	Esterase Lipase (C 8)	1	2	0
04	Lipase (C 14)	0	0	0
05	Leucine arylamidase	4	3	3
06	Valine arylamidase	0	0	0
07	Cystine arylamidase	0	0	0
08	Trypsin	0	0	0
09	α-chymotrypsin	4	3	4
10	Acid phosphatase	5	5	5
11	Naphthol-AS-BI-phosphohydrolase	4	5	4
12	α-galactosidase	0	0	0
13	ß-galactosidase	0	0	0
14	ß-glucuronidase	0	0	0
15	α-glucosidase	0	0	0
16	ß-glucosidase	0	0	0
17	N-acetyl-ß-glucosaminidase	0	0	0
18	α-mannosidase	0	0	0
19	α-fucosidase	0	0	0

A score between 0 and 5 is given by the intensity of the color change: 0 indicates a negative reaction, 5 represents the highest intensity of reaction, and scores of 1, 2, 3, or 4 reflect intermediate reactions, with scores of 3, 4, or 5 being considered positive reactions.

resistance only to Amoxicillin-clavulanic acid, Neomycin, and Trimethoprim.

Probiotic Features of Isolated LAB

Assay of low pH

Data of LAB growth under low pH conditions are depicted in the Figure 4.

The growth rate of isolates Saf2 after 3 hours of incubation is low, particularly at pH 2. As the pH increases, growth rates also increase, indicating that these isolates are less tolerant of acidic conditions.

In contrast, strains saf1 and Saf3 exhibit a high capacity to tolerate acidic conditions, with growth rates remaining high even at pH 2 compared to Saf2. All isolated strains have the ability to tolerate acidic conditions; however, under extreme acidity, the growth rate decreases, and the bacteria are unable to tolerate this pH.

Assay of NaCl Resistance

The results presented in Figure 5 show that all strains exhibited a high tolerance to NaCl concentrations of 4% and 8%, with growth rates values ranging from 42.3% to 83.5%. At 15% NaCl, the tested strains demonstrated very weak growth with rates not exceeding 31%.

Survival Under Conditions Mimicking Fish GI Tract

The isolated strains demonstrated resistance to pepsin at both pH 2 and pH 3, as well as to trypsin at pH 8, after being exposed for 3 and 4 hours, respectively (Table 5). Their viability fluctuated from 28.73% to 92.69% for pepsin tolerance.

SAF2 showed the highest resistance to pepsin at pH 2 (86.21%), while SAF1 exhibited the best resistance at pH 3 (92.69%), indicating strong resilience to conditions in the stomach. In contrast, pepsin at pH 2 had a

Table 4. Antibiotic sensitivity profile of lactic acid bacteria

Antibiotic	Disk load (µg)	Saf1	Saf2	Saf3
Oxytetracycline(OT)	30 µg	S	S	S
Tetracycline(T)	30 µg	S	S	S
Ciprofloxacin(CIP)	5 µg	S	S	S
Chloramphenicol(C)	30 µg	S	S	S
Ampicillin(AMP)	5 µg	S	S	S
Enrofloxacin(ENF)	5 µg	S	S	S
Amoxicillin-clavulanicacid (AUG)	30 µg	R	R	R
Trimethoprim (TM)	5 µg	R	R	R
Neomycin(NEO)	30 µg	R	R	R
Florfenicol(FFC)	30 µg	S	S	S

