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First Report on Probiotic Potential Characterization and Clustering Using Unsupervised Algorithm of Lactic Acid Bacteria Isolated from Air Breathing Fish

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ABSTRACT

This study represents the first effort in Northeast India to isolate lactic acid bacteria from the gut of *Channa punctata* and to assess their probiotic attributes, as well as clustering them based on their probiotic potential. Following isolation, a comprehensive analysis was conducted, including morphological differentiation, catalase activity, IMViC tests, acid and bile tolerance, autoaggregation and coaggregation, hydrophobicity, hemolytic activity, and biosafety assays, to evaluate their probiotic potential. The most potent isolates were identified through 16S rRNA sequencing and tested for pathogen antagonism, antibiotic susceptibility, growth performance, and coexistence between the isolated probiotic strains, as well as the antagonism of the consortia against pathogens. For cluster analysis, heat maps and principal component analysis were performed. Two isolates, exhibiting the most promising probiotic characteristics among the screened isolates, were identified via Sanger's dideoxy sequencing of the 16S rRNA gene as *Leuconostoc pseudomesenteroides* strain BICP3 and *Streptococcus equinus* strain BICP2. These two strains effectively inhibited pathogens *Aeromonas hydrophila* and *Aeromonas jandaei* and exhibited sensitivity to all the antibiotics tested, except for streptomycin. Both strains were found to be compatible and demonstrated higher *in vitro* inhibition against pathogens. This investigation successfully screened the probiotic potential of lactic acid bacteria colonizing the gut of *Channa punctata* and isolated two safe, potential probiotic strains for use in the aquaculture industry.

INTRODUCTION

As the expansion of aquaculture systems strives to meet the increasing global demand, farmers face the potential risks of disease outbreaks and financial losses. In order to address this issue, antibiotics and other chemotherapeutic drugs have been employed for a significant period of time. Nevertheless, the repeated use of antibiotics in aquaculture systems leads to significant alterations in the microbiota, resulting in the emergence of antimicrobial resistant bacteria **(Resende *et al.*, 2012)**. Due to numerous

disadvantages, antibiotics are prohibited or subject to strict limitations in aquaculture. Probiotics have been identified as effective alternatives to antibiotics in these situations **(Fjellheim *et al.*, 2010)**. Probiotics are live microorganisms that, when administered in adequate amounts, enhance the health of the host **(Kesarcodi-Watson *et al.*, 2008)**. Probiotics exert beneficial effects on the body by enhancing the function of the epithelial barrier, augmenting their ability to attach to the intestinal lining, generating antimicrobial compounds, and modulating the immune system **(Lyons *et al.*, 2010; Bermudez-Brito *et al.*, 2012)**. Probiotics exert positive impact on nutrition, feed utilization, gut biology, and host functioning **(Cerezuela *et al.*, 2012; Hoseinifar *et al.*, 2018)**. Furthermore, research has shown that probiotic formulations containing multiple strains or species can increase their effectiveness due to synergistic beneficial effects on the host's well-being, such as prolonging or enhancing the desired effects **(Timmerman *et al.*, 2004)**.

In the drive for sustainable development, scientists have concentrated on discovering new probiotic strains from land-based sources. However, probiotics isolated from aquatic environments may demonstrate superior efficacy in their natural habitats, resulting in enhanced colonization and the ability to restore balanced conditions **(Lazado *et al.*, 2015; Van Doan *et al.*, 2019)**. Furthermore, as indigenous probiotics are already adapted to the fish intestinal environment, making them more promising as potential probiotics **(Kotzent *et al.*, 2020)**.

Assam, a state of Northeast India, possesses the highest abundance of freshwater aquatic resources and biodiversity **(Goswami *et al.*, 2002; Kashyap *et al.*, 2012)**. The Northeast region of India is considered a biodiversity hotspot due to its wide range of plant and animal species, including economically significant microorganisms that have not been extensively studied **(Banerjee *et al.*, 2015)**. The gastrointestinal tract (GI) of aquatic animals in Northeastern India harbor unexplored microorganisms specific to this region, as they contain a high concentration of bacteria derived from the water and food they ingest **(Muthukumar *et al.*, 2015)**. The composition of the intestinal microbiota is affected by several physicochemical factors, including intestinal movement, pH levels, redox potential, nutrient availability, and substances produced by the host (such as digestive enzymes, hydrochloric acid, bile, and mucus) **(Booijink *et al.*, 2007)**. Hence, the GI tract comprises various distinct environments, each harboring a multitude of microbial ecosystems that exhibit increasing diversity as they progress through the GI tract **(Gerritsen *et al.*, 2011)**. In addition, wild fish have a more varied gut microbiome compared to farmed fish, primarily because of the differences in nutritional resources and other environmental factors **(Karl *et al.*, 2018)**. Isolating bacteria from the GI tract of wild fish from such a diverse environment can uncover unique probiotic strains.

