






Probiotic Potential of *Bacillus pumilus* COFAHE_Pro08 and *Lysinibacillus macroides* COFAHE_Pro06 Isolated from the Intestine of *Labeo catla*

Wangkheimayum Malemnganbi Devi¹ , Surajkumar Irungbam¹ , Monalisha Kumar¹ , Tanmoy Gon Choudhury^{1,*} , Dibyendu Kamilya^{2,*} 

¹Central Agricultural University (Imphal), Dept. of Aquatic Health and Environment, College of Fisheries, Lembucherra – 799210, Tripura, India.

²Indian Institute of Technology Kharagpur, Agricultural and Food Engineering Department, Kharagpur – 721302, West Bengal, India.

How to Cite

Devi, W.M., Irungbam, S., Kumar, M., Choudry, T.G., Kamilya, D. (2024). Probiotic Potential of *Bacillus pumilus* COFAHE_Pro08 and *Lysinibacillus macroides* COFAHE_Pro06 Isolated from the Intestine of *Labeo catla*. *Turkish Journal of Fisheries and Aquatic Sciences*, 24(1), TRJFAS24144. <https://doi.org/10.4194/TRJFAS24144>

Article History

Received 02 June 2023

Accepted 08 August 2023

First Online 11 August 2023

Corresponding Author

Tel.: +919615232420

E-mail: tanmoygc@gmail.com

dibyendu@agfe.iitkgp.ac.in

Keywords

Aquaculture

Autochthonous bacteria

In vitro

Probiotic

Abstract

This study aimed to evaluate the autochthonous bacterial strains of intestinal origin from the *Labeo catla* for their prospective use as probiotic. COFAHE_Pro08 and COFAHE_Pro06 were selected (out of 31 bacteria strains) for their potent antimicrobial activity against pathogenic *Aeromonas hydrophila* ATCC 7965. The Pro08 and Pro06 strains were identified as *Bacillus pumilus* and *Lysinibacillus macroides*, respectively, by 16S rRNA sequencing. The Pro08 showed a high multi-specific antibiosis efficiency score and a high spectrum of antibacterial activity. The Pro08 showed more pH and bile tolerance than the Pro06. In the *in vitro* adhesion study, Pro06 exhibited more cell-surface hydrophobicity while Pro08 showed a better capability of auto and co-aggregation along with biofilm-forming ability. The Pro08 significantly adhered to mucosal-surfaces. The Pro08 showed positive protease, amylase, lipase, and cellulase activity, the Pro06 was positive only for lipase activity. The radical-scavenging activity was significantly higher in Pro08 than Pro06. The Pro06 was resistant to oxacillin antibiotics, while pro08 was susceptible to all the tested antibiotics. Both strains were non-haemolytic and were non-toxic to fish embryos. No mortality or clinical-signs were observed during the challenged study in *L. catla*. *B. pumilus* exhibited better probiotic attributes than *L. macroides* for a novel application in aquaculture.

Introduction

Over the past twenty years, there has been an increasing interest in leveraging probiotics to improve the nutritional value of feed and minimize the prevalence of fish diseases (Verscheure et al., 2000; Balcázar et al., 2006). Probiotics, by and large, are live microorganisms that confer beneficial effects to the host upon feeding *ad libitum* (Hill et al., 2014). Interlacing their multifarious peculiarities, probiotics have increasingly been viewed as an alternative to antibiotic treatment (Verschuere, 2000), which can counter the exalted risk of infectious fish diseases. These

diseases are often the result of intensified or semi-intensified aquaculture systems, which create favorable conditions for the microbes to multiply with simultaneous compromise of fish immunity due to stress (Lio-Po and Lim, 2014).

In Indian freshwater aquaculture, Indian major carps (IMCs) are economically important fish and are frequently infected by motile aeromonads, with *Labeo catla* being the most susceptible to the infection (Khatri et al., 2009). To address this, there is a prudent use of chemotherapeutics, such as antibiotics, chemicals, drugs, etc., in the aquaculture system (Hall, 2011; Naylor et al., 2009). However, such indiscriminate practices

accelerate the potential accumulation of antibiotic residue and antibiotic-resistant bacteria, raising public health concerns (Sapkota et al., 2008; Sargenti et al., 2020). Herein, with a comprehensive contemplation of the available literature, probiotics have been proposed as an eco-friendly and bio-pleasant alternative to chemotherapeutics in aquaculture.

Several bacterial species from various genera have been characterized and validated for their probiotic potentials, among which many fall into the genus *Bacillus* (Kuebutornye et al., 2019), a few under the genus *Lysinibacillus* (Mani et al., 2021; Lian-Vidriales et al., 2021), etc. However, since there is no guarantee of having probiotic attributes in all the strains of a species, there is a need to refine *in vitro* tests to predict the viability of probiotics to function in the host. There are notable findings on probiotics that have claimed the health benefits relating to their consumption and this mainly resulted from the method employed during the selection, characterization, and validation of strains for their probiotic potential. Probiotics must be able to impact their benefits on the host through growth or their activities in the body (Morelli, 2000). The intestinal tract provides a complex physiological environment and harbours many microorganisms involved in the nutrition and health of the host (Ray et al., 2012). It is well-recognized that host-associated autochthonous microbes are more competent and efficient than probiotics of non-native origin (Khan et al., 2022). The physiological activities of the microbes are highest in their natural habitat, and microbes can coevolve with the host, possibly leading to beneficial bacteria mutation (Huang et al., 2020; Ibrahim, 2015).

Considering the benefits of autochthonous bacteria, the current study was undertaken to characterize probiotic attributes of bacteria isolated from the intestine of a commercially important Indian major carp species, *L. catla*. A comparative assessment of two isolated potential probiotic strains (*Bacillus pumilus* and *Lysinibacillus macrolides*) was carried out using a range of assays.

Materials and Methods

Isolation of Gut Bacteria

Healthy *L. catla* fingerlings of length 13.53 ± 0.47 cm and weight 19.36 ± 0.56 g were procured from a local fish farm (23.14'10.8" N, 91.23'26.6" E) fish farms and allowed to starve for 48h before aseptically excising the gastrointestinal tract. To isolate the endosymbionts, the excised guts were collectively homogenized and serially diluted (up to 10^{-6}) with sterile NaCl solution (0.85% w/v), spread on nutrient agar plates, and incubated for 24 h at 30°C. Morphologically discrete colonies were singled out randomly (n=31) and subcultured to acquire the pure culture of each isolate.

The bacterial isolates were first scrutinized for their anti-microbial activity against an indicator strain of

Aeromonas hydrophila ATCC 7965 using the agar well diffusion assay previously described by Nalawade et al. (2016). Briefly, the nutrient agar plates were swabbed evenly with an overnight culture of the indicator bacteria. After 5-10 min, wells of 10 mm diameter were made and a hundred microlitres of each overnight culture of test strains were loaded into each well. The plates were incubated at 30°C for 24 h, and the strains showing inhibition zone were chosen for molecular identification and characterization.

Molecular Identification of the Isolates

To identify these presumptive probiotic strains, polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene using the universal primers 27F (5' AGAGTTTGATCCTGGCTCAG 3') and 1492R (5' GGTTACCTTGTTACGACTT 3') (Weisburg et al., 1991). The PCR products were electrophoresed on an agarose gel (1.5 %), and the amplicons were observed under a gel documentation unit (Bio-Rad, USA). The amplified product was then purified using a PCR product purification kit (Thermo Fisher Scientific Inc., USA) and sequenced. The sequence alignment was carried out in the BLAST program of NCBI (National Center for Biotechnology Information). A phylogenetic (Maximum Likelihood) tree was created by the MEGA 11 software using 16S rRNA sequence from the strain and closely related species, retrieved from the GenBank. The sequence of the strain was submitted to the NCBI database, and the accession number was obtained.

Antimicrobial Efficiency

The antagonism activity of both isolates was determined by adopting the modified cross-streaking technique of Velho-Pereira et al. (2011). The severity of the antagonism was checked by a parallel streak method (Nakamura et al., 1999). In the cross-streak technique, the nutrient agar plates were prepared and ensued by inoculating test strain on the plates with a single streak (length – 7 cm, width – 4 mm) across the diameter and incubated for 48 h at 30°C. Subsequently, after 48 h of incubation, the indicator strains were streaked perpendicularly to the test strain apart by 3 mm. Notably, the indicator strain streak line had a length of 3 cm with 0.4 cm width, and 1 cm was kept in between the indicator streaks. It was re-incubated at 30°C for 48 h, and the inhibitory activity of the test strain was recorded. To express the antimicrobial efficiency in quantitative score matrices, the following equations were applied:

$$\text{PASDAAS} = (\text{AWG}/\text{TSA}) \times 100 \quad (\text{eq-1})$$

Where, PASDAAS: Percent area - specific differential antibiosis activity score; AWG: Area of indicator strain streak on the plate without growth; TSA: Total streak area of the indicator strain

$$\text{PMSAES} = (\sum \text{PASDAASTP}_{1-2} / \text{TPS}) \times 100 \quad (\text{eq-2})$$

Where, PMSAES: Percent multispecific antibiosis efficiency score; PASDAASTP: Percent area-specific differential antibiosis activity score of indicator strains 1&2; TPS: Total possible score of all the indicator strains (i.e., $100 \times 2 = 200$)

$$\text{POIES} = (\text{TNIS} / \text{TNTS}) \times 100 \quad (\text{eq-3})$$

Where, POIES: percent overall inhibition efficiency score; TNIS: total number of inhibited indicator strains/species; TNTS: Total number of test species (indicator strains/species)

Likewise, the parallel streak technique was carried out by streaking two separate parallel lines (width - 4 mm) of the test strain across the diameter of the nutrient agar plate apart by 3 cm. It was then incubated at 30°C for 48 h and later, a single streak of the indicator strain (length - 7 cm; width - 0.4 cm) was inoculated in the middle of the test streaks. Later, it was re-incubated for another 24 h at 30°C and the severity of the inhibition was recorded.

