

Probiotic Potential of Lactobacillus Strains Isolated from the Pearl Oyster (*Pinctada radiata*)

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ABSTRACT

Infectious disease outbreaks are one of the major challenges in aquaculture; therefore, there is an increasing interest in the application of probiotics for sustainable aquaculture practices to minimize the spread of infectious diseases. In this study, bacteria were isolated from *Pinctada radiata* to examine their probiotic potential. Lactic acid bacteria (LAB) are potentially good probiotic candidates for aquaculture. Five LAB strains were isolated from the gut contents of the pearl oyster *P. radiata* (Leach, 1814) on the Jeddah coast of the Red Sea. The LAB were characterized morphologically, biologically, and biochemically, examined for their probiotic properties, and identified using 16S rRNA sequencing. The five selected isolated LAB strains were Gram positive (rods and cocci) and tested negative for catalase and oxidase. The LAB strains were identified as *Bacillus valesensis* (POR1), *B. siamensis* (POR2), *Staphylococcus epidermidis* #1 and *S. epidermidis* #2 (POR3 and POR5), and *S. hominis* (POR4). POR1 and POR2 tested negative for gamma-hemolytic activity, while POR3, POR4, and POR5 tested positive for alpha-hemolytic activity. All five strains showed sensitivity to antibiotics, including erythromycin (E10), nitrofurantoin (F100), and novobiocin (NV5), and four strains showed an acid tolerance up to 2.5 pH. Among the isolates, POR1, 2, 4, 5 tolerated 3-h exposure to 0.3% bile salt. All LAB strains showed an antagonistic activity against clinical strains of *S. aureus*, *Acinetobacter baumannii*, methicillin-resistant *S. aureus*, and *Escherichia coli*. Therefore, it could be suggested that the five LAB strains isolated from the gut contents of *P. radiata* could be good candidate probiotics for aquaculture application since those strains showed significance of probiotic selection tests.

INTRODUCTION

Pinctada radiata (Leach, 1814), also known as the Gulf, Atlantic or rayed pearl oyster, is geographically distributed across the world's oceans, but is particularly abundant in the Indo-Pacific region (Aparicio & Méndez, 2021), and the Red Sea (Png-Gonzalez *et al.*, 2021). *P. radiata* occurs at various depths, but most commonly at a

shallow depth of 5– 25m, where it can attach to hard substrates (**Doğan & Nerlović, 2008**). The oysters are benthic organisms with thin-to medium-sized shells (**Yigitkurt *et al.*, 2020**). They were widely used for aesthetic purposes more than 4000 years ago (**Moutopoulos *et al.*, 2022**). Furthermore, *P. radiata* is highly adaptable to eurythermal and euryhaline habitats; thus, it can be easily introduced into new agricultural environments (**Theodorou *et al.*, 2019**). Deliberate oyster migration through mariculture, a subset of aquaculture, and shipping are vectors for their introduction (**Gavrilović *et al.*, 2017**). Aquaculture activities can be used as vectors in locating species and as a source of shellfish, especially non-native species (**Tlig-Zouari *et al.*, 2009**). Mariculture is a rapidly growing industry due to the continuous demand for marine products and seafood resulting from the continued growth of the global population (**Sahu *et al.*, 2008; Jamal *et al.*, 2019**). Mariculture addresses the seafood trade deficit, creates jobs in waterfront communities, and provides consumers with locally produced fresh products (**Azra *et al.*, 2021**).

Mollusk aquaculture is an important source of the second-most cultured seafood group after fish, with an annual production of more than 17.7 million tons (**FAO, 2022**). In addition, mollusks are a source of nutrients, an economic asset, and provide water filtration ecosystem services to aquatic systems (**Moutopoulos *et al.*, 2022**). A major challenge for aquaculture is the outbreak of infectious diseases, which can lead to significant economic losses for the industry (**Hoseinifar *et al.*, 2018; Pérez-Sánchez *et al.*, 2018**). To overcome this challenge, chemotherapy, vaccination, and good management practices have been used to combat infectious pathogens (**Azra *et al.*, 2021**). However, in recent decades, the use of probiotic has become a more sustainable practice in aquaculture (**C De *et al.*, 2014; Hai, 2015**). Probiotics are live bacteria that exert broad spectrum of activity against infectious diseases (**Akhter *et al.*, 2015**). For example, probiotics can be nutritional supplements that strengthen immunity in cultured animals, fight infectious diseases, and serve as an alternative to chemotherapy and vaccination (**Pérez-Sánchez *et al.*, 2018**). Probiotics have been successfully used in mollusc aquaculture to increase the disease control efficiency and improve water quality, feed utilization, growth, safety, and survival rates (**Pandiyan *et al.*, 2013**). Probiotics have been investigated in pearl oyster cultures isolated from commercial probiotics (**Subhash & Lipton, 2007**), while in this study the probiotic was isolated from the gut of the same pearl oyster.

Among probiotic organisms, lactic acid producing bacteria (LAB) stand out in aquaculture practices due to their diverse activities, such as contribution to water quality management, disease control, larval rearing, waste management, stress reduction, and biopreservation (**Ringø *et al.*, 2018**). Moreover, many LAB produce compounds that can defend against some pathogenic organisms (**Balcázar *et al.*, 2007**). LAB are not only harmless, but also provide various benefits in most animal systems; several studies have shown that probiotics improve the health of cultured fish (**Gatesoupe, 2008**). More

specifically, the use of native bacteria isolated from a host as a probiotic in host aquaculture is a popular practice in aquaculture that brings several immunological benefits (Ringø *et al.*, 2020). Additionally, since native bacteria are commensal to the animal host, meaning they do not cause harm to the host, they can be a good source of probiotics for aquaculture applications. The results of such studies suggest that *P. radiata* aquaculture could likely benefit from the use of probiotic, as these mollusks are filter-feeding benthic organisms whose digestive systems harbor a variety of different bacteria that may have probiotic potential. Therefore, in this study, gut bacteria of *P. radiata* from the Red Sea were isolated, identified, and examined for resistance to gastric acidity and bile salts, sensitivity to antibiotics, and antagonistic activity against pathogens. The objective of this study was to determine the suitability of LAB strains, isolated from *P. radiata*, as sustainable probiotics for use in pearl oyster aquaculture.