Channa punctata, often referred as the snakehead fish or mud fish, inhabits various environments such as inland water bodies, freshwater plains, muddy lake bottoms, canals, and swamps **(Yousuf *et al.*, 2023)**. It is cultivated by fish farmers and used as dietary source and for medicines **(Shillewar, 2021)**. Therefore, the goal of our

research was to isolate lactic acid bacteria and to assess their probiotic properties to identify a novel probiotic strain from the gut of *Channa punctata*. Characterization of the strains was conducted by using microbiological techniques: Gram staining, catalase, acid and bile tolerance, hydrophobicity, antagonism, hemolytic assay, antibiotic susceptibility, molecular identification, coexistence test, and antagonism effect of the consortium. Among the initial pool of 70 isolates, 28 were chosen for further investigation based on positive result from gram staining and negative catalase test.

MATERIALS AND METHODS

1. Sample collection

Healthy *Channa punctata* were collected (N=110) from different locations across Assam. Geographical locations of sampling sites are shown in Fig. (1). The fish samples were expeditiously conveyed to the Fish Molecular Biology Laboratory at Gauhati University, with meticulous attention to maintaining optimal aeration, to facilitate subsequent investigational endeavors.

2. Isolation and culture of gut microbes

The collected fish were subjected to a 48hr period of starvation in order to eliminate any external bacteria present. After experiencing starvation, the fish were rendered comatose by subjecting them to hypothermia and were then disinfected using a 1% iodine solution immediately **(Trust *et al.*, 1974)**. The fish were dissected, and their intestines were aseptically removed and homogenized with normal saline solution (NSS) in a ratio of 1:10 (volume) **(Das *et al.*, 1991)**. By adopting serial dilution technique, 0.25ml of each dilution was uniformly distributed on a previously dried MRS (Man, Rogosa, and Sharpe) agar plate (Himedia®, India). The plates were placed in an incubator at 34°C with carbon dioxide tension condition for 48h. Subsequently, the colonies with a milky white appearance were streaked onto MRS agar in order to isolate and purify them. Gram positive and catalase negative isolates were chosen for further analysis. The Fish Molecular Biology Laboratory, Gauhati University already possessed pathogenic bacteria, *Aeromonas hydrophila* (GenBank Accession no MN097841) and *Aeromonas jandaei* (GenBank Accession MN204041).