Tolerance to pH and Bile Salt

The pH and Bile salt tolerance of the isolates were determined by using the modified methods of Prasad et al. (1998) and Nikoskelainen et al. (2001), respectively. Briefly, bacterial suspensions with OD_{600} of 0.25 unit were prepared for each isolate from the overnight culture, which was later inoculated in nutrient broths (HiMedia, Mumbai, India) of varying pH (2- 9) and bile concentrations (0, 2.5, 5, 10, and 20%). It was then incubated at 30°C for 24 h and the changes in growth were observed at 600 nm.

Bacterial Cell Hydrophobicity

A revised technique by Rosenberg et al. (1980) was adopted to inspect the bacterial cell surface hydrophobicity using different organic solvents i.e., xylene, chloroform, and ethyl acetate. In brief, after 48 h of incubation in nutrient broth at 30°C, the bacterial suspension was centrifuged at 10,000 g for 3 min, followed by two times washing with PBS (pH 7.4). The pellet was resuspended in 2 ml of PBS (pH 7.4) and the OD_{600} (OD_a) was measured. Then, each of the three organic solvents (i.e., xylene, chloroform, and ethyl acetate) were mixed with bacterial cell suspension separately in a ratio of 1:1 (v/v) and vortexed for 5 min. The mixture was left static for 30 min to separate into two distinct layers, and the OD of the aqueous layer was taken at 600 nm (OD_b). To calculate the hydrophobicity, the following formula was used:

$$\text{Hydrophobicity (\%)} = (1 - \text{OD}_b / \text{OD}_a) \times 100$$

Auto-aggregation and Co-aggregation

The capacity of the test strains to auto-aggregate and a co-aggregate were evaluated using the methods described by Reneiro et al. (1992) and Collado et al. (2008), respectively. In brief, the 24 h-old broth cultures were centrifuged at 10000 g for 3 min, and the pellet obtained was resuspended in PBS to obtain an OD of 0.5 at 600 nm. The test bacterial-PBS aliquot was incubated at 30°C and observation was taken at 2 h and 4 h to check for auto-aggregation while 0.5 ml of each test strain and the pathogenic (*A. hydrophila*) strains were mixed and incubated without agitation for 24 h to check the co-aggregation capacity. Both the abilities were determined by the following formula:

$$\text{Auto-aggregation (\%)} = 1 - (\text{OD of the upper suspension} / \text{OD of the total bacterial suspension}) \times 100$$

$$\text{Co-aggregation (\%)} = [(\text{OD}_{\text{pathogen}} + \text{OD}_{\text{isolate}}) / 2 - (\text{OD}_{\text{mix}}) / (\text{OD}_{\text{pathogen}} + \text{OD}_{\text{isolate}}) / 2] \times 100$$

Mucosal Adherence

The ability of the probiotic strains to adhere to the fish mucus was evaluated as demonstrated by Chabrillon et al. (2005). Briefly, an anaesthetized fish was dissected to collect the mucus, which was then centrifuged at 12000 g for 15 min. It was filter sterilized, and the obtained supernatant was inoculated with the test strain at 10^6 CFU/ml. The mixture was then incubated for 24 h at 30°C, and the OD at 600 nm was recorded.

Biofilm Formation Test

The biofilm production capacity was qualitatively determined using Congo Red Agar (CRA) medium (HiMedia) [brain heart infusion (BHI) broth - 37 g/L, sucrose - 50 g/L, agar - 10 g/L and Congo Red indicator - 8 g/L] (Freeman et al. 1989). The test strains were inoculated on the CRA plates and incubated for 24 h at 30°C to see for any colour changes.

Extracellular Enzymes Production Assay

The ability of the selected strains to produce extracellular enzymes was assessed qualitatively (Gross & Morell, 1971). The appearance of a clear zone around the colonies of test strains on peptone-gelatin agar (HiMedia) flooded with 15% HgCl_2 indicated positive protease activity. While the affirmation of amylase activity was indicated by a whitish appearance on the periphery of colonies on carboxy methyl cellulose agar (HiMedia) flooded with gram's iodine, the positive lipolytic enzyme activity was illustrated in a similar manner on 1% tributyrin agar (HiMedia). The positive amylase test was determined by the appearance of whitish-yellow discolouration over the starch agar

(HiMedia) media plates flooded with Lugol's iodine solution.

Antioxidant Assay

To determine antioxidant activity, cell-free supernatant was initially prepared by centrifuging the bacterial broth cultures at 8000 g for 10 min (Afify et al., 2012). Ascorbic acid was used as a control in all the measurements. The DPPH and H₂O₂ scavenging activity of the strains were evaluated following the methods described by Elmastas et al. (2006) and Ruch et al. (1989) respectively, and calculated as follows:

$$\text{DPPH scavenging rate (\%)} = 100 - \left[\frac{\text{A}_{\text{sample}} - \text{A}_{\text{blank}}}{\text{A}_{\text{control}}} \times 100 \right]$$

$$\text{H}_2\text{O}_2 \text{ scavenging rate (\%)} = \left[\frac{\text{A}_{\text{control}} - \text{A}_{\text{sample}}}{\text{A}_{\text{control}}} \right] \times 100$$

In vitro Safety Assessment

Antibiotic Susceptibility Test and Haemolytic Test

The antibiotic susceptibility of the test strains was evaluated by Kirby-Bauer's disc diffusion method (Kirby, 2009). The strains were tested against 12 antibiotics [kanamycin (30 mcg), tetracycline (30 mcg), Azithromycin (15mcg), Ampicillin (10 mcg), Erythromycin (15 mcg), Penicillin-G (10 units), Vancomycin (30 mcg), Amoxiclav (30 mcg), Streptomycin (10 mcg), Polymyxin-B (300 units), Gentamicin (10 mcg), Oxacillin (1 mcg)]. The inhibition zones were measured, and the results were interpreted according to the guidelines provided by the Clinical and Laboratory Standard Procedure (CLSI).

The haemolytic ability of the test strains was determined by evaluating their ability to lyse red blood cells (Joseph et al., 1982).

In vivo Safety Assessment

Fish Embryo Toxicity Test

The *in vivo* toxicity test of the strains was conducted according to the guidelines provided by Organisation for Economic Co-operation and Development (OECD, 2006) with some modifications. First, the cell-free supernatants of *B. pumilus* and *L. macroides* were prepared from 24 h-old broth culture by centrifuging at 4000 g for 10 min and proceeded for freeze-drying at -40°C (Prasad et al., 1998).

The *in vivo* toxicity test of the stains was done on the fertilized egg of the common carp *Cyprinus carpio*. The lyophilized supernatants of the two selected strains were applied to the healthy *C. carpio* embryos in separate Petri dishes at a concentration of 2.5 mg/ml each, equivalent to the supernatants obtained from a bacterial concentration of 10⁶ CFU/ml of the strains.

Embryos were incubated at 26±1°C and observed directly under a stereo microscope after 24 h. The acute toxicity was recorded at 4 apical points i.e., coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac, and lack of heart-beat.

Challenge Study

To evaluate the safety of the test strains, an *in vivo* challenge study was conducted. Thirty *L. catla* fingerlings were equally and randomly distributed in three tanks, and the fish were intraperitoneally injected with 100 µl of the bacterial suspension (10¹⁰ CFU/ml). An equal volume of sterile PBS (pH 7.2) was injected into each control group of fish. Fish were observed for 2 weeks for mortalities or clinical signs, if any.

Statistical Analysis

The data were analyzed using the SPSS-29.0.1.0 for Windows program (SPSS Inc., Illinois, USA). The student t-test and one-way analysis of variance (ANOVA) were used to compare mean values. The threshold for probability was set at 0.05 to evaluate significance. The outcomes were represented using a mean and a standard error (SE).

Results

Molecular Identification of the Strains

A total of 25 strains were obtained from *L. catla* gut samples, among which two isolates of different colony morphology and high inhibition zones in the agar well diffusion method were selected. The two strains, COFAHE_Pro08 and COFAHE_Pro06 were identified as *Bacillus pumilus* and *Lysinibacillus macroides*, respectively. The GenBank accession numbers (OP295494 and OP295593) were obtained after submitting the gene sequence of the strains to the NCBI database. The phylogenetic tree showed that the strains were in the same group as other *Bacillus* and *Lysinibacillus* strains (Figure 1).