S: sensitive, R: resistant

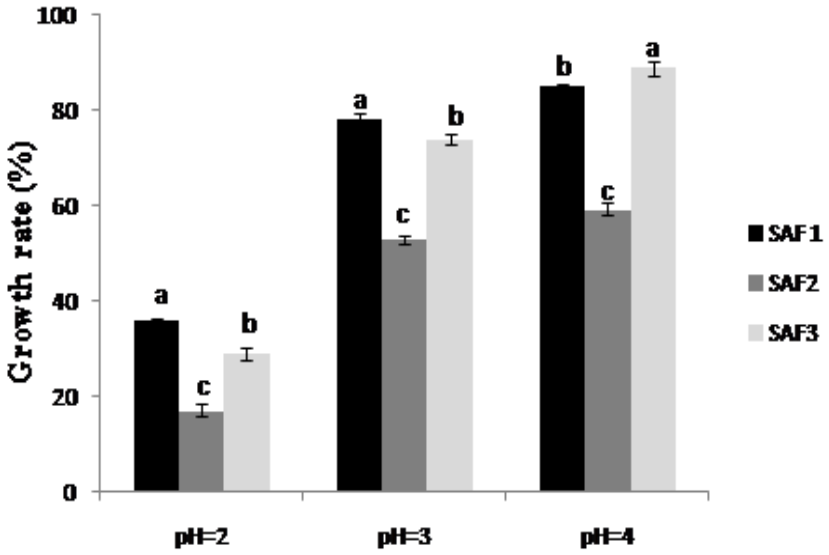

Figure 4. Survival percentage (%) of lactic acid bacteria incubated for 3 hours at 37°C in MRS broth adapted to various pH levels. Bars labeled with the letters (a–c) represent statistically significant differences in survival rates (P<0.05) for *L. paracasei* (SAF1), *P. pentosaceus* (SAF2), and *P. acidilactici* (SAF3).

significant effect (P<0.05) on reducing the viability of SAF3 (28.73%). Under trypsin at pH 8, the SAF1 strain exhibited the highest resistance at 80.19%, succeeded by SAF2 at 77.45% and SAF3 at 72.77%.

Antibacterial Activity

The antimicrobial properties of the isolates were assessed against various pathogenic fish bacteria, is presented in Table 6. Data showed varying inhibition zone diameters for the tested probiotic species. All three examined probiotic isolates showed broad-spectrum antagonistic activity opposed to the both isolated and reference pathogens. The inhibition zones ranged from 6±0.00 mm to 20±0.71 mm. Isolated *Vibrio alginolyticus* and *Vibrio vulnificus* were the most sensitive strains to the three probiotic bacteria, with inhibition zone diameters between 13±0.71 mm and 20±0.71 mm.

Biofilm Formation and Adhesion

The potential probiotic bacteria tested, including *L. paracasei* (SAF1), *P. pentosaceus* (SAF2), and *P. acidilactici* (SAF3), displayed strong adhesion ability, as indicated by the formation of blackish colonies on Congo red medium.

The biofilm formation capacity of these bacteria is illustrated in Figure 6, with SAF1 showing the highest biofilm production, followed by SAF2 and SAF3 (OD570>1).