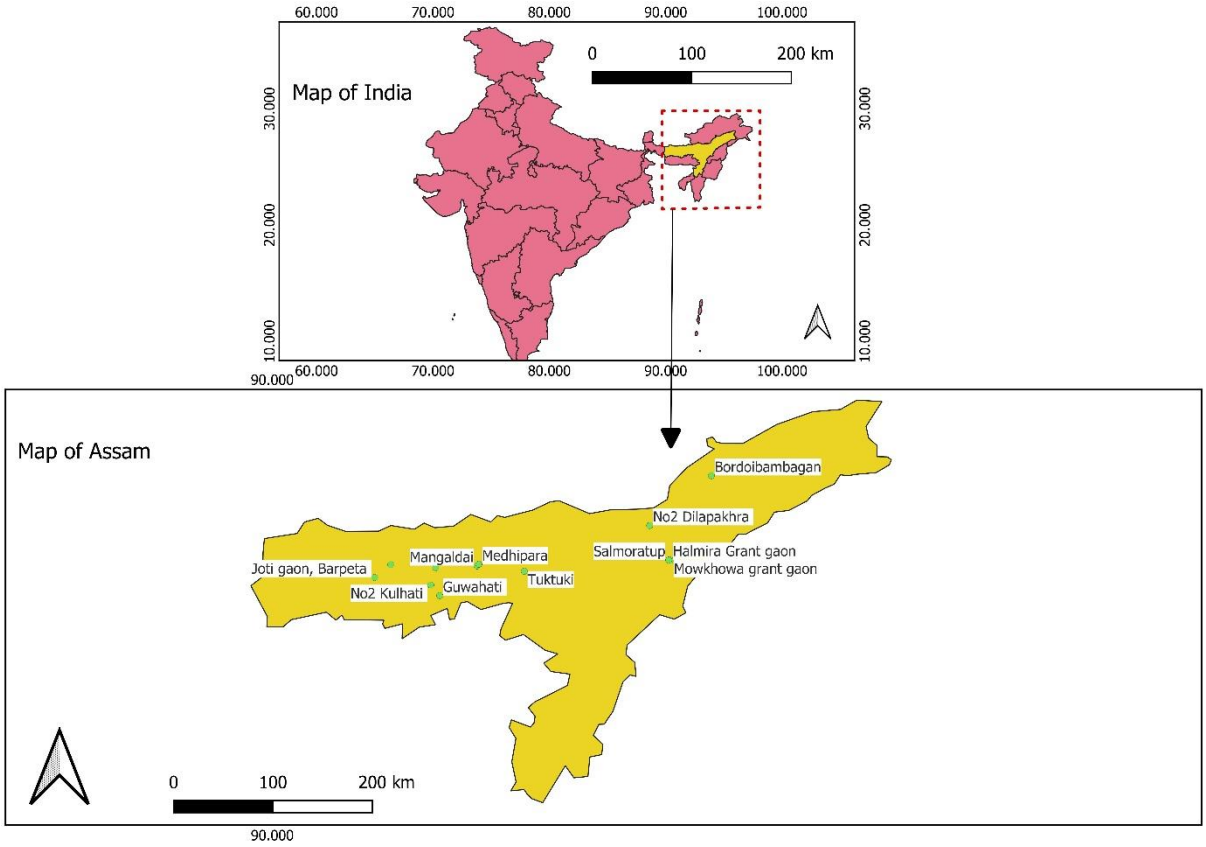

Fig. 1. Geographical locations of sample collection sites: Mowkhowa grant gaon(Lat 26.502307° Long 93.936047°), No2 Dilapakhra (Lat 26.845844° Long 93.729661°), Bordoibambagan(Lat 27.338317° Long 94.339815°), Mangaldai (Lat 26.447179° Long 92.023178°), Halmira Grant gaon (Lat 26.507637° Long 93.925143°), Guwahati (Lat 26.152517° Long 91.654968°), Salmoratup (Long 26.504996° Long 93.918528°), No2 Kulhati (Lat 26.25874° Long 91.566737°), Joti gaon, Barpeta (Lat 26.333343° Long 91.011658°), Tuktuki (Lat 26.394006° Long 92.491187°), Medhipara (Lat 26.463612° Long 92.041519°)

3. Morphological and biochemical characterization

The investigation focused on analyzing colony morphology, performing Gram staining, biochemical characterization tests including the catalase production and the IMViC series (Indole, Methyl Red, Voges-Proskauer, and Citrate utilization tests). These procedures were conducted according to the guidelines outlined in Berger's Manual of Determinative Bacteriology **(Holt *et al.*, 1994)**. Additionally, carbohydrate utilization tests were carried out using the HiCarbo™ Kit (KB009A, KB009B1) Himedia®, India, adhering strictly to the manufacturer's instructions.

4. Acid and bile tolerance test

The capability to survive in diverse intestinal environments including low pH and bile salts, is an important attribute for a probiotic organism **(Sánchez *et al.*, 2013)**. The ability to tolerate the acidic pH and bile salts were evaluated following the protocol described by **Tan *et al.* (2013)**. The isolates were cultivated in MRS broth at a concentration of 10^8CFU/ml, followed by centrifugation at 6000rpm for 10 minutes, washed and resuspended in MRS broth. The pH of the MRS broth was adjusted to 1, 2, 3, 4, 5, 6, and 7 using sterile 1N HCl (Labsynth, Diadema, Brazil), with a control broth maintained without pH adjustment. The samples were incubated at 34°C, and after 4h, 100µL aliquots were extracted for determining the colony-forming units (CFUs) on MRS agar plates containing 1.5% (w/v) agar.