Antimicrobial Activity

The differential antibacterial activity of the two isolates was deduced against 8 indicator pathogenic stains in cross streaking, and their zones of inhibition were expressed in terms of percent area-specific differential antibiosis activity score (PASDAAS). The highest PASDAAS of 58.3% was recorded in *B. pumilus* against *A. veronii* (MK907586) (Figure 2). The percent multi-specific antibiosis efficiency score (PMSAES) was evaluated using each PASDAAS. Here, the multispecific antibiosis efficiency was more in *B. pumilus* with a score of 32.67%, while *L. macroides* showed a score of only 10.12% (Figure 3). Further, the percent overall inhibition

efficiency score indicated that *B. pumilus* was active against all the indicator pathogen strains (100%) and *L. macroides* were active against only 66.7% of the indicator pathogenic stains (Figure 3). The parallel streaking revealed that *B. pumilus* could inhibit *A. hydrophila* (ATCC 49140 and 35645), and *A. veronii* (MK907586) completely (Table 1).

pH and Bile Tolerance

The strains *B. pumilus* and *L. macroides* were exposed to different pH levels (2.0 – 9.0), and it was observed that both the strains tolerated the pH range with maximum proliferation occurring at pH 8.0 for *B. pumilus* and at pH 7.0 for *L. macroides* (Figure 4). With

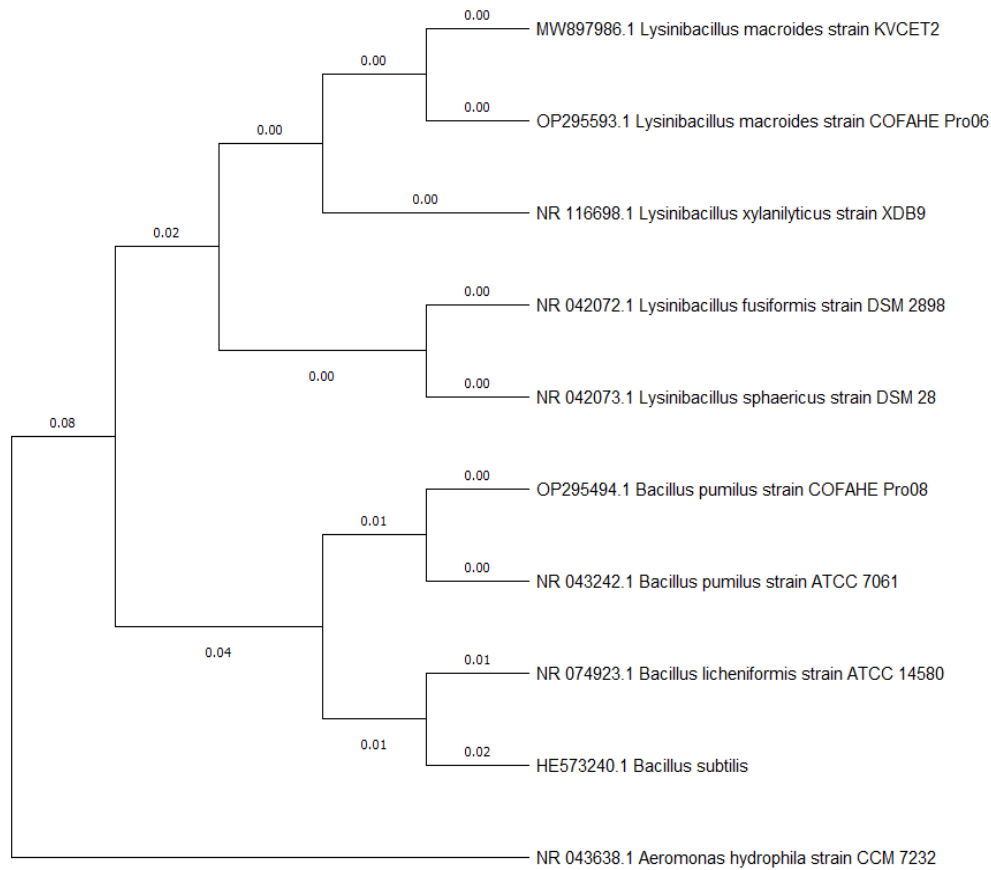


Figure 1. Dendrogram showing the phylogenetic relationship of *Bacillus pumilus* COFAHE_Pro08 and *Lysinibacillus macroides* COFAHE_Pro06 with other closely related strains retrieved from NCBI

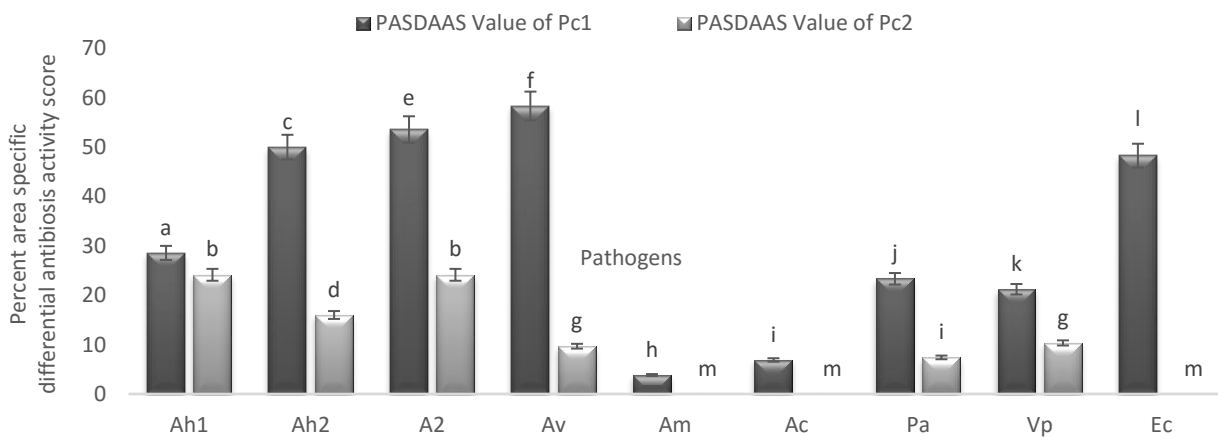


Figure 2. Percent area-specific differential antibiosis activity score (PASDAAS) of the test strains; Data are expressed as mean ± SE. Different superscript lowercase letters in the bar chart imply a significant difference ($P < 0.05$). Pc1: *Bacillus pumilus* COFAHE_Pro08; Pc2: *Lysinibacillus macroides* COFAHE_Pro06; Ah1: *Aeromonas hydrophila* (ATCC 7965); Ah2: *A. hydrophila* (ATCC 49140); A2: *A. hydrophila* (ATCC 35645); Av: *A. veronii* (MK907586); Am: *A. media* (MK907592); Ac: *A. cavaiae* (MK907593); Pa: *Pseudomonas aeruginosa*; Vp: *Vibrio parahaemolyticus* (ATCC 17802); Ec: *Escherichia coli* (ATCC 10536).

the increasing bile concentration, there was a gradual reduction in the growth of the isolates (Figure 5). *B. pumilus* showed a significantly higher ($P<0.05$) bile tolerance compared to *L. macroides*.

Hydrophobicity

The adhering property of the isolates towards the hydrophobic surfaces was evaluated using three solvents i.e., chloroform, xylene, and ethyl acetate. The

hydrophobicity promoted by chloroform and xylene was significantly ($P<0.05$) higher in *L. macroides* than the other strain, while *B. pumilus* showed higher ethyl acetate hydrophobicity (Figure 6).

Auto- and Co-aggregation

The degree of adhesive interaction between the cells of the same isolate (auto-aggregation capacity) was significantly higher ($P<0.05$) in both the test strains than

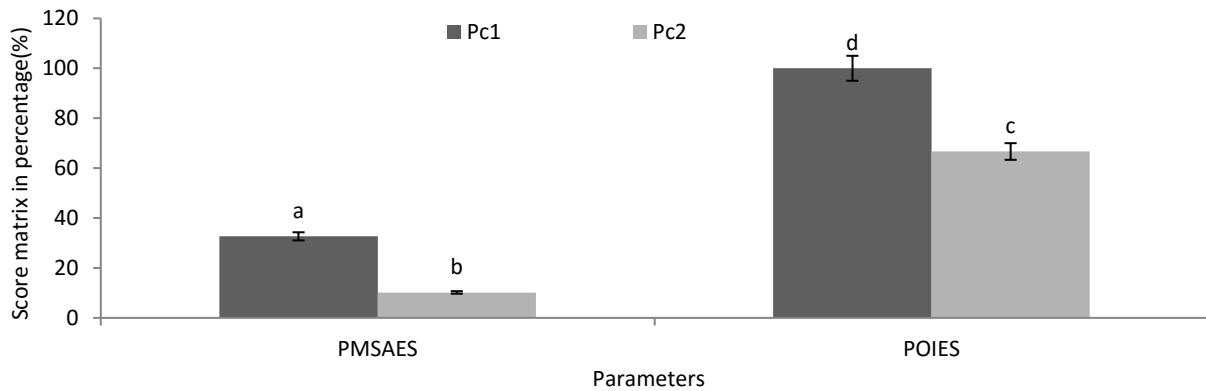


Figure 3. Inhibition efficiency score by the test strains; Data are expressed as mean \pm SE. Different superscript lowercase letters in the bar chart imply a significant difference ($P<0.05$). PMSAES: Percent multispecific antibiosis efficiency score; POIES: Percent overall inhibition efficiency score; Pc1: *Bacillus pumilus* COFAHE_Pro08; Pc2: *Lysinibacillus macroides* COFAHE_Pro06.

Table 1. Inhibition efficiency score of *Bacillus pumilus* COFAHE_Pro08 and *Lysinibacillus macroides* COFAHE_Pro06

Indicator bacteria	<i>Bacillus pumilus</i>	<i>Lysinibacillus macroides</i>
<i>Aeromonas hydrophila</i> (ATCC 7965)	++	++
<i>A. hydrophila</i> (ATCC 49140)	+++	++
<i>A. hydrophila</i> (ATCC 35645)	+++	++
<i>A. veronii</i> (MK907586)	+++	++
<i>A. media</i> (MK907592)	+	+
<i>A. caviae</i> (MK907593)	+	+
<i>Pseudomonas aeruginosa</i>	++	++
<i>Vibrio parahaemolyticus</i> (ATCC 17802)	++	++

Note: ###, complete inhibition; ##, moderate; inhibition; #, low inhibition.