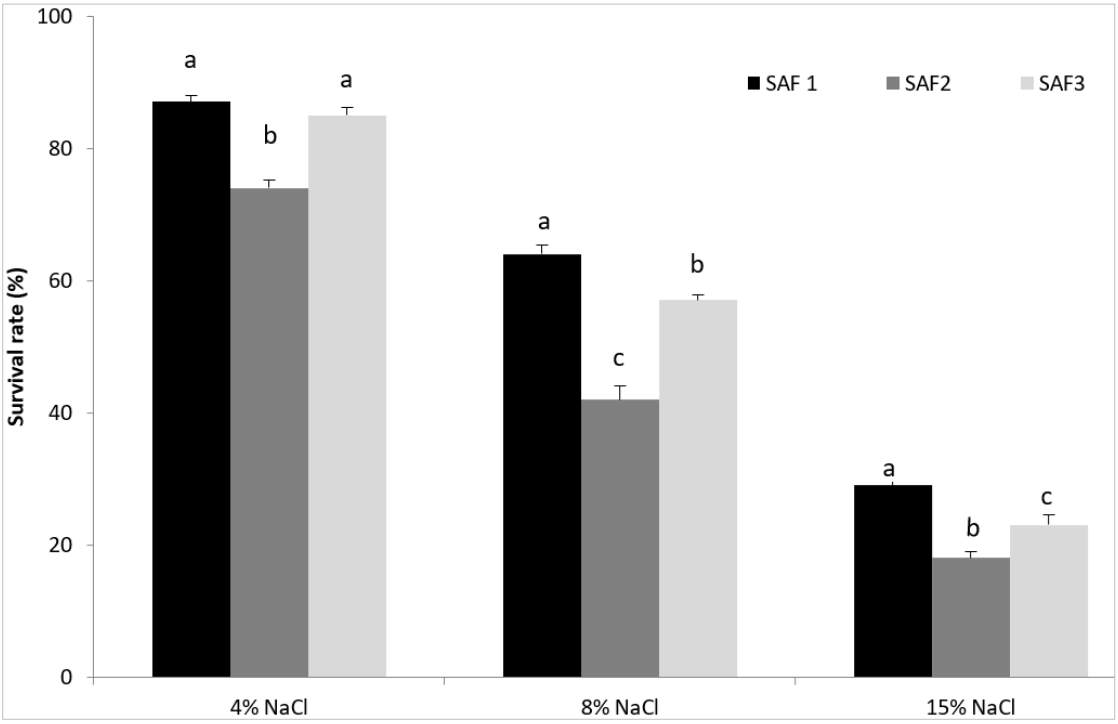

Figure 5. Survival rate (%) of lactic acid bacteria cultured for 48 hours at 37°C in MRS broth with varying salt concentrations. Bars marked with different letters (a–c) denote statistically significant differences in survival rates (P<0.05) for *L. paracasei* (SAF1), *P. pentosaceus* (SAF2), and *P. acidilactici* (SAF3).

Table 5. Impact of pepsin and trypsin on the survival of isolated LAB bacteria

LAB Bacteria	Pepsin (pH= 2)			Pepsin (pH= 3)			Trypsin (pH= 8)		
	T0	T3	Survival rate (%)	T0	T3	Survival rate (%)	T0	T4	Survival rate (%)
L. paracasei SAF1	3.17±0.09	1.30±0.031	41.01	7.25±0.2	6.72±0.061	92.69	8.28±0.16	6.64±0.49	80.19
P.pentosaceus SAF2	3.48±0.57	3±0.04	86.21	6.87±0.42	5.30±1.12	77.15	8.47±0.04	6.56±0.32	77.45
P. acidilactici SAF3	3.48±0.45	1±0.07	28.73	5.85±0.03	4.33±0.79	74.02	8.41±0.08	6.12±0.52	72.77

* Number of viable cells (log CFU/mL) ± standard deviation is presented, representing the average of three independent experiments. T0 refers to the viable cell count (log CFU/mL) of each strain at 0 hours. t3 and t4 indicate the viable counts (log CFU/mL) of each strain at 3 and 4 hours, respectively, along with the % viability of the selected LAB. Mean values (with standard deviation) within the same row that are followed by different letters are significantly different (P<0.05) according to Tukey's test.

Cell Surface Hydrophobicity Assay

Cellular adhesion to hydrocarbons was estimated to determine the isolated LAB's level of hydrophobicity (Table 7).

Three different solvents were used for the hydrophobicity test of the lactic acid bacteria strains: chloroform, ethyl acetate, and n-hexadecane. The results indicated that *Lacticaseibacillus paracasei* (SAF1) exhibited the highest hydrophobicity with n-hexadecane (97.77%), followed by ethyl acetate (83.21±0.89%). However, moderate hydrophobicity values were observed with chloroform, ranging from 36.28% to 66% for *Pediococcus pentosaceus* and *Pediococcus acidilactici*, respectively.

Auto and Co-aggregation Capacities

Data depicted in Table 8 demonstrated a high auto-aggregation capacity, with *Pediococcus pentosaceus* showing 87.22% and *Pediococcus acidilactici* displaying 86.10%. Meanwhile, *Lacticaseibacillus paracasei* exhibited a significantly the highest auto-aggregation percentage of 90.7%.

The co-aggregation activity of the isolates was tested against eight pathogenic strains: *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Photobacterium damselae*, *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Vibrio alginolyticus* ATCC 17749, *Vibrio parahaemolyticus* ATCC 17802, and *Aeromonas hydrophila* ATCC 7966.

As depicted in Table 8, the three LAB bacteria displayed notable co-aggregation capacities with pathogenic microorganisms, with percentages ranging from 8% to 96%, highlighting a strong potential for interaction and aggregation with these pathogens.