MATERIALS AND METHODS

1. Samples collection

Samples of *P. radiata* (n=10) were collected by scuba diving at a depth of 10m at the Sharm Obour (Obour Creak) station on the Red Sea (N21°42.562' E39°05.764') (Fig. 1) at King Abdulaziz University in December 2019. The samples were collected and stored in a bucket at the same sampling site and with aeration to keep the samples alive during transport to the Marine Microbiology Laboratory. Before dissection, natural *P. radiata* seawater were filtered with 0.22-µm MF filter paper (Millipore®, France), after which the filtered water was sterilized in an autoclave. Subsequently, sterilized sea water (SSW) with a salinity of 35ppt was stored and used to obtain gut material. The gut contents of *P. radiata* were aseptically siphoned with a syringe (Nasr, 1984) and transferred to a 15-mL sterilized glass tube with FSNSW for homogenization.

2. Isolation and culture of presumptive LAB

Serial dilution was carried out by transferring 1.0mL of homogenate (see section 1.) into 9mL of FSNSW for dilutions up to 10^{-6} . An aliquot (0.1mL) of the diluted samples was inoculated onto prepared Zobell Marine Agar plates (HiMedia M384-500G Zobell Marine Agar 2216, Maharashtra, India) from the last three dilutions, which were then incubated at 37°C for 120h under aerobic and anaerobic conditions (Maresca *et al.*, 2018). Distinct colonies were observed under aerobic conditions and then streaked and subcultured on de Man, Rogosa, and Sharpe (HiMedia M641-500G Lactobacillus MRS Agar) agar plates for 24h at 37°C (Dubey & Maheshwari, 2012; Forhad, 2015). The MRS agar was composed of a tryptic digest of casein, beef extract, yeast extract, glucose, sorbitan monooleate, dipotassium hydrogen orthophosphate, magnesium sulfate, manganese sulfate, ammonium citrate, sodium acetate, sorbic acid, agar (Tharmaraj & Shah, 2003), and SSW. The plates were then incubated under aerobic conditions at 37°C for 24h.

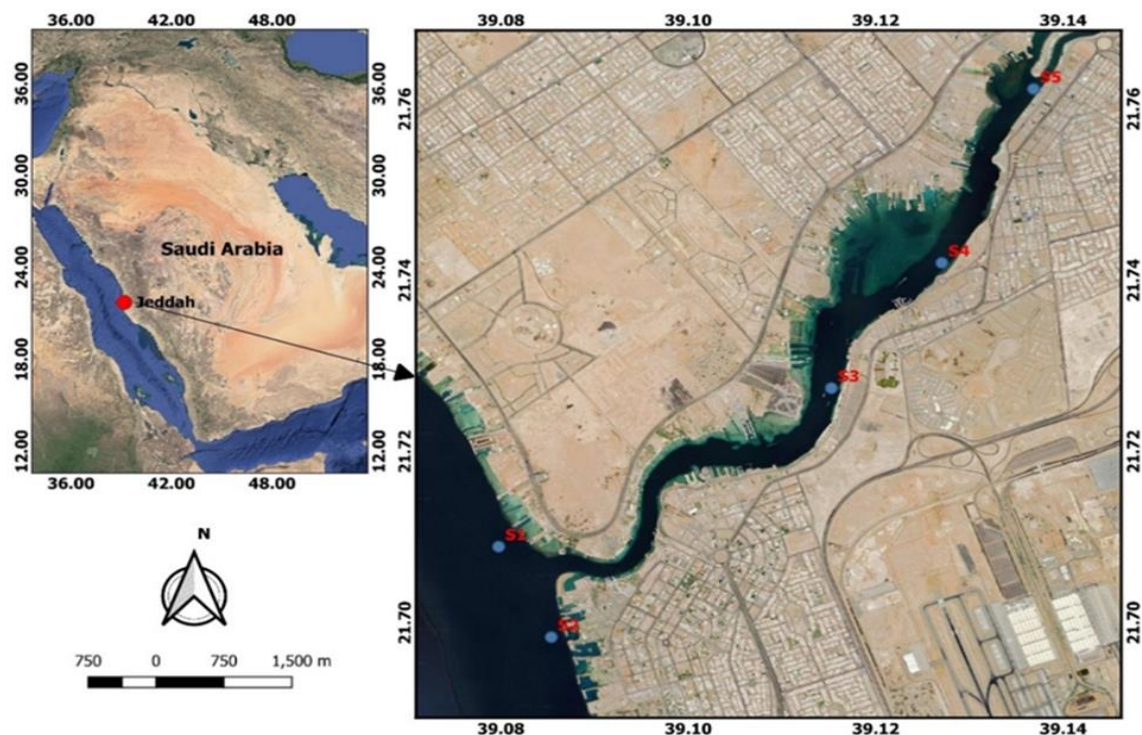


Fig. 1. Sampling stations

3. Characterization of LAB isolates

Gram staining and biochemical testing of the pure isolates were performed according to the methods described by **Reiner (2010)** and **Shields and Cathcart (2010)**, respectively. For the catalase test, a LAB colony was placed on a glass slide, and a drop of 3% hydrogen peroxide was added to completely cover the colony. The immediate appearance of bubbles indicated a catalase-positive result. Catalase-negative LAB isolates were selected for the evaluation of probiotic properties. For the oxidase test, a colony of each isolated LAB strain was smeared on Whatman no. 1 filter paper previously soaked with a freshly prepared 1% m/v aqueous solution of N, N, N, N-tetramethyl-p-phenylene diamine dihydrochloride; the dark purple that appeared within 10s indicated that the result was oxidase-positive.

4. Screening for potential probiotics

4.1. Hemolytic assay

Five LAB were grown in MRS agar culture. These five LAB isolates were subjected to tests for hemolytic activity, acid tolerance, and bile salt solubility to determine probiotic potential. The hemolytic assay was carried out on Columbia agar supplemented with 5% (v/v) human blood according to the procedure described by **Buxton (2005)** and **Chahad *et al.* (2012)**. Each isolate was first cultured overnight in MRS broth to obtain a fresh culture. Subsequently, a loop of MRS broth culture was

adjusted to an optical density (OD) of 0.4 at 600nm using a spectrophotometer (Shimadzu UV mini-1240 CE, Watertown, Massachusetts, USA) and was then used to inoculate a Columbia agar plate incubated at 37°C for 24h. Hemolysis was observed by color changes, with the appearance of a clear zone considered a positive result (Masalam *et al.*, 2018).