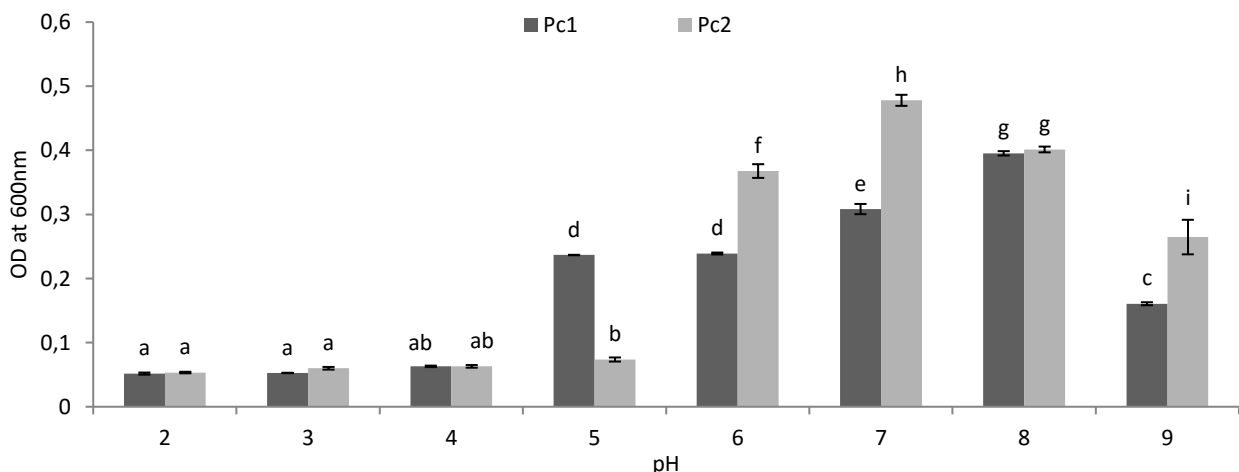


Figure 4. pH tolerance of the test strains. Data are expressed as mean \pm SE. Different superscript lowercase letters in the bar chart imply a significant difference ($P<0.05$). Pc1: *Bacillus pumilus* COFAHE_Pro08; Pc2: *Lysinibacillus macroides* COFAHE_Pro06.

in the pathogenic *A. hydrophila* (ATCC 49140), a non-probiotic control (Figure 7). *B. pumilus* showed a significantly higher ($P<0.05$) auto-aggregation capacity than *L. macroides*. Like auto-aggregation, *B. pumilus* exhibited a significantly higher ($P<0.05$) co-aggregating capacity than *L. macroides* (Figure 8).

Mucosal Adherence

The adhering capacity of *B. pumilus* to mucosal surfaces was significantly higher ($P<0.05$) compared to *L. macroides* and the pathogenic control *A. hydrophila* (ATCC 49140) (Figure 9). However, the adhering capacity

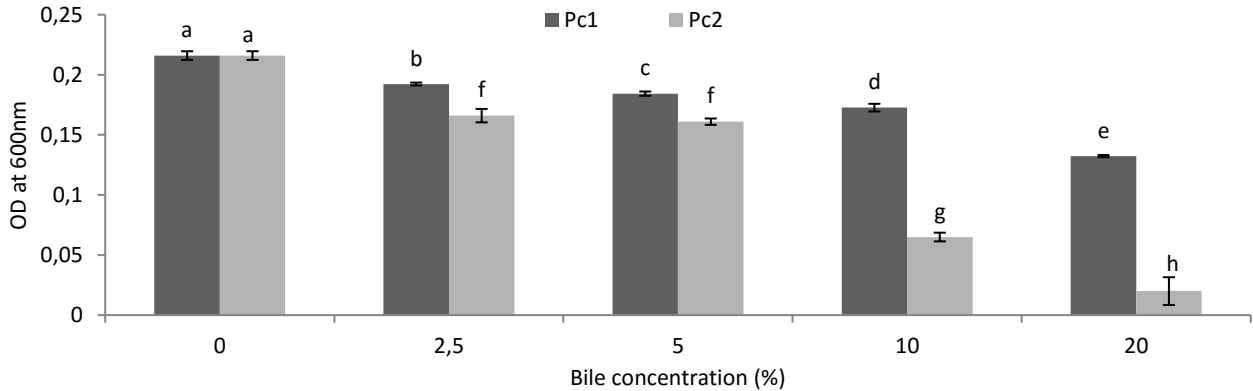


Figure 5. Bile tolerance of the test strains. Data are expressed as mean ± SE. Different superscript lowercase letters in the bar chart imply a significant difference ($P<0.05$). Pc1: *Bacillus pumilus* COFAHE_Pro08; Pc2: *Lysinibacillus macroides* COFAHE_Pro06.

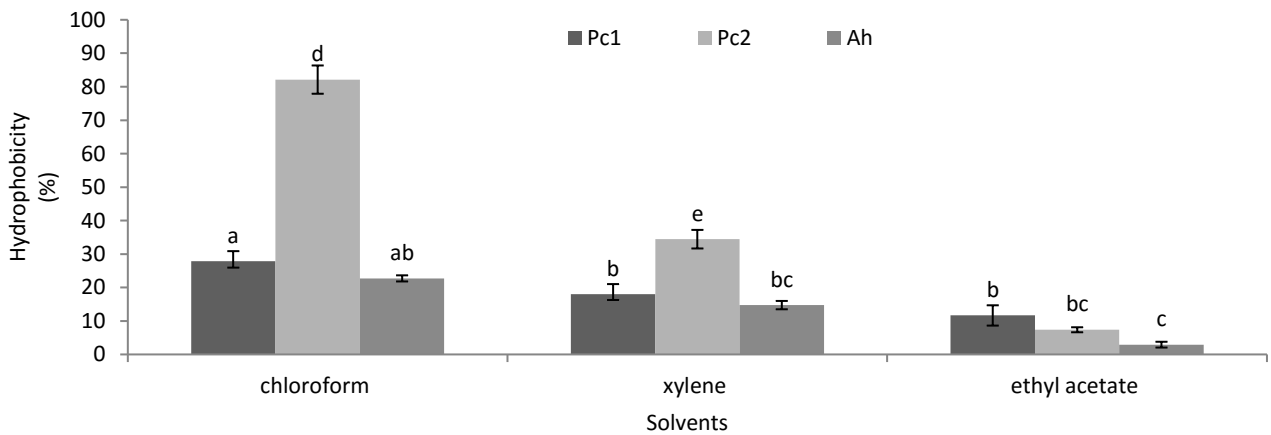


Figure 6. Bacterial cell hydrophobicity of the test strains; Data are expressed as mean ± SE. Different superscript lowercase letters in the bar chart imply a significant difference ($P<0.05$). Pc1: *Bacillus pumilus* COFAHE_Pro08; Pc2: *Lysinibacillus macroides* COFAHE_Pro06; Ah: *Aeromonas hydrophila* (ATCC 49140).

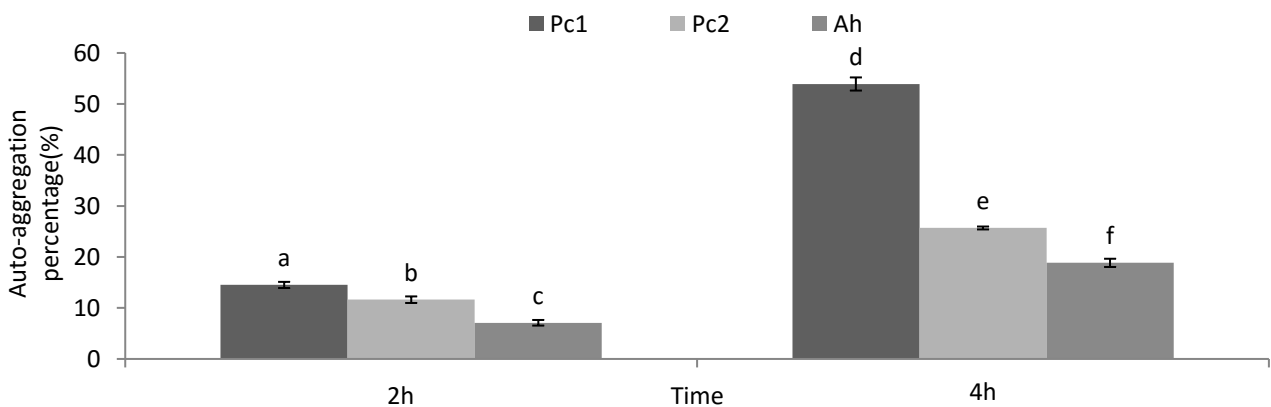


Figure 7. Auto-aggregating capacity by the test strains; Data are expressed as mean ± SE. Different superscript lowercase letters in the bar chart imply a significant difference ($P<0.05$). Pc1: *Bacillus pumilus* COFAHE_Pro08; Pc2: *Lysinibacillus macroides* COFAHE_Pro06; Ah: *Aeromonas hydrophila* (ATCC 49140).

of *L. macroides* was not significantly different ($P>0.05$) than the pathogenic control.

Biofilm Formation Test

In the congo red agar method, the *B. pumilus* strain formed black colonies, which indicated positive biofilm formation capacity, while *L. macroides* did not show any biofilm-forming capacity.

Antioxidant and Extracellular Enzymes Production Assay

The *B. pumilus* strain showed a significantly higher ($P<0.05$) radical scavenging activity than *L. macroides*. However, in comparison to the positive control (L-ascorbic acid), the bacterial strains showed significantly lower ($P<0.05$) scavenging activity (Figure 10).