The *Lacticaseibacillus paracasei* strain demonstrated the highest and most significant co-aggregation rates with all pathogenic bacteria, with percentages ranging from 82% to 96%, especially against *V. alginolyticus* (91.76%), *Aeromonas hydrophila* (96.45%), and *Aeromonas salmonicida*. *Pediococcus*

Table 6. Antibacterial activity of probiotic bacteria against isolated and reference marine pathogens

LAB Strain / Pathogenic Strain	SAF1	SAF2	SAF3
Isolated pathogens			
Vibrio parahaemolyticus	10±0.33	12±1.3	10±0.33
Vibrio alginolyticus	13±0.71	15±0.71	18±0.2
Vibrio vulnificus	18±0.00	16±0.5	20±0.71
Aeromonas hydrophila	11±0.00	12±0.33	12±0.33
Photobacterium damsela	8±0.71	7±0.2	6±0.00
Reference pathogens			
Vibrio parahaemolyticus ATCC 17802	10±0.03	10±0.00	11±1.2
Vibrio alginolyticus ATCC 17749	10±00	12±0.71	11±0.77
Aeromonas hydrophila ATCC 7966	12±0.33	12±0.03	11±0.5

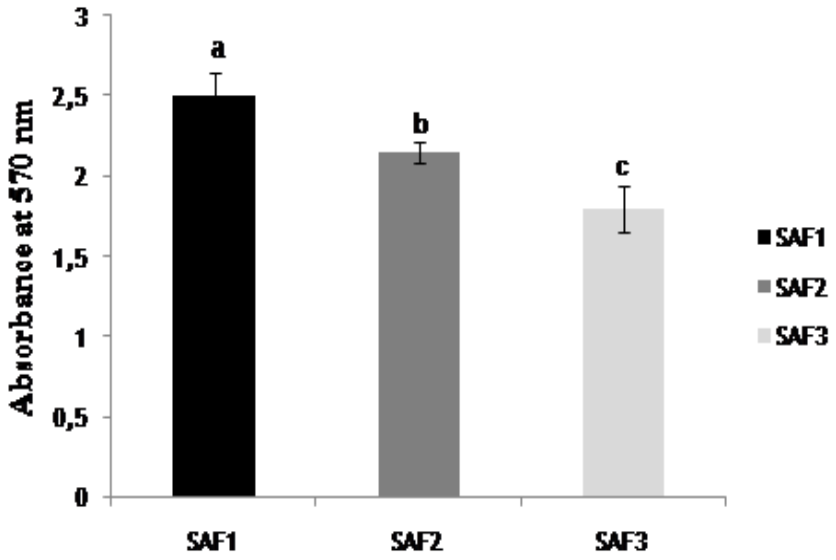

Figure 6. Biofilm production capacity of lactic acid bacteria was measured by absorbance at 570 nm after crystal violet staining. Bars labeled with letters (a–b) represent statistically significant differences in values ($P<0.05$) for *L. paracasei* (SAF1), *P. pentosaceus* (SAF2), and *P. acidilactici* (SAF3).

pentosaceus also exhibited strong co-aggregation capacity, particularly against *V. parahaemolyticus* and *A. salmonicida*. It showed notable activity against *Vibrio parahaemolyticus* (81.7%), *Vibrio alginolyticus* ATCC 17749 (78.47%), and *Aeromonas salmonicida* (74.62%) (Table 8).

Discussion

Although lactic acid bacteria (LAB) are a well-studied group, there has been limited research on LAB originating from marine environments. The involvement of marine-derived LAB in organic matter decomposition and their potential uses in the food, pharmaceutical, and aquaculture industries are still not fully explored (Kathiresan & Thiruneelakandan, 2008; Hwanhlem et al., 2011).

Research on the probiotic properties of LAB for aquaculture and biopreservation is an emerging area of interest. Several studies have identified LAB bacteria such as *Pediococcus*, *Enterococcus*, *Lactococcus lactis*, *Lactobacillus casei*, and *Carnobacterium* from Tunisian marine fish, such as seabass and seabream (Riahi et al*.,* 2024; Jlidi et al*.,* 2022; El-Jeni et al., 2016, 2019; Das et al., 2016; Chahad et al., 2012; Boulares et al., 2011, 2012; Felix et al., 2019; Medina et al., 2020; Lamari et al., 2014). In this current investigation, the probiotic potential of three LAB strains isolated from marine aquaculture samples of seabream, seabass, and shrimp was evaluated.

The initial screening, conducted using *in vitro* assays, represents a preliminary step before further *in vivo* assessment for aquaculture applications.