4.2. Acid tolerance test

The acid tolerance test was performed using a modified version of the method described by Conway *et al.* (1987). LAB strains were subcultured into tubes containing 10mL of freshly prepared MRS broth with SSW at 8.2 pH, and incubated aerobically overnight at 37°C. MRS broth was also prepared separately, and the pH was adjusted to 2.5, 3.0, and 3.5 by gradual addition of HCl. An aliquot of each LAB strain (1mL at an OD₆₀₀ = 0.4) was transferred to each of the MRS broths (9mL) at different pH and analyzed in triplicate. The pH of the control broth and FSNSW was 8.2. The resulting cultures were incubated at 37°C, and cell viability was determined by inoculating MRS agar plates with 0.1ml of the cultures after 1, 2, 3, and 4h of incubation sequentially. The plates were incubated for 24h at 37°C under aerobic conditions before counting the cells. The experiment was repeated three times, and the mean of viable cells was calculated.

4.3. Bile salt solubility test

A bile salt solution was prepared by dissolving 0.30 g of bile salt (Oxoid®B8756-10G, Bile Salts for microbiology, Sigma Aldrich, New Zealand) in 1.0L of FSNSW (Shehata *et al.*, 2016). Two types of MRS broth culture media were then prepared, one with bile salt solution and one with FSNSW. The medium was autoclaved and allowed to cool to an ambient temperature before inoculation. For testing, 10mL of either bile salt MRS or FSNSW-MRS broth was added to each of the two test tubes the isolate was inoculated into both media and incubated at 37°C for 24h. Both tubes of each strain were incubated at 37°C for up to 3h, and the OD₆₀₀ was sequentially measured according to Jin *et al.* (1998) after 0, 1, 2, and 3h to determine the viability of the cells.

5. Antagonistic activity of the LAB isolates

The LAB strains were evaluated for their antagonistic activity to test for antimicrobial properties. Overnight cultures of four clinical bacterial strains, including *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *Acinetobacter baumannii*, and *Escherichia coli*, were prepared, and the turbidity was adjusted to 0.5 McFarland standard using the method of Zapata and Ramirez-Arcos (2015). The suspensions were inoculated onto Müller Hinton agar plates (Himedia, M173-500G, Müller Hinton Agar, India) and spread evenly using a sterile swab stick. The wells were perforated using a sterile cork borer (diameter: 8mm). A 100µl aliquot of the supernatant from each of the LAB strains or the control (distilled water) was added to each well, allowed to stand in a container for 30min, and then left in the refrigerator at 4°C. Incubation then took place at

37°C for 18h. The diameter of the inhibition zone was then measured with a ruler and recorded. Experiments were performed in triplicate, and the mean value across the trials was calculated.

6. Antibiotic sensitivity of the LAB isolates

The antimicrobial susceptibility of the LAB isolates was determined using the Kirby-Bauer disk diffusion method (**Hudzicki, 2009**) and antibiotic-impregnated discs (Oxoid, UK). The following antibiotics were used: erythromycin (E10; 10µg), tetracycline (TE50; 50µg), penicillin (P1.5; 1.5IU), gentamycin (CN30; 30µg), nitrofurantoin (F100; 100 µg), novobiocin (NV5; 5µg), streptomycin (S25; 25µg), and oxytetracycline (OT30; 30µg).

7. 16S rRNA sequencing of the LAB isolates

Genomic DNA from each LAB isolate was extracted and purified using a Quick-DNATM Miniprep Plus Kit (catalog numbers D4068 & D4069; Zymo Research, Irvine, CA 92614, USA), according to the manufacturer's protocol. The universal primers 27F 5'-AGAGTTTGATCTGGCTCAG-3' and 1100R 5'-GGGTTGCGCTCGTTG-3' were used to amplify the 16S rRNA gene region. The PCR conditions were as follows: one cycle at 95°C for 5min, 28 cycles at 95°C for 45sec, annealing at 58°C for 45sec with extension at 72°C for 2min, and a final extension step at 72°C for 10min. The amplified PCR products of the LAB strains were analyzed by agarose gel electrophoresis, purified, and then sequenced commercially by Macrogen Inc (Republic of Korea). The obtained sequences were analyzed using the online Basic Local Alignment Search Tool (BLAST) available at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. The sequences were deposited in the National Center for Biotechnology Information (NCBI) GenBank, and each sequence was assigned accession numbers (OQ568887, OQ568888, OQ568889, OQ568890, and OQ568891). A phylogenetic tree was constructed using the neighbor-joining method developed by **Saitou and Nei (1987)** in the MEGAX standalone application (**Kumar *et al.*, 2018**), then exported in a new format and annotated using Interactive Tree of Life (iTOL) web version 6.7 (**Letunic & Bork, 2021**).

8. Data analysis

SPSS was used to perform statistical analysis on the data (Ver. 19.0 SPSS, Chicago, IL, USA). A one-way ANOVA with a significance level of $P < 0.05$ was employed to compare variations in treatment means. All tests were carried out in three replicates, and the data were presented as the mean plus standard deviations.

SPSS statistical software package (version 23.0) was used for statistical significance in the data analysis of isolates. The analysis of variance (ANOVA) post hoc was used for statistical significances among isolates to evaluate their activities based on probiotic parameters. Differences were considered statistically significant at $P < 0.05$.

RESULTS

1. Isolation of LAB strains

A total of 32 different bacteria were isolated from the gut content of pearl oysters. Potential probiotic LAB strains were screened for growth in MRS medium, gram staining, oxidase, and catalase assays. Of the 32 LAB strains isolated, five LAB strains grew in MRS medium and were Gram positive (rods and cocci), and tested negative for catalase and oxidase. These five LAB strains were selected for identification, screening, and evaluation of presumptive probiotic properties. The strains were identified by comparison with species-level sequences available in the database and were shown to belong to two genera: *Bacillus* (*B. valezensis* and *B. amyloliquefaciens*) and *Staphylococcus* (*S. epidermidis* #1, *S. epidermidis* #2, and *S. hominis*). The percentage identities for the five LAB strains are presented in Table (1).