Based on the qualitative evaluation (Table 2), it was summated that the *B. pumilus* strain had the ability

to produce all four extracellular enzymes (protease, amylase, lipase, and cellulase) while *L. macroides* could produce only lipase.

Antibiotic Susceptibility and Haemolytic Activity

Of the twelve antibiotics tested in this study, *B. pumilus* showed susceptibility to ten antibiotics and intermediate breakpoint status against Polymyxin-B and Oxacillin. On the other hand, *L. macroides* showed resistance to Oxacillin and ‘intermediate breakpoint’ status to Polymyxin-B and Kanamycin (Table 2).

The test isolates did not lyse the blood cells indicating their non-haemolytic (γ -haemolysis) characteristic.

Fish Embryo Toxicity Test and Challenge Study

In the embryo toxicity test of the strains, it was observed that there was growth of fungus on coagulated eggs in the control group and 30% of the embryo were

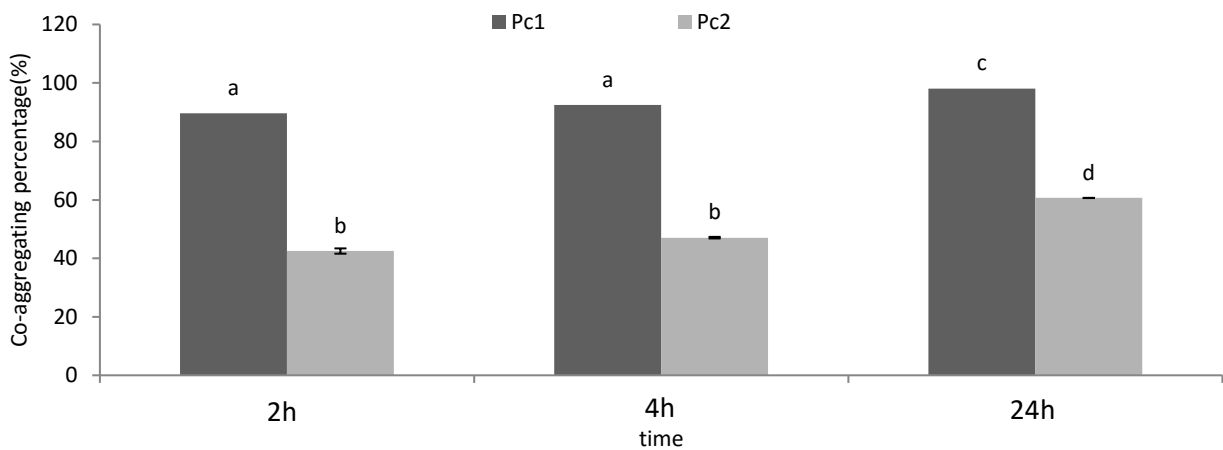


Figure 8. Co-aggregating capacity of the test strains; Data are expressed as mean \pm SE. Different superscript lowercase letters in the bar chart imply a significant difference ($P<0.05$). Pc1: *Bacillus pumilus* COFAHE_Pro08; Pc2: *Lysinibacillus macroides* COFAHE_Pro06.

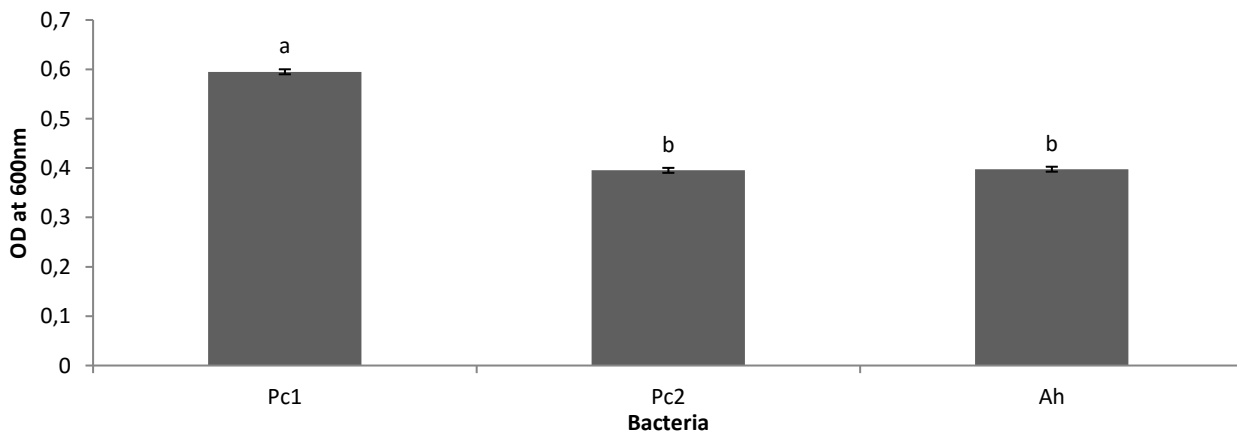


Figure 9. Mucosal adhesion capacity of the test strains. Data are expressed as mean \pm SE. Different superscript lowercase letters in the bar chart imply a significant difference ($P<0.05$). Pc1: *Bacillus pumilus* COFAHE_Pro08; Pc2: *Lysinibacillus macroides* COFAHE_Pro06; Ah: *Aeromonas hydrophila* (ATCC 49140).

at the stage of blastophore closure and somite appearance. While, in the case of embryo treated with *B. pumilus* and *L. macroides*, there were fewer coagulated eggs and no fungal growth. Moreover, these treated eggs were at the advanced stages of embryonic development i.e., detachment of tail, beginning of heartbeat, and upper head directed at the front end of the egg. In this study, none of the four apical endpoints except for the coagulation of fertilized eggs were observed. This feature was observed both in the control and treatment groups. The results indicated that both isolates were not toxic to common carp embryos at a concentration of 10⁶ CFU/ml.

Moreover, after injecting the bacterial suspension of the test strains intraperitoneally, no mortality or clinical signs were observed in the challenged fingerlings which indicated the safety of the strains.

Discussion

In this study, two strains, COFAHE_Pro08, and COFAHE_Pro06, isolated from the intestine of *L. catla* were evaluated for their probiotic potential using a

range of *in vitro* phenotypic assays, *in vitro* safety assessments, *in vivo* fish embryo toxicity test, and challenge study. The 16S rRNA sequencing-based molecular identification determined that COFAHE_Pro08 and COFAHE_Pro06 strains were *B. pumilus* and *L. macroides*, respectively. Both the genera *Bacillus* and *Lysinibacillus* have been reported to have several species of constituent gut symbionts in fish (Huang et al., 2020; Kumar et al., 2008; Mani et al., 2021; Moradi et al., 2019; Seelam et al., 2017; Thankappan et al., 2015).

Both the methods of antimicrobial efficacy detection, i.e., cross- and parallel streaking, revealed that both the test strains repressed all the eight indicator bacteria, and quantitative score matrices for determining the antimicrobial efficiency (PASDASS, PMSAES, and POIES) were significantly higher in *B. pumilus* than that of *L. macroides*. The inhibitory activity of the *Bacillus* spp. can be accounted for their secretion of anti-microbial substances such as nisin (Gross & Morell, 1971), subtylin (Gross & Kiltz, 1973), plantaricin S (Upreti & Hinsdill, 1975), iturin, subtilin, and bacilysin (Chung et al., 2008). *Lysinibacillus* spp. has

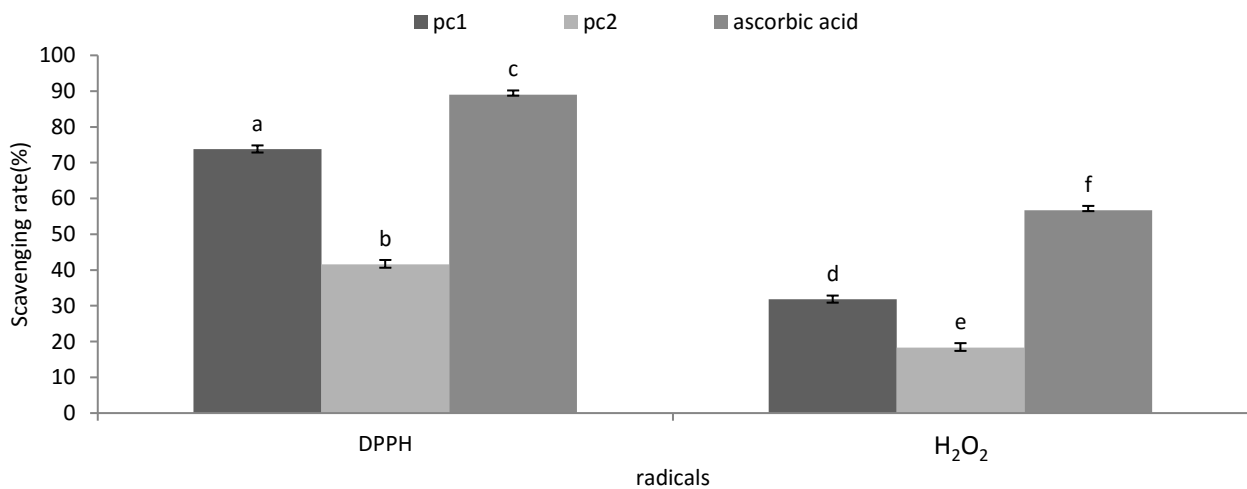


Figure 10. Radical scavenging activity of the test strains. Data are expressed as mean ± SE. Different superscript lowercase letters in the bar chart imply a significant difference (P<0.05). Pc1: *Bacillus pumilus* COFAHE_Pro08; Pc2: *Lysinibacillus macroides* COFAHE_Pro06.