Molecular identification through 16S rDNA gene sequencing revealed that Saf1, Saf2, and Saf3, isolated respectively from seabream (*Sparus aurata*), shrimp (*Penaeus vannamei*), and seabass (*Dicentrarchus labrax*), were homologous with *Lacticaseibacillus paracasei*, *Pediococcus pentosaceus*, and *Pediococcus acidilactici*, respectively. The phylogenetic tree and genetic distance analysis confirm the morphological, enzymatic, and molecular identification of the isolates.

The absence of DNase production and hemolytic activity meets key safety criteria for the use of LAB as probiotics, supporting their potential for therapeutic or dietary applications (Dbeibia et al., 2023). Our findings confirm the safety of the isolated strains, as they do not produce DNase and exhibit non-hemolytic behavior.

Ensuring the lack of detrimental enzymes like β-glucosidase and β-glucuronidase is also vital, as these enzymes facilitate the bioconversion of glycosides into aglycone, which can be harmful (Lamari et al., 2014). The three LAB strains produced alkaline phosphatase, acid phosphatase, and phosphohydrolase, enzymes critical to both bacterial metabolism and host health.

These enzymes play a key role in enhancing growth, health, and immune response in aquaculture species like fish and shrimp. They assist in breaking down phosphate-containing compounds, improving phosphate and mineral absorption, supporting skeletal development, and contributing to energy metabolism (Renuka, 2024; Merrifield et al., 2010a, 2010b).

Antibiotic susceptibility is a key factor in determining whether a strain can be considered an effective probiotic, as highlighted in the literature. Zhang et al. (2018) emphasized that probiotic bacteria

Table 7. The LAB cell surface's hydrophobicity toward n-hexadecane, ethyl acetate, and chloroform

LAB	Chloroform	Ethyl acetate	n-hexadecane
SAF1	36,28±0.71*^{b}(M)	83,21±0.77^{b}(H)	97,77±0.03^{a}(H)
SAF2	66±0.31^{a}(M)	83,05±0.31^{b}(H)	87,81±0.52^{c}(H)
SAF3	66,02±0.5^{a}(M)	85,58±0.05^{a}(H)	90,84±0.04^{b}(H)

H: highly hydrophobic (71–100%); M: moderately hydrophobic (36–70%); L: low hydrophobic (0–35%). *% mean adhesion±standard deviation. Mean values (with standard deviation) within the same column that are followed by different letters are significantly different (P<0.05) according to Tukey's test. *L. paracasei* (SAF1), *P. pentosaceus* (SAF2), and *P. acidilactici* (SAF3).

Table 8. The Auto and Co-Aggregation capacities of LAB Strains with Pathogens

LAB STRAIN	SAF1	SAF2	SAF3
	Lacticaseibacillus paracasei	*Pediococcus pentosaceus*	*Pediococcus acidilactici*
Auto-aggregation (%)	90.70*±0.81^{a}	87.22±0.21^{b}	86.10±0.77^{b}
Co-aggregation (%)			
Isolates fish pathogens			
Vibrio parahaemolyticus	86.64±0.48^{a}	81.70±1.1^{b}	38.29±0.21^{c}
Vibrio alginolyticus	91.76±0.19^{a}	44.57±0.24^{b}	18.05±0.23^{c}
Photobacterium damsela	88.17±0.64^{a}	38.52±1.55^{c}	42.01±0.25^{b}
Aeromonas hydrophila	96.45±0.31^{a}	51.05±0.13^{b}	8.54±0.27^{c}
Aeromonas salmonicidae	94.30±0.06^{a}	74±0.77^{b}	23.98±0.28^{c}
Reference pathogenes			
Vibrio alginolyticus ATCC 17749	82.51±0.63^{a}	78.47±0.21^{b}	20.14±0.34^{c}
Vibrio parahaemolyticus ATCC 17802	90.23±0.27^{a}	28.17±0.23^{c}	73.20±0.45^{b}
Aeromonas hydrophila ATCC 7966	88.64±0.28^{a}	29.84±0.16^{b}	8.68±0.86^{c}

*(%)±standard deviation. The data represent the average of three independent experiments. Mean values (with standard deviation) in the same row that are followed by different letters are significantly different (P<0.05) according to Tukey's test.

with inherent resistance to antibiotic character play a crucial role in reestablishing microbial equilibrium in the gastrointestinal tract following antibiotic treatment for harmful bacteria caused gastrointestinal illness. Thus, the resistance of our LAB bacteria to certain antibiotics suggests that these isolates could persist in the gut even after antibiotic use, potentially aiding in maintaining the natural equilibrium of intestinal microflora in these conditions. Furthermore, both the antibiotic susceptibility and inherent resistance of these LAB strains support their safety as potential probiotics and could contribute to the development of safe probiotic products for human use.