Table 1. Identification of LAB isolates in the gut content of *P. radiata* from the Red Sea, Saudi Arabia, based on 16S rRNA sequence comparisons

Strain	BLAST analysis	Identity (%)	Identity of species	GenBank accession number
POR1	<i>Bacillus valezensis</i> HMOS16	98.90	<i>B. valezensis</i>	OQ568887
POR2	<i>B. siamensis</i> KCTC 13613	99.02	<i>B. siamensis</i>	OQ568891
POR3	<i>Staphylococcus epidermidis</i> DSM 20044 ^T	99.58	<i>S. epidermidis</i> #1	OQ568888
POR4	<i>S. hominis subsp. hominis</i> DSM 20328	97.18	<i>S. hominis</i>	OQ568889
POR5	<i>S. epidermidis</i> ISP111B	99.58	<i>S. epidermidis</i> #2	OQ568890

2. Probiotic properties

Three of the five LAB strains (*S. epidermidis* #1 POR3, *S. hominis* POR4, and *S. epidermidis* #2 POR5) were positive for α -hemolytic activity, while the remaining two strains exhibited no hemolytic activity (Table 2).

Table 2. Hemolytic activity of LAB strains isolated from the digestive tract of *Pinctada radiata*

LAB strain	Identity	Hemolytic activity
POR1	<i>B. valezensis</i> POR1	No hemolysis
POR2	<i>B. siamensis</i> POR2	No hemolysis
POR3	<i>S. epidermidis</i> #1 POR3	α -hemolysis
POR4	<i>S. hominis</i> POR4	α -hemolysis
POR5	<i>S. epidermidis</i> #2 POR5	α -hemolysis

All five LAB strains grew well in the control pH of value 8.2, and at pH values of 2.5, 3.0, 3.5, and 4.0, even after 3h exposure (Fig. 2). However, at 2.5 pH, the LAB strains

POR2, POR4, and POR5 were significantly different from the other strains ($F=13.4$, $P < 0.05$). At a bile salt concentration of 0.3%, the number of colony forming units (CFU) for POR4 was the highest, followed by those of POR2, POR5, and POR3 with high significant variation ($F=35.4$, $P < 0.05$) (Fig. 2).

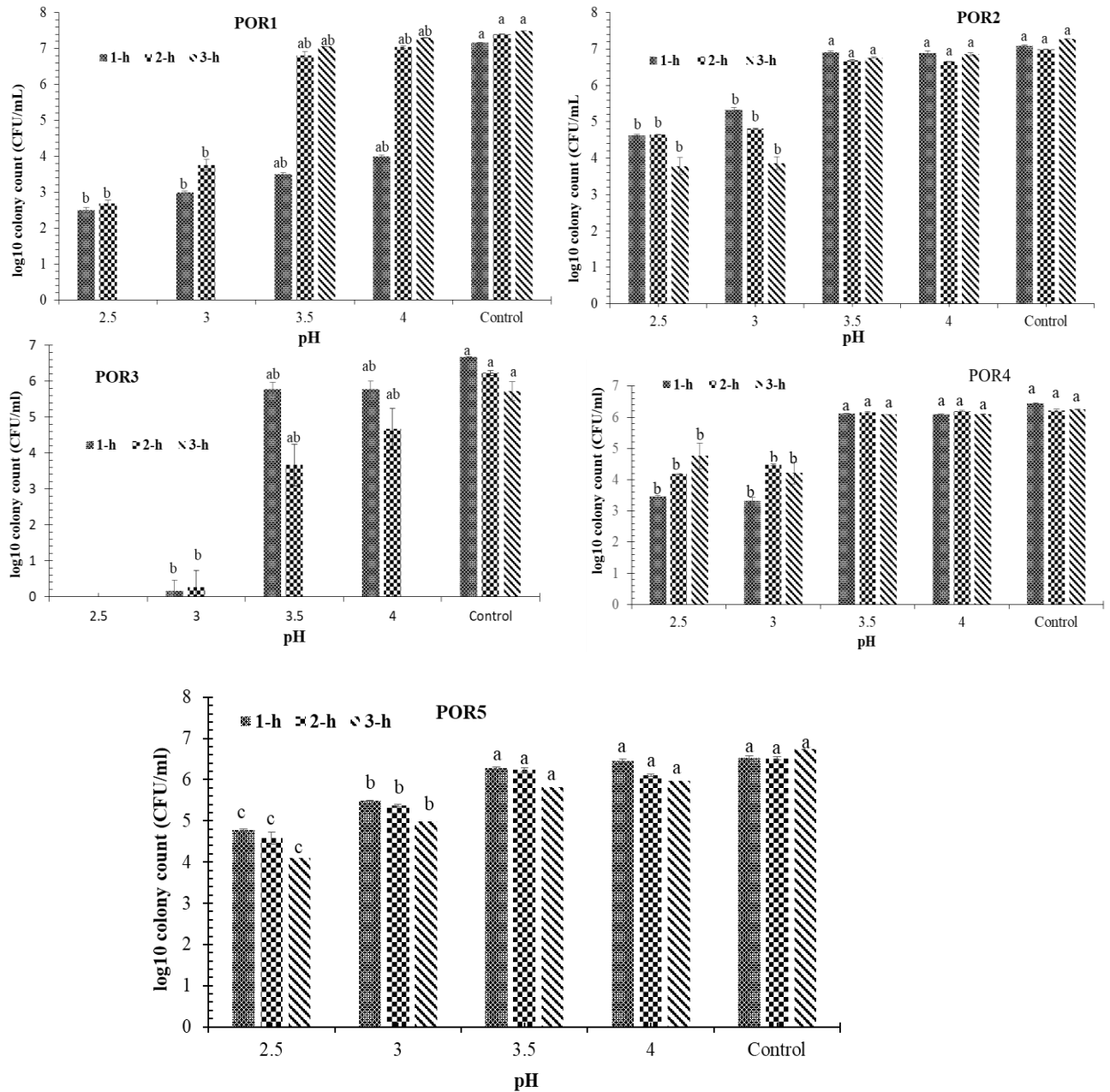


Fig. 2. Acid tolerance test for the five selected LAB strains at 1, 2, and 3h

3. Antagonistic activity of LAB strains

Each of the five LAB strains showed activity against both Gram-positive (*S. aureus* and MRSA) and Gram-negative (*A. baumannii* and *E. coli*) bacteria. The highest

inhibition zones against *S. aureus* were those of POR1 and POR3, followed by POR5, POR4, and the lowest was POR2 although they were all lower than that of the control (Neomycin, 30 μ g) (Table 3). The largest zone of inhibition against methicillin-resistant *S. aureus* (MRSA) was that of POR5, and the smallest was that of POR4. POR2 had the largest zone of inhibition against *A. baumannii*, and it was equal to the zone of inhibition of the control. POR2 also exhibited the highest inhibitory activity against *E. coli*, followed by control and POR4. Analysis of variance (ANOVA) showed that there was a significant difference between strains ($F=1.3$, $P< 0.05$).