Table 3. Antibiotic susceptibility of *Bacillus pumilus* COFAHE_Pro08 and *Lysinibacillus macroides* COFAHE_Pro06

Antibiotic (concentration/disc)	<i>Bacillus pumilus</i>	<i>Lysinibacillus macroides</i>
Kanamycin (30 mcg)	S	I
Tetracycline (30 mcg)	S	S
Azithromycin (15 mcg)	S	S
Ampicillin (10 mcg)	S	S
Erythromycin(15 mcg)	S	S
Penicillin-G (10 units)	S	S
Vancomycin(30 mcg)	S	S
Amoxiclav (30 mcg)	S	S
Streptomycin (10 mcg)	S	S
Polymyxin-B (300 units)	I	I
Gentamicin (10 mcg)	S	S
Oxacillin (1 mcg)	I	R

Note: S, susceptible; I, intermediate; R, resistant

also been reported to produce anti-microbial substances such as bacilysin, fengycin, and surfactin (Sakthivel et al., 2018). Several studies have reported the antagonism activity of both *Bacillus* spp. and *Lysinibacillus* spp. against fish pathogenic bacteria such as *Vibrio* spp., *Pseudomonas aeruginosa*, *A. hydrophila*, etc. (Kuebutornye et al., 2020; Mani et al., 2021; Ramesh et al., 2015; Reda et al., 2018; Vaseeharan & Ramasamy, 2003)

To manifest this antagonistic ability of these combative strains, they must first be able to survive and colonize in the hostile environment (low pH and high bile content) of the gastrointestinal tract (Nikoskelainen et al., 2001). Comparatively, *B. pumilus* began to proliferate profusely at a pH of 5.0, whereas *L. macroides* showed little change in growth. This demonstrates that *B. pumilus* has a broader pH tolerance than *L. macroides*. Moreover, both strains could sustain up to 20% bile salt concentration, although there was a substantial reduction in bacterial growth with the increasing bile concentration. The bile tolerance capability of *B. pumilus* was significantly higher than that of the *L. macroides* strain indicating the former's potential to better survive in the hostile environment of the gastrointestinal tract.

Besides their potential to resist hostile intestinal conditions, the isolates should have potent adherence capacity on the intestinal mucosal surfaces to seize the available spaces from the pathogenic bacteria (Kos et al., 2003). This adherence property can be determined by assessing bacterial cell surface hydrophobicity as it directly corresponds to the electron-accepting and donating ability of the isolates (Bellon-Fontaine et al., 1996). In this study, both test strains showed significantly higher hydrophobicity than the non-probiotic control. Among the selected strains, *L. macroides* had a significantly higher affinity for xylene and chloroform, while the affinity for ethyl acetate was significantly higher in *B. pumilus*. The cell surface hydrophobicity is reflected by the bacterial adhesion to xylene, while chloroform and ethyl acetate are the indicators of bacterial electron donating (basic) and electron-accepting (acidic) characteristics, respectively Bellon-Fontaine et al., 1996). This attribute will not only help in proliferation but also inhibit pathogens via competing for host cell binding sites and nutrients available (Nikoskelainen et al., 2003; Tuomola et al., 2001). This affinity is governed by different passive forces like hydrophobic interaction and electrostatic interaction, which eventually have a significant role in the complex adhesion processes between bacterial cell membranes and adhesion-promoting proteins of the epithelial mucosal layer (Servin & Coconnier, 2003; Van Loosdrecht et al., 1987).

Following the mucosal adhesion, the ability to aggregate is a must-have property for a probiotic to proliferate in the intestinal environment to deliver its beneficial effects and inhibit pathogens (Collado et al., 2008). In this study, both the test strains showed

significant auto- and co-aggregation ability. In both cases, *B. pumilus* showed better aggregating potential than *L. macroides*, indicating a better adherence potential of the strain.

Biofilm formation by probiotics is another redeeming feature that enables them to establish successfully in the adverse environment of the host intestine (Lepargneur & Rousseau, 2002). Previous studies have reported the improved survival of probiotic bacteria in the host intestine in and post-biofilm formation (Salas-Jara et al., 2016). In this study, *B. pumilus* showed the ability to form a biofilm, whereas *L. macroides* failed to do so. This indicated a better colonizing ability of *B. pumilus*.

As far as the extracellular enzyme production capability of both strains is concerned, it was found that *L. macroides* could produce only lipase while *B. pumilus* strain produced all the enzymes tested. Proficiency in extracellular enzyme production by potential probiotics is an additional advantage in terms of improving the digestion and nutrient utilization of the host (Balcázar et al., 2006). Here also, *B. pumilus* was better than *L. macroides* in producing exoenzymes, suggesting the strain's potency towards better food utilization in the host.

Like any other animals, probiotics also have their own system of antioxidant enzymes which help in the stimulation and elevation of antioxidant enzymes in the host system. Consequently, it reduces the damages caused by oxidation stresses of endogenous and exogenous sources such as pathogens, cytokines, etc. This positive effect can be attributed to their antioxidant metabolites and ability to chelate ions (Wang et al., 2017). In this study, H₂O₂ and DPPH radicals scavenging activity by *B. pumilus* was significantly higher than *L. macroides*. However, these scavenging activities were not significantly superior to the positive control.

Though probiotics are praised for their beneficial effects, safety assessment of the probiotic is necessary from the perspective of its ability to express virulence factors or acquire antibiotic resistance genes. The harmful probiotics can cause septic conditions in immunocompromised hosts (Mater et al., 2017). According to European Food Safety Authority (EFSA, 2012), it is abominable to possess antibiotic resistance properties in probiotic bacteria. However, with the consideration that antibiotic resistance due to intrinsic factor or mutation has a lesser risk of horizontal gene transfer compared to those carried by mobile genetic elements or added genes, the bacteria with intrinsic resistance can be considered as a potential probiotic candidate (WHO, 2006). The present study has evaluated the antibiotic sensitivity of both strains against 12 commercial antibiotics, and it was evident that *B. pumilus* was susceptible to all while *L. macroides* were resistant to oxacillin and intermediately susceptible to polymyxin-B and Kanamycin. However, a strain of *B. pumilus* isolated from milk was found to be resistant to cefotaxime (Adamski et al., 2023), and a

mutation in codon 56 of the rpsL gene caused *B. pumilus* 3-19 to develop streptomycin resistance (Danilova et al., 2017). Apart from this, the haemolytic assay is another way to check the *in vitro* pathogenicity of probiotic candidate strains, as the haemolytic strain can harm the host (Pradhan et al., 2023). In this study, both strains tested negative for any haemolytic ability, hence safe.

In addition to *in vitro* safety assessment, the *in vivo* embryo toxicity and challenged study were conducted. Here, the embryotoxicity of both the isolates was conducted on common carp fertilized eggs and observed for any changes in four epical points of acute toxicity. The number of coagulated eggs recorded in the probiotic-treated groups and the control group had no significant difference. Moreover, a surplus property was also evident that there is no fungal growth on the coagulated eggs of probiotic-treated groups, which was reversed in the control group. According to OECD (2006), there are four apical endpoints to be recorded as indicators of acute toxicity in the fish embryo after 24 h, i.e., coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac, and lack of heart-beat. However, these apical points were not observed, and the characteristics shown in the treated groups and the control were synonymous, indicating that both the test strains were not toxic. Moreover, the challenged study revealed that both strains were non-pathogenic and safe for the test fish.

In all-inclusive, *B. pumilus* showed better *in vitro* beneficial features than *L. macroides*. Considering the overall efficiency in antagonistic activity, tolerance to extreme pH and bile salt concentration, adhesion and aggregating capability, biofilm production, extracellular enzyme production ability, free radical scavenging potential, and safety attributes, *B. pumilus* COFAHE_Pro08 can be regarded as a potential probiotic strain for use in sustainable aquaculture. However, the use of this strain as a dietary supplement needs to be elucidated for further application.

Ethical Statement

Not applicable.

Funding Information

Not applicable.

Author Contribution

WMD: Investigation, analysis, and original draft preparation; SI: Investigation, phenotypic analysis, and draft editing; MK: Investigation, molecular analysis, and manuscript review; TGC: Methodology, supervision, and review and editing of the draft; DK: Conceptualization, supervision, methodology, and review and editing of the draft.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors gratefully acknowledge Central Agricultural University, Imphal, India for providing the required funding and research facilities.