To evaluate the impact of bile salts, these isolates were cultured in MRS broth supplemented with varying concentrations of bile salts (1, 2, 3, 4, and 5%) along with a control broth, free from bile salts, maintained at 34°C. Following a 4h incubation period, 100µL aliquots were extracted for determining the CFU on previously dried MRS 1.5% (w/v) agar plates. The survival rates of these isolates were calculated using the equation delineated by **Govindaraj *et al*. (2021)**.

$$survival\% = \frac{logCFU\ of\ viable\ cells\ after\ 4hr\ of\ incubation}{logCFU\ of\ initial\ viable\ cells} * 100$$

5. Autoaggregation and coaggregation assay

The autoaggregation capabilities of the selected isolates were assessed following the protocol of **Angmo *et al*. (2016)**. The selected isolates were grown in MRS broth, and collected by centrifugation at 8756rpm for 10 minutes. Subsequently, the cells were washed and resuspended in phosphate-buffered saline (PBS, NaCl, KCl, Na_2HPO_4, and KH_2PO_4) and adjusted to a pH of 7.4. The cell suspensions were adjusted to an optical density (OD) of 1.0 and incubated at 34°C. Absorbance was measured at 2, 4, 8, 12, and 24h interval at 600nm. The autoaggregation percentage was calculated using the specified formula:

$$Aggregation\% = \left(1 - \frac{At}{Ao}\right) * 100$$

Where, A_0 represents absorbance at 0h and A_t represents absorbance at different time points.

The coaggregation assay was performed according to the methodology described by **Zuo *et al.* (2015)**. Equal volumes (1×10^8 CFU/ml) of suspension of the selected isolates and of the pathogenic bacterium *Aeromonas hydrophila* (GenBank Accession No. MN097841) were mixed and incubated for periods of 12 and 24h. Optical density (OD) at 600nm was taken at 0h and after 12 and 24h of incubation. The percentage of coaggregation was calculated by using the formula of **Nagaoka *et al.* (2008)** as follows:

$$Coaggregation\% = \left(\frac{Ao - At}{Ao}\right) * 100$$

Where, A_0 represents O.D at 0h, and A_t represents O.D at different time points.

6. Hydrophobicity assay

Hydrophobicity was assessed as described by **Li *et al.* (2014)**, utilizing xylene, chloroform, and ethyl acetate. 1ml aliquot of the bacterial suspension (1 x 10^8 CFU/ml) was combined with an equal volume of each solvent individually. The resulting biphasic mixtures were vigorously agitated with a vortex mixer for 60 seconds. Following this, the suspensions were allowed to settle at room temperature for intervals of 2, 4, and 8h. Absorbance of the aqueous phase was subsequently measured at 600nm. A decrease in absorbance of the aqueous phase indicated cell surface hydrophobicity. The percentage of hydrophobicity was calculated using the specified formula:

$$Hydrophobicity\,\% = \left(\frac{Ao - At}{Ao}\right) * 100$$

Where, A_t represents OD at different time points, and A_o represents initial OD of the mixtures.

7. Antagonistic assay

Following the well diffusion method described by **Magaldi *et al.* (2004)** and **Valgas *et al.* (2007)**, the antimicrobial activity of cell-free supernatant (CFS) of the selected isolates was evaluated. The CFS was collected by centrifuging the broth cultures of the isolates at 6000rpm for 10 minutes, followed by filtration through a 0.2µm membrane filter (Millipore, Bedford, MA, USA). Broth Cultures of *Aeromonas hydrophila* and *Aeromonas jandaei* (1×10^8 CFU/ml) were individually spread on pre-dried Mueller-Hinton Agar (MHA) plates. Wells were punched into the agar with the help of fine pipette tip, and 20µL of the CFS from each potential probiotic isolate were poured into each well, one well left as a control. The plates were incubated at 34°C for 48h, and zones of inhibition (ZOI) were measured. Isolates demonstrating the highest ZOI against the pathogens, along with the most promising probiotic characteristics, were selected for further analysis.

8. Hemolytic activity

The hemolytic activity test was carried out according to the protocols outlined by **Gerhardt *et al.* (1982)** and **Buxton (2005)**. Broth cultures of the selected isolates were grown overnight, then streaked onto blood agar plates supplemented with 5% sheep blood and incubated at 34°C for 48 hours. The presence or absence of clear zones around the bacterial colonies indicated their hemolytic activity.