In line with our results, prior research has shown that most antibiotics are typically effective against *Pediococcus* species (Temmerman et al., 2003; Ruiz-Moyano et al., 2010; Ribeiro et al., 2014). Uymaz et al. (2009) found that a human isolate of *Pediococcus pentosaceus* BH105 was susceptible to both penicillin and chloramphenicol. Sensitivity to ampicillin, amoxicillin, and penicillin was also demonstrated by Venkateshwari et al. (2010), Mandal et al. (2011), and Ribeiro et al. (2014). Furthermore, Vidhyasagar and Jeevaratnam (2013) found that *Pediococcus pentosaceus* was sensitive to tetracycline, chloramphenicol, and erythromycin.

In aquaculture, probiotics must survive the acidic conditions of the digestive tract to apply their advantageous effects. The pH of the digestive tract is typically 2–3, with food traveling through the digestive system for 2–3 hours (Maragkoudakis et al., 2006). To preliminarily assess LAB viability, growing absorbance at pH 3 is used (Bao et al., 2010). The identified LAB bacteria did not survive pH 2 after 3 hours of exposure, consistent with findings by Osmanagaoglu et al. (2010), though contrary to Vidhyasagar and Jeevaratnam (2013), whose reported survival of *Pediococcus pentosaceus* at pH 2.

The capacity of LAB to thrive in seawater is a crucial condition for probiotic selection in marine aquaculture systems (Vazquez et al., 2003). All tested strains thrived in environments containing 4–8% NaCl, although none grew at 15% NaCl. Similar findings by Abriouel et al. (2012) and Das et al. (2016) demonstrated that most *Lactobacillus* strains can grow in environments with up to 6.5% NaCl, while *Pediococcus* strains thrive in conditions containing up to 5% NaCl.

Probiotics must also withstand digestive enzymes like pepsin and trypsin to grow and colonize the gastrointestinal tract (Charteris et al., 2001). All evaluated strains demonstrated tolerance to both pepsin and trypsin, suggesting that they are able to resist digestive conditions while passing through the host's small intestine and stomach.

In this study, all strains exhibited strong antagonistic activity against the *Vibrionaceae* family, which includes some of the most significant pathogens affecting Mediterranean aquaculture farms (Dopazo et al., 1988; Muniesa et al., 2020; Barefoot & Klaenhammer, 1983).

The antimicrobial proprieties of LAB are primarily attributed to the releasing of organic acids, which create an acidic environment (Reinheimer et al., 1990; Tejero-Sariñena et al*.,* 2012). However, LABs also produce hydrogen peroxide and bacteriocins, which contribute to their antimicrobial properties (Dimitonova et al., 2007; Schillinger et al., 1989).

The next step in the selection process involved testing the adhesion of LAB to an abiotic surface, which may indicate their potential to colonize the gut and inhibit undesirable strains (Servin & Coconnier, 2003). The identified bacteria exhibited strong adhesion capabilities, with *L. paracasei* showing high adherence, while *P. acidilactici* demonstrated weaker adherence. This adhesion ability may rely on interactions among the bacterial cell membrane and the surfaces involved (Kos et al., 2003; Vinderola and Reinheimer, 2003).

Our results indicate that all three LAB bacteria displayed *in vitro* adhesion properties, implying their potential to colonize the gastrointestinal (GI) mucosa. Further research is necessary to investigate the adhesion of different LAB bacteria to the intestinal epithelial cells of aquaculture organisms and to validate their *in vivo* probiotic potential (El-Jeni et al., 2016).

Hydrophobicity is a physicochemical property of the cell surface that can influence bacterial auto-aggregation and adhesion to different surfaces.