Table 3. Antagonistic activity (zone of inhibition) of LAB strains against *S. aureus*, methicillin-resistant *S. aureus* (MRSA), *A. baumannii*, and *E. coli*

	<i>S. aureus</i>	MRSA	<i>A. baumannii</i>	<i>E. coli</i>
POR1	18 \pm 4.2 ^a	15 \pm 3.0 ^b	10 \pm 2.0 ^c	13 \pm 1.0 ^c
POR2	12 \pm 0.6 ^c	14 \pm 3.0 ^c	18.3 \pm 4.5 ^a	24.3 \pm 1.5 ^a
POR3	18 \pm 1.0 ^a	14 \pm 1.0 ^c	11 \pm 2.0 ^c	15 \pm 0.0 ^b
POR4	15 \pm 2.0 ^b	12 \pm 3.0 ^c	16 \pm 1.0 ^b	17.3 \pm 2.5 ^{ab}
POR5	15 \pm 1.0 ^b	16 \pm 2.0 ^b	11 \pm 1.0 ^c	15.3 \pm 1.5 ^b
(Neo30) ¹	21 \pm 1.0 ^a	15 \pm 4.0 ^b	18 \pm 2.0 ^a	22 \pm 2.0 ^a

¹Neomycin 30 μ g (Control). \bar{X} = mean; SD = standard deviation and SE= standard error (n=3).

4. Antibiotic sensitivity and resistant of LAB strains

All five LAB strains were sensitive to erythromycin (E10), nitrofurantoin (F100), and novobiocin (NV5) with significant variation ($F= 5.7$, $P< 0.05$) (Fig. 4). POR1 was sensitive to all antibiotics, except streptomycin (S25). In addition, the resistance of LABs to antibiotics such as gentamycin (CN30), penicillin (P1.5), and streptomycin (S25) was evaluated. POR2 was resistant to penicillin (P1.5), streptomycin (S25), and oxytetracycline (OT30); POR3 was resistant to CN30, P1.5, and S25; POR2 was resistant to P1.5, S25, and OT30; POR5 was sensitive to E10, F100, and NV5; and POR4 was 100% sensitive to all eight antibiotics, with a high significant variation ($F=20.76$, $P< 0.05$). Two-way ANOVA showed that the interaction between antibiotics and probiotics was significantly different ($F=313.57$, $P< 0.05$).

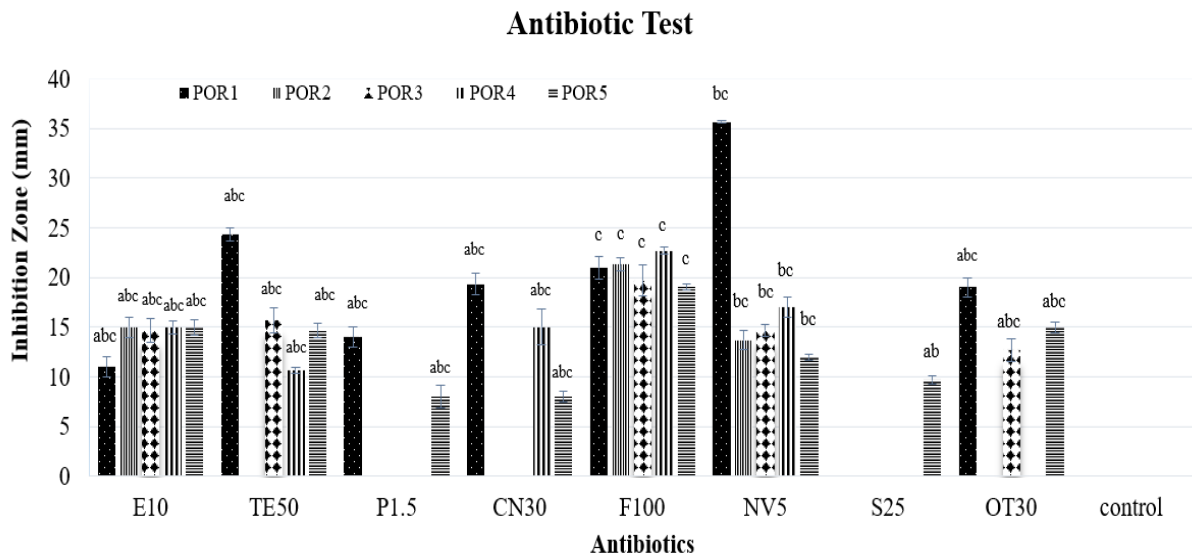


Fig. 4. Sensitivity of the five LAB strains (POR1–5) to the antibiotics erythromycin (E10), tetracyclin (TE50), penicillin (P1.5), gentamycin (CN30), nitrofurantoin (F100), novobiocin (NV5), streptomycin (S25), oxytetracycline (OT30) and the control neomycin 30 (Neo 30)

5. Molecular identification of LAB strains

Clean 1500bp 16S rRNA nucleotide sequence reads for the five strains were obtained after removing ambiguous sequences near the primer ends. The BLAST search results for the obtained sequences showed high similarities (97.18– 99.58%) between the LAB strains and those detected in GenBank (Table 1). Phylogenetic analysis of the identified strains revealed a monophyletic relationship with conspecific or congeneric strains (Fig. 5). These analyses confirmed the identification of these strains as members of the genus *Lactobacillus*.