9. Molecular identification

9.1 Genomic DNA extraction

Genomic DNA of the isolates with highest antagonistic activity against *Aeromonas hydrophila* and *Aeromonas jandaei*, along with other probiotic properties, were isolated by culturing in MRS broth at 30°C for 48hr under CO_2 tension, centrifuged at 10,000 rpm for 5 minutes at 4°C. The resultant pellets were collected while the supernatant was discarded. Genomic DNA was extracted using the QIAamp® DNA Mini Kit (Qiagen, Germany) following the manufacturer's protocol. The extracted DNA was quantified in nanograms per microliter (ng/µL) by using a Nanodrop Lite Spectrophotometer (Thermo Scientific, USA). Additionally, a qualitative assessment was carried out by running the DNA on a 1% (w/v) agarose gel electrophoresis.

9.2 Amplification of 16S rRNA gene

According to **Weisburg *et al.* (1991)**, the amplification of the 16S rRNA gene of the isolates was done by using primers (5′-AGAGTTTGATCCTGGCTCAG-3′, 5′-TACGGTTACCTTGTTACGACTT-3′) in a T100™ Thermal Cycler (Bio-Rad, Berkeley). The PCR reaction mixture comprised 25µL of a pre-made PCR master mix (R2523-100RXN, Sigma, USA), 2.5µL of both forward and reverse primers, 5µL of DNA template (100ng), and 15µL of sterile nuclease-free water, resulting in a total volume of 50µL. In addition, a negative control (lacking a DNA template) was included. A negative control (without DNA template) was also kept. The PCR was carried out with an initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 45 seconds, with a final extension at 72°C for 3 minutes. The quality of the PCR-amplified products was checked by 2% (w/v) agarose gel electrophoresis with ethidium bromide staining. The amplified 16S rRNA gene was purified by gel extraction process and subsequently sent to Mediomix Diagnosis and Bioresearch in Bengaluru, India, for Sanger sequencing using identical primers as in the amplification process.

10. Species identification and phylogenetic analysis

To identify the individual(s) with the closest genetic resemblance of the potential probiotic isolates, the obtained 16S rRNA partial sequences from Sanger sequencing were refined by aligning forward and reverse reads using BIOEDIT version 7.0.5.3 software alignment editor **(Hall, 1999)**. The modified sequences were compared for similarity using the Basic Local Alignment Search Tool (BLAST) available in the National Centre for Biotechnology Information (NCBI) database located in Rockville Pike, Bethesda, USA against the repository of deposited partial 16S rRNA sequences. The modified sequences of the two isolates were subsequently submitted to the GenBank database (NCBI).

To construct the phylogenetic tree, the sequences were aligned using the CLUSTAL W algorithm **(Thompson *et al.*, 1994)** with the default settings in the

Molecular Evolutionary Genetic Analysis 11 (MEGA Ver 11) software **(Kumar *et al.*, 2016)**. The phylogenetic tree was generated using the neighbor-joining method **(Saitou *et al.*, 1987)** in MEGA Version 11, on the basis of evolutionary distances. The bootstrap value of 1000 replicates, signifying the proportion of replicate trees in which the corresponding taxa are grouped together, are shown next to the branches **(Felsenstein, 1985)**. The tree is represented with branch lengths measured in the same units as the evolutionary distances used to construct the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method **(Tamura *et al.*, 2004)** and are presented as the number of base substitutions per site. Positions with ambiguous information were removed for each pair of sequences using the pairwise deletion option.

11. Determination of antibiotic susceptibility

The antibiotic susceptibility profiling of the two selected isolates were done using the Kirby-Bauer disc diffusion assay **(Bauer *et al.*, 1966)**. The antibiotic discs employed in this analysis are Gentamicin (10µg), Streptomycin (10µg), Tetracycline (30µg), and Ampicillin (10µg), all sourced from Himedia®. The susceptibility results were interpreted following the standards delineated by the Clinical and Laboratory Standards Institute (CLSI) guidelines **(Wayne *et al.*, 2010)**.