A strong correlation has been demonstrated between the auto-aggregation of LAB and their adhesive potential (Zuo et al., 2016). Our results indicated that *Lacticaseibacillus paracasei* (SAF1), *Pediococcus pentosaceus* (SAF2), and *Pediococcus acidilactici* (SAF3) exhibited the highest hydrophobicity with n-hexadecane and ethyl acetate.

However, moderate hydrophobicity values were observed with chloroform. These results contrast with those of Dbeibia et al. (2023), who found that the most hydrophobic strain responded to chloroform.

Auto-aggregation mentions the clustering of bacterial cells from the same strain. A strong correlation has been observed between the auto-aggregation of a probiotic strain and its capacity to attach to intestinal epithelial cells. This suggests that auto-aggregation is a crucial factor to facilitate successful colonization and improved persistence in the gastrointestinal tract, allowing effective binding to the intestinal epithelium and blocking pathogen adhesion (Aslim et al., 2005 and 2007; Boris et al., 1997).

In this paper, *Lacticaseibacillus paracasei* demonstrated a high aggregation rate of 90.70%, indicating strong clumping of cells, while the two *Pediococcus* species showed aggregation rates of 87.22% and 86.10%, respectively. Similar results were found by Vidhyasagar and Jeevaratnam (2013), where the *Pediococcus pentosaceus* strain exhibited a maximum aggregation rate of ≈89%. In contrast,

Abbasiliasi et al. (2017) reported an auto-aggregation rate of only 35.2% for *Pediococcus acidilactici*.

As a result, it is hard to establish a standard benchmark for high auto-aggregation rates among these bacteria. In a related study, Dos Santos Leandro et al. (2021) observed auto-aggregation rates ranging from 82.68% to 89.80% across different Lactobacillus strains. The capacity of probiotic strains to aggregate with potential pathogens provides additional advantages by creating a protective barrier that hinders pathogen colonization (Bao et al., 2010; Rokana et al., 2017). Our findings regarding the variation in co-aggregation capabilities among LAB strains are consistent with preceding studies, indicating that co-aggregation traits were affected by the composition and structure of the bacterial surface (Castagliuolo et al., 2005; Rokana et al., 2017). Previous research has also indicated that extracellular polysaccharides on the cell surface may play a role in the aggregation process (Sabir et al., 2010).

Conclusion

In this study, LAB were successfully isolated and identified from the gastrointestinal tracts of marine aquaculture species, including seabream, seabass, and *Penaeus vannamei* (shrimp). The LAB strains Saf1, Saf2, and Saf3 were identified as *Lacticaseibacillus paracasei*, *Pediococcus pentosaceus*, and *Pediococcus acidilactici*, respectively. These isolates demonstrated significant probiotic potential, showing tolerance to acidic pH, gastrointestinal (GI) conditions, and varying salt concentrations. They also exhibited antimicrobial activity, biofilm formation, adhesion properties, antibiotic sensitivity, and the ability to auto-aggregate, co-aggregate, and display hydrophobicity. Additionally, these strains were capable of phosphatase production, further enhancing their potential for industrial applications. The results of this study highlight aquatic organisms as a viable source for isolating LAB strains. *Lacticaseibacillus paracasei* (Saf1) emerged as the most promising candidate, exhibiting the strongest probiotic characteristics, particularly in terms of its antimicrobial activity. Due to its significant probiotic potential, SAF1 appears to be a stronger strain than the others tested. However, additional in vivo studies are needed to evaluate the safety and colonization ability of these isolated strains, with special attention to *Lacticaseibacillus paracasei*.

Ethical Statement

No ethical approval was required, as the animal samples were obtained directly from farms during routine practices and from local markets, with no experimental handling or sacrifice performed.

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This research received no external funding.

Author Contribution

S.J: Conceptualization, Writing -review and editing; A.D: Data Curation, Formal Analysis, Investigation, Methodology, Visualization and Writing; A.B.N: Analysis; T.Z and L.A: Methodology; M.M.M: Fomal analysis; K.H.M: Funding Acquisition; R.M; Administraition and C.J: Visualization, Supervision and Validation.

Conflict of Interest

The authors declare that they do not have any conflicts of interest.

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