References

- Adamski, P., Byczkowska-Rostkowska, Z., Gajewska, J., Zakrzewski, A. J., & Kłębukowska, L. (2023). Prevalence and Antibiotic Resistance of *Bacillus* sp. Isolated from Raw Milk. *Microorganisms*, *11*(4), 1065. <https://doi.org/10.3390/microorganisms11041065>
- Afify, A. E. M. M., Romeilah, R. M., Sultan, S. I., & Hussein, M. M. (2012). Antioxidant activity and biological evaluations of probiotic bacteria strains. *International Journal of Academic Research*, *4*(6). <http://doi.org/10.7813/2075-4124.2012/4-6/A.18>
- Balcázar, J. L., De Blas, I., Ruiz-Zarzuola, I., Cunningham, D., Vendrell, D., & Múzquiz, J. L. (2006). The role of probiotics in aquaculture. *Veterinary microbiology*, *114*(3-4), 173-186. <https://www.sciencedirect.com/science/article/pii/S0378113506000265>
- Bellon-Fontaine, M. N., Rault, J., & Van Oss, C. J. (1996). Microbial adhesion to solvents: a novel method to determine the electron-donor/electron-acceptor or Lewis acid-base properties of microbial cells. *Colloids and surfaces B: Biointerfaces*, *7*(1-2), 47-53. <https://www.sciencedirect.com/science/article/pii/0927776596012726>
- Chabrilón, M., Rico, R. M., Balebona, M. C., & Moriñigo, M. A. (2005). Adhesion to sole, *Solea senegalensis* Kaup, mucus of microorganisms isolated from farmed fish, and their interaction with *Photobacterium damsela* subsp. *piscicida*. *Journal of Fish Diseases*, *28*(4), 229-237. <https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1365-2761.2005.00623>
- Chung, S., Kong, H., Buyer, J. S., Lakshman, D. K., Lydon, J., Kim, S. D., & Roberts, D. P. (2008). Isolation and partial characterization of *Bacillus subtilis* ME488 for suppression of soilborne pathogens of cucumber and pepper. *Applied Microbiology and Biotechnology*, *80*, 115-123. <https://link.springer.com/article/10.1007/s00253-008-1520-4>
- Collado, M. C., Meriluoto, J., & Salminen, S. (2008). Adhesion and aggregation properties of probiotic and pathogen strains. *European food research and technology*, *226*, 1065-1073. <https://link.springer.com/article/10.1007/s00217-007-0632>
- Danilova, I. V., Toymentseva, A. A., Baranova, D. S., & Sharipova, M. R. (2017). The genetic mechanism of resistance to antibiotics in *Bacillus pumilus* 3-19 strain. *BioNanoScience*, *7*, 88-91. <https://doi.org/10.1007/s12668-016-0295-9>
- EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). (2012). Guidance on the

- assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. *EFSA Journal*, 10(6), 2740.
<https://efsa.onlinelibrary.wiley.com/doi/abs/10.2903/j.efs.2012.2740>
- Elmastaş, M., Gülçin, I., Işildak, Ö., Küfrevioğlu, Ö. İ., İbanoğlu, K., & Aboul-Enein, H. Y. (2006). Radical scavenging activity and antioxidant capacity of bay leaf extracts. *Journal of the Iranian Chemical Society*, 3, 258-266.
<https://doi.org/10.1007/BF03247217>
- FAO/WHO (2001). Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Acid Bacteria. Report of a Joint FAO/WHO Expert Consultation, Córdoba, Argentina.
http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf?ua=1
- FAO (2012). The State of World Fisheries and Aquaculture 2012. Rome. 209.
<https://www.fao.org/publications/card/fr/c/81ea54da-0f65-56c6-91d2-8257b8ac4136>
- Freeman, D. J., Falkiner, F. R., & Keane, C. T. (1989). New method for detecting slime production by coagulase negative staphylococci. *Journal of clinical pathology*, 42(8), 872-874. <https://jcp.bmj.com/content/42/8/872>
- Gross, E., & Morell, J. L. (1971). Structure of nisin. *Journal of the American Chemical Society*, 93(18), 4634-4635. <https://pubs.acs.org/doi/pdf/10.1021/ja00747a073>
- Gross, E., & Kiltz, H. H. (1973). The number and nature of α , β -unsaturated amino acids in subtilin. *Biochemical and biophysical research communications*, 50(2), 559-565. <https://www.sciencedirect.com/science/article/pii/0006291X73908760>
- Hall, S. J. (2011). *Blue frontiers: managing the environmental costs of aquaculture*. WorldFish.
<https://books.google.com/books?hl=en&lr=&id=C6EoywET0D4C&oi=fnd&pg=PR2&dq=7.%0>
- Henriksson, P. J. G., Belton, B., Jahan, K. M. E., & Rico, A. (2018). Measuring the potential for sustainable intensification of aquaculture in Bangladesh using life cycle assessment. *Proceedings of the National Academy of Sciences*, 115(12), 2958-2963.
<https://doi.org/10.1073/pnas.1716530115>
- Helmi, A. M., Mukti, A. T., Soegianto, A., Mahardika, K., Mastuti, I., Effendi, M. H., & Plumeriastuti, H. (2020). A review of bacterial zoonoses and antimicrobial resistant (AMR) on grouper fish (*Epinephelus* sp.). *Sys Rev Pharm*, 11(9), 79-88.
- Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., & Sanders, M. E. (2014). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature reviews Gastroenterology & hepatology*, 11, 506-514. <https://www.nature.com/articles/nrgastro.2014.66>
- Huang, Q., Sham, R. C., Deng, Y., Mao, Y., Wang, C., Zhang, T., & Leung, K. M. (2020). Diversity of gut microbiomes in marine fishes is shaped by host-related factors. *Molecular ecology*, 29(24), 5019-5034.
<https://onlinelibrary.wiley.com/doi/abs/10.1111/mec.15699>
- Ibrahem, M. D. (2015). Evolution of probiotics in aquatic world: Potential effects, the current status in Egypt and recent prospectives. *Journal of advanced research*, 6(6), 765-791.
<https://www.sciencedirect.com/science/article/pii/S2090123213001483>
- Joseph, S. W., Colwell, R. R., & Kaper, J. B. (1982). *Vibrio parahaemolyticus* and related halophilic vibrios. *CRC Critical Reviews in Microbiology*, 10(1), 77-124.
<https://doi.org/10.3109/1040841820911350>
- Khan, M. I., Kamilya, D., Choudhury, T. G., & Rathore, G. (2022). Dietary administration of a host-gut derived probiotic *Bacillus amyloliquefaciens* COFCAU_P1 modulates immune-biochemical response, immune-related gene expression, and resistance of *Labeo rohita* to *Aeromonas hydrophila* infection. *Aquaculture*, 546, 737390.
<https://doi.org/10.1016/j.aquaculture.2021.737390>
- Khatri, S., Sahu, J., & Prasad, M. M. (2009). Prevalence of fish diseases in Sambalpur, Orissa, India.
<https://krishi.icar.gov.in/jspui/handle/123456789/35374>
- Kirby, B. (2009). Kirby-Bauer disk diffusion susceptibility test protocol. *American Society for Microbiology*, 66, 208. <https://asm.org/getattachment/2594ce26-bd44-47f6-8287-0657aa9185ad/Kirby-Bauer-Disk-DiffusionSusceptibility-Test-Protocol-pdf.pdf>
- Kos, B. V. Z. E., Šušković, J., Vuković, S., Šimpraga, M., Frece, J., & Matošić, S. (2003). Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *Journal of applied microbiology*, 94(6), 981-987.
<https://academic.oup.com/jambio/article-abstract/94/6/981/6722207>
- Kuebutornye, F. K., Wang, Z., Lu, Y., Abarike, E. D., Sakyi, M. E., Li, Y., ... & Hlordzi, V. (2020). Effects of three host-associated *Bacillus* species on mucosal immunity and gut health of Nile tilapia, *Oreochromis niloticus* and its resistance against *Aeromonas hydrophila* infection. *Fish & shellfish immunology*, 97, 83-95.
<https://www.sciencedirect.com/science/article/pii/S1050464819311751>
- Kumar, R., Mukherjee, S. C., Ranjan, R., & Nayak, S. K. (2008). Enhanced innate immune parameters in *Labeo rohita* (Ham.) following oral administration of *Bacillus subtilis*. *Fish & shellfish immunology*, 24(2), 168-172. <https://www.sciencedirect.com/science/article/pii/S1050464807001829>
- Lepargneur, J. P., & Rousseau, V. (2002). Protective role of the Doderlein flora. *Journal de gynecologie, obstetrique et biologie de la reproduction*, 31(5), 485-494.
<https://europepmc.org/article/med/12379833>
- Liñan-Vidriales, M. A., Peña-Rodríguez, A., Tovar-Ramírez, D., Elizondo-González, R., Barajas-Sandoval, D. R., Ponce-Gracia, E. I., ... & Quiroz-Guzmán, E. (2021). Effect of rice bran fermented with *Bacillus* and *Lysinibacillus* species on dynamic microbial activity of Pacific white shrimp (*Penaeus vannamei*). *Aquaculture*, 531, 735958. <https://doi.org/10.1016/j.aquaculture.2020.735958>
- Lio-Po, G. D., & Lim, L. S. (2014). Infectious diseases of warmwater fish in fresh water. In *Diseases and disorders of finfish in cage culture* (pp. 193-253). Wallingford UK: CAB International.
- Mani, S. R., Vijayan, K., Jacob, J. P., Vijayakumar, S., & Kandhasamy, S. (2021). Evaluation of probiotic properties of *Lysinibacillus macroides* under in vitro conditions and culture of *Cyprinus carpio* on growth parameters. *Archives of microbiology*, 203(7), 4705-4714.
<https://link.springer.com/article/10.1007/s00203-021-02452>