12. Growth performance

Evaluation of the growth performance was conducted by inoculating the pure bacterial isolates (1mL, 1×10^8 CFU/mL) in MRS broth (Himedia®, India). The cultures were incubated under CO_2 tension conditions at 34°C, and the optical density (O.D.) was measured (n=3) every 2 hours up to 24 hours at 600nm.

13. Coexistence test

The feasibility of co-culturing the two bacterial strains was assessed following the methodology described by **Guo *et al.* (2009)**. The bacteria were cultivated under their respective optimal growth conditions for 48 hours. Afterward, samples were streaked perpendicularly on the surface of 1.5% MRS (w/v) agar plates and incubated for 24 hours. The plates were then examined for any potential antagonistic interactions **(James *et al.*, 2017; Al-Hussini *et al.*, 2018)**.

14. Preparation and antagonistic activity of the consortia

Consortia of the compatible probiotic isolates were prepared using the Direct Mixing method, as this approach is more effective than monoculture in achieving its targets **(Brenner *et al.*, 2008; Kapoore *et al.*, 2021)**. The antagonistic activity of the consortia against the pathogens *A. hydrophila* and *A. jandaei* was evaluated using the well diffusion method **(Magaldi *et al.*, 2004; Valgas *et al.*, 2007)**. The cell-free supernatant (CFS) of the consortia was obtained by centrifuging the culture at 6000rpm for 10 minutes, followed by filtration through a 0.22-micrometer filter (Millipore, Bedford, MA, USA). A 0.25µL suspension of *A. hydrophila* and *A. jandaei* was spread individually on pre-dried MHA plates. The supernatant of the bacterial consortia was

added to one well on each MHA plate, while another well was left as a control. Plates were incubated at 34°C for 24 hours and subsequently examined for the appearance of zones of inhibition (ZOI).

15. Unsupervised clustering and statistical analysis

The heat map of all bacterial isolates based on their probiotic characteristics was generated using GraphPad Prism 10.1.0 (316). Unsupervised clustering of the probiotic attributes of these isolates was performed using principal component analysis (PCA), which facilitated dimensionality reduction and unbiased clustering, utilizing OriginPro (2019b) software **(Farhadian *et al.*, 2021)**. To determine statistically significant differences among the parameters of the isolates, one-way ANOVA was conducted followed by a post hoc Tukey test using SPSS (IBM SPSS Version 29.0.2.0(20)) software **(Sola *et al.*, 2022)**. Additionally, the Holm-Sidak test **(Avican *et al.*, 2021)** was performed in GraphPad Prism 10.1.0 (316) to identify any significant differences among the parameters of each isolate **(Govindaraj *et al.*, 2021)**. All experiments were performed in triplicate, and the results are presented as mean ± S.D.

RESULTS

1. Isolation, morphological and biochemical characterization of bacterial isolates

Initially, 42 isolates with round, milky white colonies were selected from a pool of 130 isolates. Among 42 isolates, 28 have been selected for further analysis based on positive gram staining and negative catalase test. These 28 isolates were named as PSB2, PSC2, PSA3, PSA4, PSA5, PSB1, PSB6, PSB7, PSB8, PSB9, PSC5, PSC7, PSC8, PSC9, PSD1, PSD2, PSD3, PSD5, PSD7, PSP1, PSQ21, PSY12, PSX5, PSZ1, PSZ12, PSZ25, PSZ26 and PSZ27.

Table 1. Biochemical characterization of all the 28 isolates from gut of *Channa punctata* from different locations of Assam, North East India

Isolates	Shape	MR test	VP test	Indole test	Citrate utilization test	Catalase test
PSB2	Round	+	-	+	-	-
PSC2	Round	+	-	+	-	-
PSA3	Rod	-	+	-	-	-
PSA4	Round	+	-	-	-	-
PSA5	Rod	+	-	-	-	-
PSB1	Round	+	-	-	-	-
PSB6	Round	+	-	-	-	-
PSB7	Round	+	-	+	+	-
PSB8	Round	+	-	-	+	-
PSB9	Round	+	-	+	+	-
PSC5	Round	+	-	-	-	-
PSC7	Round	+	-	+	+	-