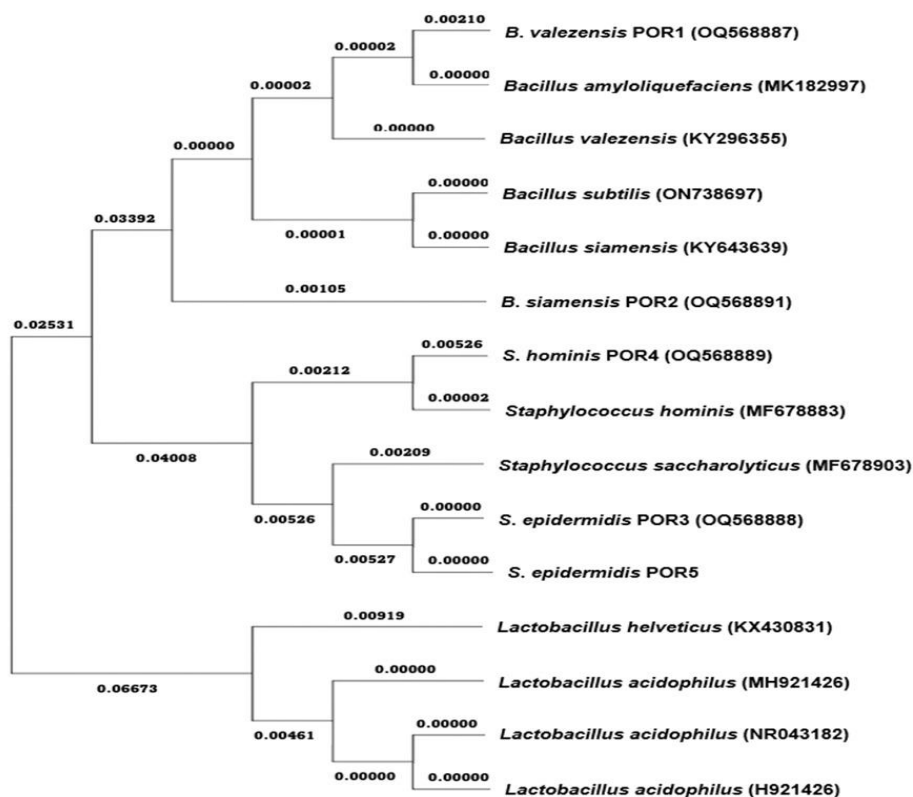


Fig. 5. Phylogenetic tree showing the evolutionary relatedness among the LAB strains (POR1–5) and closely related strains from the NCBI database

DISCUSSION

The use of probiotics in mollusc aquaculture is important for strengthening the immune system, which in turn increases the survival rate and ensures the growth of target animals (C De *et al.*, 2014). The use of native bacteria as probiotic candidates is a sustainable strategy to overcome the adverse effects of commercial probiotics, such as Aquastar®, EM® and MicroPan®. Probiotic bacteria used in aquaculture are expected to function and survive under various physiological conditions, including those of the host gastric region. All organisms in this study were isolated from the gut of *P. radiata*, and the five selected isolates were generally more tolerant to stressful conditions (e.g., low pH, bile salt exposure, antagonistic microbial activity, and antibiotic resistance), which makes them prospective probiotic candidates. Jose *et al.* (2015a) reported that LAB isolated from the gut of a cow can withstand many adverse conditions such as gastric acidity, bile salts, and competition with other microbiota. The LAB strains selected for screening as presumptive probiotics were gram positive, similar to other LAB strains shown to have potential probiotic applications. For example, Escamilla-Montes *et al.* (2015) reported that Gram-positive bacteria are preferred in mollusk aquaculture due to their safety, which is a major criterion in aquaculture to avoid food-borne illnesses and avoid undesirable side effects when consumed.

LAB strains also possess other probiotic properties useful for aquaculture applications. Based on their hemolytic activity, three of the five strains (POR3, POR4, and POR5) showed no hemolysis (gamma hemolysis), indicating that they are non-toxic and harmless when used as probiotics. Hemolytic strains are usually avoided in aquaculture because lysis of the animal's red blood cells occurs (Dàvila *et al.*, 2006). Furthermore, strains with high tolerance to acidity and bile salts are presumed to be good probiotic candidates due to their ability to survive in the small intestine, containing bile salts, and the acidic stomach environment (Abhisingha *et al.*, 2018). The ability to resist these two conditions is an important criterion for selecting potential probiotic bacterial strains (Shehata *et al.*, 2016). In the present study, four LAB isolates of *P. radiata* showed high resistance to bile salt solubility after 3h and high acidic tolerance at a pH as low as 2.5.

The antagonistic activity of probiotic bacteria is an important property that expresses the ability of probiotic strains to protect cultured animals against opportunistic pathogens and prevent spoilage and disease (Chizhayeva *et al.*, 2022). This feature suggests the ability of organisms to produce compounds such as bacteriocins, which are commonly produced by bacteria and have antibiotic properties. The production of antagonistic compounds by LAB strains is a promising replacement for chemotherapeutic agents used in aquaculture (Balcázar *et al.*, 2007). LAB isolates from *P. radiata* showed antibiotic activity against both Gram-positive and Gram-negative bacteria, suggesting that the organisms have antagonistic inhibitory advantages against opportunistic pathogens.

The LAB strains of *P. radiata* were also sensitive to some commonly used antibiotics, which is an important requirement for reducing the effects of antibiotic resistance in aquaculture. A major requirement of probiotics is that resistance genes are naturally innate, and these genes cannot be transferred to other bacteria through probiotic ingestion (Jose *et al.*, 2015b). Therefore, prospective probiotic candidates should be screened to prevent the spread of resistance genes in aquaculture environments. The five LAB strains analyzed in this study were sensitive to at least three different antibiotics (erythromycin [E10], nitrofurantoin [F100], and novobiocin [NV5]).

CONCLUSION

In this study, five LAB strains were isolated from the gut of *P. radiata*, and their probiotic properties were analyzed relative to their potential application in the aquaculture of the host organism, the rayed pearl oyster. The LAB probiotic strains exhibited desirable properties, including safety, tolerance to stomach and small intestine, antagonism in pathogens, and susceptibility to antibiotics, which make them suitable for use in aquaculture, promoting a healthy environment and minimizing the risk of antibiotic resistance. LAB isolates POR1 and POR5, in particular, possessed the strongest relevant probiotic properties, suggesting they would promote the growth and health of pearl oysters in aquaculture. Future experiments should assess the effects of POR1 and POR5 using *in vivo* pearl oyster aquaculture experiments.