- Mater, D. D., Langella, P., Corthier, G., & Flores, M. J. (2007). A probiotic *Lactobacillus* strain can acquire vancomycin resistance during digestive transit in mice. *Microbial Physiology*, 14(1-3), 123-127.
<https://www.karger.com/Article/Abstract/106091>
- Moradi, M., Tajik, H., Mardani, K., & Ezati, P. (2019). Efficacy of lyophilized cell-free supernatant of *Lactobacillus salivarius* (Ls-BU2) on *Escherichia coli* and shelf life of ground beef. In *Veterinary Research Forum* (Vol. 10, No. 3, p. 193). Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6828169/>
- Morelli, L. (2000). In vitro selection of probiotic lactobacilli: a critical appraisal. *Current Issues in Intestinal Microbiology*, 1(2), 59-67.
<https://www.caister.com/backlist/ciim/v/v1/06.pdf>
- Nalawade, T. M., Bhat, K. G., & Sogi, S. (2016). Antimicrobial activity of endodontic medicaments and vehicles using agar well diffusion method on facultative and obligate anaerobes. *International journal of clinical pediatric dentistry*, 9(4), 335.
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5233701>
- Nakamura, A., Takahashi, K. G., & Mori, K. (1999). Vibriostatic bacteria isolated from rearing seawater of oyster brood stock: potentiality as biocontrol agents for vibriosis in oyster larvae. *Fish Pathology*, 34(3), 139-144.
https://www.jstage.jst.go.jp/article/jsfp1966/34/3/34_3_139/_article/-char/ja
- Naylor, R. L., Hardy, R. W., Bureau, D. P., Chiu, A., Elliott, M., Farrell, A. P., & Nichols, P. D. (2009). Feeding aquaculture in an era of finite resources. *Proceedings of the National Academy of Sciences*, 106(36), 15103-15110.
<https://doi.org/10.1073/pnas.0905235106>
- Nikoskelainen, S., Salminen, S., Bylund, G., & Ouwehand, A. C. (2001). Characterization of the properties of human- and dairy-derived probiotics for prevention of infectious diseases in fish. *Applied and Environmental Microbiology*, 67(6), 2430-2435.
<https://journals.asm.org/doi/abs/10.1128/AEM.67.6.2430-2435.2001>
- Nikoskelainen, S., Ouwehand, A. C., Bylund, G., Salminen, S., & Lilius, E. M. (2003). Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria (*Lactobacillus rhamnosus*). *Fish & shellfish immunology*, 15(5), 443-452.
<https://www.sciencedirect.com/science/article/pii/S1050464803000238>
- OECD (2006) Draft Proposal for a New Guideline, Fish Embryo Toxicity (FET) Test. OECD Guideline for the Testing of Chemicals. Organization for Economic Cooperation and Development, Paris, France.
<https://www.oecd.org/chemicalsafety/testing/36817070.pdf>
- Pradhan, S. K., Devi, R., Khan, M. I. R., Kamilya, D., Choudhury, T. G., & Parhi, J., (2023) Isolation of *Aeromonas salmonicida* subspecies *salmonicida* from aquaculture environment in India: Polyphasic identification, virulence characterization, and antibiotic susceptibility. *Microbial Pathogenesis*, 179, 106100.
<https://doi.org/10.1016/j.micpath.2023.106100>
- Prasad, J., Gill, H., Smart, J., & Gopal, P. K. (1998). Selection and characterisation of *Lactobacillus* and *Bifidobacterium* strains for use as probiotics. *International Dairy Journal*, 8(12), 993-1002.
<https://www.sciencedirect.com/science/article/pii/S0958694699000242>
- Ramesh, D., Vinothkanna, A., Rai, A. K., & Vignesh, V. S. (2015). Isolation of potential probiotic *Bacillus* spp. and assessment of their subcellular components to induce immune responses in *Labeo rohita* against *Aeromonas hydrophila*. *Fish & shellfish immunology*, 45(2), 268-276.
<https://www.sciencedirect.com/science/article/pii/S105046481500176>
- Ray, A. K., Ghosh, K., & Ringø, E. J. A. N. (2012). Enzyme-producing bacteria isolated from fish gut: a review. *Aquaculture Nutrition*, 18(5), 465-492.
<https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1365-2095.2012>
- Reda, R. M., Selim, K. M., El-Sayed, H. M., & El-Hady, M. A. (2018). In vitro selection and identification of potential probiotics isolated from the gastrointestinal tract of Nile tilapia, *Oreochromis niloticus*. *Probiotics and antimicrobial proteins*, 10, 692-703.
<https://link.springer.com/article/10.1007/s12602-017-9314-6>
- Reniero, R., Cocconcelli, P., Bottazzi, V., & Morelli, L. (1992). High frequency of conjugation in *Lactobacillus* mediated by an aggregation-promoting factor. *Microbiology*, 138(4), 763-768.
<https://www.microbiologyresearch.org/content/journal/micro/10.1099/00221287-138-4-763>
- Rosenberg, M., Gutnick, D., & Rosenberg, E. (1980). Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS microbiology letters*, 9(1), 29-33.
<https://academic.oup.com/femsle/article-abstract/9/1/29/498157>
- Ruch, R. J., Cheng, S. J., & Klaunig, J. E. (1989). Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis*, 10(6), 1003-1008.
<https://academic.oup.com/carcin/article-abstract/10/6/1003/404012>
- Sakthivel, K., Manigundan, K., Gautam, R. K., Singh, P. K., Subramani, T., Sharma, S. K., & Khande, R. (2018). Bioefficacy of antimicrobial peptide biosynthesis-gene-linked antagonistic *Lysinibacillus sphaericus* strains for management of bacterial plant diseases in Andaman and Nicobar Islands, India. *Journal of Environmental Biology*, 39(4), 517-521.
<https://search.proquest.com/openview/018132389fd72c42b535e29a1b1346b6/1?pq-origsite=gscholar&cbl=636374>
- Salas-Jara, M. J., Ilabaca, A., Vega, M., & García, A. (2016). Biofilm forming *Lactobacillus*: new challenges for the development of probiotics. *Microorganisms*, 4(3), 35.
<https://www.mdpi.com/156946>
- Sapkota, A., Sapkota, A. R., Kucharski, M., Burke, J., McKenzie, S., Walker, P., & Lawrence, R. (2008). Aquaculture practices and potential human health risks: current knowledge and future priorities. *Environment international*, 34(8), 1215-1226.
<https://doi.org/10.1016/j.envint.2008.04.009>
- Sargenti, M., Bartolacci, S., Luciani, A., Di Biagio, K., Baldini, M., Galarini, R., & Capuccella, M. (2020). Investigation of the correlation between the use of antibiotics in aquaculture systems and their detection in aquatic environments: a case study of the nera river aquafarms in Italy. *Sustainability*, 12(12), 5176.
<https://doi.org/10.3390/su12125176>

- Seelam, N. S., Akula, H., Katike, U., & Obulam, V. S. R. Isolation, identification and characterization of *Lysinibacillus sphaericus* from fresh water fish intestinal tract. *International Journal of Biology Research* 2(1): 9-13. <https://www.academia.edu/download/52027806/2-1-17-370.pdf>
- Servin, A. L., & Coconnier, M. H. (2003). Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. *Best Practice & Research Clinical Gastroenterology*, 17(5), 741-754. <https://www.sciencedirect.com/science/article/pii/S1521691803000520>
- Thankappan, B., Ramesh, D., Ramkumar, S., Natarajaseenivasan, K., & Anbarasu, K. (2015). Characterization of *Bacillus* spp. from the gastrointestinal tract of *Labeo rohita*—towards to identify novel probiotics against fish pathogens. *Applied biochemistry and biotechnology*, 175, 340-353. <https://link.springer.com/article/10.1007/s12010-014-1270>
- Tuomola, E., Crittenden, R., Playne, M., Isolauri, E., & Salminen, S. (2001). Quality assurance criteria for probiotic bacteria. *The American journal of clinical nutrition*, 73(2), 393s-398s. <https://academic.oup.com/ajcn/article-abstract/73/2/393s/4737568>
- Upreti, G. C., & Hinsdill, R. D. (1975). Production and mode of action of lactocin 27: bacteriocin from a homofermentative *Lactobacillus*. *Antimicrobial agents and chemotherapy*, 7(2), 139-145. <https://journals.asm.org/doi/abs/10.1128/AAC.7.2.139>
- Van Loosdrecht, M. C., Lyklema, J., Norde, W., Schraa, G., & Zehnder, A. (1987). The role of bacterial cell wall hydrophobicity in adhesion. *Applied and environmental microbiology*, 53(8), 1893-1897. <https://journals.asm.org/doi/abs/10.1128/aem.53.8.1893-1897.1987>
- Vaseeharan, B. A. R. P., & Ramasamy, P. (2003). Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. *Letters in applied microbiology*, 36(2), 83-87. <https://academic.oup.com/lambio/article-abstract/36/2/83/6704697>
- Velho-Pereira, S., & Kamat, N. M. (2011). Antimicrobial screening of actinobacteria using a modified cross-streak method. *Indian journal of pharmaceutical sciences*, 73(2), 223. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3267309>
- Verschuere, L., Rombaut, G., Sorgeloos, P., & Verstraete, W. (2000). Probiotic bacteria as biological control agents in aquaculture. *Microbiology and molecular biology reviews*, 64(4), 655-671. <https://doi.org/10.1128/MMBR.64.4.655-671.2000>
- Wang, Y., Wu, Y., Wang, Y., Xu, H., Mei, X., Yu, D., ... & Li, W. (2017). Antioxidant properties of probiotic bacteria. *Nutrients*, 9(5), 521. <https://www.mdpi.com/198174>
- Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of bacteriology*, 173(2), 697-703. <https://journals.asm.org/doi/abs/10.1128/jb.173.2.697-703.1991>
- World Health Organization, Food and Agriculture Organization of the United Nations & International Office of Epizootics. (2006). Report of a joint FAO/OIE/WHO Expert Consultation on antimicrobial use in aquaculture and antimicrobial resistance, Seoul, Republic of Korea. *World Health Organization*. <https://apps.who.int/iris/handle/10665/133869>
- Ziarati, M., Zorriehzaha, M. J., Hassantabar, F., Mehrabi, Z., Dhawan, M., Sharun, K., ... & Shamsi, S. (2022). Zoonotic diseases of fish and their prevention and control. *Veterinary Quarterly*, 42(1), 95-118. <https://doi.org/10.1080/01652176.2022.2080298>