PSC8	Round	+	-	-	-	-
PSC9	Round	+	-	-	-	-
PSD1	Round	+	-	-	+	-
PSD2	Rod	-	-	+	-	-
PSD3	Round	-	+	+	+	-
PSD5	Round	-	+	-	+	-
PSD7	Round	+	+	+	-	-
PSP1	Round	+	-	-	-	-
PSQ21	Round	+	-	+	+	-
PSY12	Round	-	-	+	-	-
PSX5	Round	+	-	-	+	-
PSZ1	Rod	-	-	-	-	-
PSZ12	Rod	-	+	+	+	-
PSZ25	Round	-	-	-	-	-
PSZ26	Rod	+	-	+	-	-
PSZ27	Round	+	-	+	+	-

+ positive; - negative

2. Acid and bile test

Table (2) displays the survival rates of 28 round milky white isolates following a 4-hour incubation period across a pH gradient of 1–7. Isolates PSB2 and PSC2 demonstrated the highest resistance to low pH conditions, maintaining survival rates of 86.37% and 85.52%, respectively, at pH 3. In contrast, isolates PSA3 and PSB7 exhibited the lowest viability after 4 hours, with survival rates of 41.79% and 42.52%, respectively. No cellular proliferation was observed at pH 1 and pH 2.

Table (3) presents the viability of these isolates across a bile salt concentration gradient. All 28 isolates showed no growth at a 5% bile salt concentration, although there were differences in their viability levels. Isolate PSB2 demonstrated the highest tolerance, with a survival rate of 86.37% at 1% bile concentration and 45.83% at 4%. Isolate PSC2 exhibited slightly lower tolerance, with survival rates of 85.22% at 1% bile concentration and 44.68% at 4%.

A survival rate of 75% or higher in simulated gastric juice and bile salt conditions is considered the threshold for qualification as a probiotic bacterium **(Suwannaphan *et al.*, 2021)**.

Table 2. Survivability of the screened 28 isolates at different pH

Isolates	pH3		pH4		pH5		pH6		pH7	
	logCFU/ml	Survival%	logCFU/ml	Survival%	logCFU/ml	Survival%	logCFU/ml	Survival%	logCFU/ml	Survival %
PSB2	7.32± 0.01	86.37	7.44± 0.01	87.86	7.78± 0.00	91.89	7.82	92.27	8.41± 0.01	99.28
PS	7.36±	85.52	7.47±	86.83	7.79±	90.64	7.91±	92.02	8.44±	98.0

C2	0.02		0.02		0.00		0.01		0.03	9
PS A3	3.36± 0.10	41.79	4.55± 0.13	56.61	4.73± 0.05	58.77	4.96± 0.21	61.75	4.94± 0.03	61.4 1
PS A4	3.97± 0.03	46.70	5.12± 0.02	60.29	5.27± 0.01	62.01	5.39± 0.01	63.37	5.47± 0.01	64.3 2
PS A5	3.42± 0.10	43	4.56± 0.07	57.36	4.69± 0.09	59.03	4.80± 0.08	60.33	4.88± 0.03	61.4 3
PS B1	3.86± 0.03	46.73	4.94± 0.03	59.70	5.01± 0.02	60.63	5.18± 0.03	62.58	5.24± 0.01	63.3 5
PS B6	3.75± 0.08	45.43	4.94± 0.03	59.84	5.01± 0.02	60.77	5.12± 0.02	62.12	5.19± 0.03	62.9 6
PS B7	3.42± 0.10	42.52	4.63± 0.13	57.53	4.80± 0.04	59.71	4.88± 0.03	60.74	4.98± 0.03	62.0 0
PS B8	4.17± 0.02	48.39	5.29± 0.03	61.39	5.40± 0.02	62.69	5.50± 0.02	63.83	5.59± 0.01	64.8 9
PS B9	3.83± 0.13	45.96	5.05± 0.05	60.58	5.16± 0.02	61.82	5.24± 0.01	62.81	5.29± 0.01	63.3 8
PS C5	4.00± 0.04	47.26	5.21± 0.03	61.61	5.26± 0.01	62.21	5.32± 0.02	62.91	5.44± 0.01	64.2 6
PS C7	3.63± 0.06	44.48	4.84± 0.06	59.27	4.94± 0.03	60.43	5.03± 0.05	61.52	5.14± 0.02	62.8 6
PS C8	4.32± 0.01	49.66	5.44± 0.02	62.62	5.54± 0.01	63.75	5.59± 0.01	64.38	5.66± 0.01	65.1 3
PS C9	3.86± 0.03	46.34	4.97± 0.03	59.59	5.10± 0.04	61.17	5.24± 0.01	62.81	5.32± 0.01	63.7 3
PS D1	3.88± 0.03	46.46	4.97± 0.03	59.44	5.16± 0.02	61.68	5.24± 0.01	62.66	5.22± 0.16	62.4 6
PS D2	3.55± 0.13	43.31	4.86± 0.03	59.32	4.98± 0.03	60.79	5.10± 0.04	62.21	5.19± 0.02	63.2 4
PS D3	3.55± 0.13	43.15	4.88± 0.06	59.31	4.97± 0.03	60.38	5.05± 0.05	61.39	5.19± 0.02	63.0 1
PS D5	4.39± 0.01	50.60	5.45± 0.01	62.81	5.54± 0.01	63.78	5.62± 0.01	64.70	5.67± 0.01	65.2 7
PS D7	4.16± 0.02	48.05	5.46± 0.01	63.09	5.53± 0.02	63.94	5.60± 0.01	64.72	5.63± 0.01	65.0 9
PSP 1	4.16± 0.02	47.99	5.46± 0.01	63.02	5.53± 0.02	63.87	5.61± 0.01	64.77	5.65± 0.01	65.2 4
PS Q2 1	3.94± 0.03	46.32	5.07± 0.02	59.61	5.29± 0.01	62.19	5.39± 0.01	63.44	5.49± 0.01	64.5 5
PS Y1 2	3.96± 0.12	46.75	5.16± 0.02	60.88	5.25± 0.02	62.04	5.37± 0.02	63.45	5.46± 0.01	64.4 3
PS X5	3.97± 0.03	46.43	5.25± 0.03	61.36	5.39± 0.01	63.06	5.50± 0.02	64.28	5.54± 0.01	64.7 9