Abbreviations: CFU, colony forming units; CN30, gentamycin; E10, erythromycin; F100, Nitrofurantoin; FSNSW, filtered sterilized natural seawater; LAB, lactic acid bacteria; MRS agar, Man, Rogosa, and Sharpe agar; NV5, novobiocin; OD, optical density; OT30, oxytetracycline; P1.5, penicillin; S25, streptomycin; TE50, tetracycline.

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REFERENCES

- Abhisingha, M.; Dumnil, J. and Pitaksutheepong, C.** (2018). Selection of potential probiotic *Lactobacillus* with inhibitory activity against *Salmonella* and fecal coliform bacteria. *Pro.Antimicrobial. Prot.*, 10: 218-227.
- Akhter, N.; Wu, B.; Memon, A.M. and Mohsin, M.** (2015). Probiotics and prebiotics associated with aquaculture: a review. *Fish. shellfish. Immunol.*, 45: 733-741.
- Aparicio, B.C. and Méndez, I.M.** (2021). First record of the potentially invasive *Pinctada imbricata fucata* (Gould, 1850) (*Bivalvia Pteriidae*) along the Mediterranean coasts of Spain. *Bio. J.*, 12: 585–588.
- Azra, M.N.; Okomoda, V.T.; Tabatabaei, M.; Hassan, M., and Ikhwanuddin, M.** (2021). The contributions of shellfish aquaculture to global food security: assessing its characteristics from a future food perspective. *Front. Mar. Sci.*, 8: 654897.
- Balcázar, J.L.; Vendrell, D.; de Blas, I.; Ruiz-Zarzuela, I.; Gironés, O. and Múzquiz, J.L.** (2007). In vitro competitive adhesion and production of antagonistic compounds by lactic acid bacteria against fish pathogens. *Vet. Microbiol.*, 122: 373-380.
- Bin Masalam, M.S.; Bahieldin, A.; Alharbi, M.G.; Al-Masaudi, S.; Al-Jaouni, S.K.; Harakeh, S.M. and Al-Hindi, R.R.** (2018). Isolation, molecular characterization and probiotic potential of lactic acid bacteria in Saudi raw and fermented milk. <https://doi.org/10.1155/2018/7970463>.
- Buxton, R.** (2005). Blood agar plates and hemolysis protocols. *A. S. M.*, 1-9.
- De, B.C.; Meena, D.K.; Behera, B.K.; Das, P.; Das Mohapatra, P.K., and Sharma, A.P.** (2014). Probiotics in fish and shellfish culture: immunomodulatory and ecophysiological responses. *Fish Physiol. Biochem.*, 40: 921-971.
- Chahad, O.B.; El Bour, M.; Calo-Mata, P.; Boudabous, A. and Barros-Velázquez, J.** (2012). Discovery of novel biopreservation agents with inhibitory effects on growth of food-borne pathogens and their application to seafood products. *Res. Microbiol.*, 163: 44-54.

- Chizhayeva, A.; Amangeldi, A.; Oleinikova, Y.; Alybaeva, A. and Sadanov, A.** (2022). Lactic acid bacteria as probiotics in sustainable development of aquaculture. *Aquat. Living Resour.*, 35: 1-17.
- Conway, P.L.; Gorbach, S.L. and Goldin, B.R.** (1987). Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *J. Dairy Sci.*, 70: 1-12.
- Dàvila, E.; Zamora, L.M.; Pla, M.; Carretero, C. and Parés, D.** (2006). Identification and antagonistic activity of lactic acid bacteria occurring in porcine blood from industrial slaughterhouses—a preliminary study. *Int. J. Food Microbiol.*, 107: 207-211.
- Doğan, A. and Nerlović, V.** (2008). On the occurrence of *Pinctada radiata* (Mollusca: Bivalvia: Pteriidae), an alien species in Croatian waters. *Acta Adriat: Int. J. Mar. Sci.*, 49: 155-158.
- Dubey, R.C. and Maheshwari, D.K.** (2012). *Practical Microbiology*. 2nd ed., S. Chand Co. P Ltd, New Delhi, pp. 413.
- Escamilla-Montes, R.; Luna-Gonzalez, A.; Flores-Miranda, M.; Alvarez-Ruiz, P.; Fierro-Coronado, J.A.; Sánchez-Ortiz, A.C. and Avila-Leal, J.** (2015). Isolation and characterization of potential probiotic bacteria suitable for mollusk larvae cultures. *Thai. J. Vet. Med.*, 45: 11-21.
- Forhad, M.H.; Rahman, S.M.K.; Md. Rahman, S.; Saikot, F.K. and Biswas, K.C.** (2015). Probiotic Properties Analysis of Isolated Lactic Acid Bacteria from Buffalo Milk. *Arch. Clin. Microbiol.*, 7: 5-10.
- FAO.** (2022). *The State of World Fisheries and Aquaculture. Towards Blue Transformation*. Rome, FAO., 266 p.
- Gatesoupe, F.J.** (2008). Updating the importance of lactic acid bacteria in fish farming: natural occurrence and probiotic treatments. *Microb. Physiol.*, 14: 107-114.
- Gavrilović, A.; Piria, M.; Guo, X.-Z.; Jug-Dujaković, J.; Ljubučić, A., Krkić, A.; Iveša, N.; Marshall, B.A. and Gardner, J.P.A.** (2017). First evidence of establishment of the rayed pearl oyster, *Pinctada imbricata radiata* (Leach, 1814), in the eastern Adriatic Sea. *Mar. Pollut. Bull.*, 125: 556-560.
- Hai, N.V.** (2015). The use of probiotics in aquaculture. *J. Appl. Microbiol.*, 119: 917-935.
- Hoseinifar, S.H.; Sun, Y.Z.; Wang, A. and Zhou, Z.** (2018). Probiotics as means of diseases control in aquaculture, a review of current knowledge and future perspectives. *Front. Microbiol.*, 9: 2429.
- Hudzicki, J.** (2009). Kirby-Bauer disk diffusion susceptibility test protocol. *A. S. M.* 15: 55-63.
- Jamal, M.T.; Abdulrahman, I.A.; Al Harbi, M. and Chithambaran, S.** (2019) Probiotics as alternative control measures in shrimp aquaculture: A review. *J. Appl. Biol. Biotech.*, 7: 69-77.

- Jin, L.Z.; Ho, Y.W.; Abdullah, N. and Jalaludin, S.** (1998). Acid and bile tolerance of *Lactobacillus* isolated from chicken intestine. *Lett. Appl. Microbiol.*, 27: 183-185.
- Jose, N.M., Bunt, C.R., and Hussain, M.A.** (2015a). Comparison of Microbiological and Probiotic Characteristics of *Lactobacilli* Isolates from Dairy Food Products and Animal Rumen Contents. *Microorganisms.*, 3: 198-212.
- Jose, N.M.; Bunt, C.R. and Hussain, M.A.** (2015b). Implications of Antibiotic Resistance in Probiotics. *Food Rev. Int.*, 31: 52-62.
- Kumar, P.; Nagarajan, A. and Uchil, P.D.** (2018). Analysis of cell viability by the lactate dehydrogenase assay. *Cold Spring Harb. Protoc.*, 6: 465-468.
- Letunic, I. and Bork, P.** (2021). Interactive Tree of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res.*, 49: 293-296.
- Maresca, D.; Zotta, T. and Mauriello, G.** (2018). Adaptation to aerobic environment of *Lactobacillus johnsonii/gasseri* strains. *Front. Microbiol.*, 9: 157.
- Moutopoulos, D.K.; Minasidis, V.; Ziou, A.; Douligeri, A.S.;Katselis, G. and Theodorou, J.A.** (2022). Investigating the Acceptance of a New Bivalve Product in the Greek Shellfish Market: The Non-Indigenous Pearl Oyster *Pinctada imbricata radiata*. *J. mar. sci. eng.*, 10: 251.
- Nasr, D.H.** (1984). Feeding and growth of the pearl oyster *Pinctada margaritifera* (L.) in Dongonab Bay, Red Sea. *Limnology and marine biology in the Sudan, Develop. Hydrobiol.*, 21: 241-245.
- Pandiyan, P.; Balaraman, D.; Thirunavukkarasu, R.; George, E.G.J.; Subaramaniyan, K.; Manikkam, S. and Sadayappan, B.** (2013). Probiotics in aquaculture. *Drug Invent. Today.*, 5: 55-59.
- Pérez-Sánchez, T.; Mora-Sánchez, B. and Balcázar, J.L.** (2018). Biological approaches for disease control in aquaculture: advantages, limitations and challenges. *Trends Microbiol.*, 26: 896-903.
- Png-Gonzalez, L.; Aguilo-Arce, J.; Vázquez-Luis, M. and Carbonell, A.,** (2021). New occurrence of *Pinctada imbricata radiata* (Leach, 1814) in the Balearic Archipelago (NW Mediterranean Sea). *Bioinvasions Rec.*, 10: 853–858.
- Reiner, K.** (2010). Catalase test protocol. *A. S. M.*, 1-6.
- Ringø, E.; Hoseinifar, S.H.; Ghosh, K.; Doan, H.V.; Beck, B.R. and Song, S.K.** (2018a). Lactic Acid Bacteria in Finfish—An Update *Front. Microbiol.*, 9: 1-37.
- Ringø, E.; Van Doan, H.; Lee, S.H.; Soltani, M.; Hoseinifar, S.H.; Harikrishnan, R. and Song, S.K.** (2020b). Probiotics, lactic acid bacteria and bacilli: interesting supplementation for aquaculture. *J. Appl. Microbiol.*, 129: 116-36.
- Saitou, N. ad Nei, M.** (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, 4: 406-425.
- Sahu, M.K.; Swarnakumar, N.S.; Sivakumar, K.; Thangaradjou, T. and Kannan, L.** (2008). Probiotics in aquaculture: importance and future perspectives. *Indian J. Microbiol.*, 48: 299-308.

- Shehata, M. G.; El Sohaimy, S.A.; El-Sahn, M.A. and Youssef, M.M.** (2016). Screening of isolated potential probiotic lactic acid bacteria for cholesterol lowering property and bile salt hydrolase activity. *Ann. Agric. Sci.*, 61: 65-75
- Shields, P. and Cathcart, L.** (2010) Oxidase test protocol. *A. S. M.*, 1-9.
- Subhash, S.K. and Lipton, A.P.** (2007). Effects of a probiotic bacterium, *Lactobacillus acidophilus*, on the growth and survival of pearl oyster (*Pinctada margaritifera*) Spat. *Isr J Aquacult-Bamid.*, 59: 201-205.
- Tharmaraj, N. and Shah, N.P.** (2003). Selective enumeration of *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, bifidobacteria, *Lactobacillus casei*, *Lactobacillus rhamnosus*, and propionibacteria. *J.Dairy. Sci.*, 86: 2288-2296.
- Theodorou, J.A.; Perdikaris, C. and Spinos, E.** (2019). On the occurrence of rayed pearl oyster *Pinctada imbricata radiata* (Leach, 1814) in Western Greece (Ionian Sea) and its biofouling potential. *Biharean Biol.*, 13: 4-7.
- Tlig-Zouari, S.; Rabaoui, L.; Irathni, I. and Ben Hassine, O.K.** (2009). Distribution, habitat and population densities of the invasive species *Pinctada radiata* (Molluca: Bivalvia) along the Northern and Eastern coasts of Tunisia. *Cah. Biol. Mar.*, 50: 131-142.
- Yigitkurt, S.; Lök, A.; Kirtik, A.; Acarli, S.; Kurtay, E.; Küçükdermenci, A. and Durmaz, Y.** (2020). Spat efficiency in the pearl oyster (Leach, 1814) in the surface and bottom water at Karantina Island. *Oceanol. Hydrobiol. Stud.*, 49: 184-192.
- Zapata, A. and Ramirez-Arcos, S.** (2015). A comparative study of McFarland turbidity standards and the Densimat photometer to determine bacterial cell density. *Curr. Microbiol.*, 70: 907-909.