PSZ1	3.78±0.15	44.73	5.04±0.04	59.58	5.23±0.05	61.80	5.34±0.02	63.15	5.44±0.01	64.32
PSZ12	3.97±0.07	45.70	5.39±0.01	62.05	5.58±0.01	64.33	5.64±0.01	64.94	5.67±0.01	65.31
PSZ25	3.82±0.11	45.05	5.00±0.04	59.01	5.25±0.03	61.94	5.31±0.02	62.75	5.45±0.01	64.37
PSZ26	3.63±0.13	42.85	5.01±0.02	59.26	5.27±0.01	62.30	5.36±0.01	63.30	5.44±0.02	64.32
PSZ27	3.82±0.07	44.78	5.17±0.02	60.56	5.30±0.04	62.13	5.47±0.02	64.09	5.51±0.01	64.59

Values are average of three replicates.

Table 3. Survivability of the selected 28 isolates at different bile concentration

Isolates	1% bile		2% bile		3% bile		4 % bile	
	logCFU/ml	Survival%	logCFU/ml	Survival%	logCFU/ml	Survival%	logCFU/ml	Survival%
PSB2	7.32±0.01	86.37	7.25±0.01	85.56	6.10±0.04	72.04	3.88±0.06	45.83
PSC2	7.33±0.01	85.22	7.29±0.01	84.72	6.16±0.02	71.58	3.84±0.06	44.68
PSA3	5.27±0.03	65.55	5.22±0.02	64.95	3.98±0.05	49.54	3.57±0.23	44.36
PSA4	5.17±0.02	60.78	4.94±0.03	58.08	3.30±0.00	38.84	1.00±1.73	11.76
PSA5	5.12±0.02	64.46	4.94±0.03	62.10	3.52±0.07	44.26	2.10±1.83	26.42
PSB1	4.94±0.03	59.70	4.75±0.08	57.41	2.10±1.83	25.40	0.00±0.00	0.00
PSB6	5.22±0.03	63.29	5.03±0.05	60.93	3.63±0.06	44.05	2.00±1.73	24.24
PSB7	4.98±0.05	61.98	4.63±0.06	57.64	2.20±1.91	27.37	2.00±1.73	24.88
PSB8	5.07±0.02	58.85	4.97±0.03	57.72	3.82±0.07	44.36	2.33±2.03	27.10
PSB9	5.17±0.03	61.94	5.07±0.02	60.75	3.73±0.05	44.67	2.10±1.83	25.18
PSC5	4.98±0.03	58.92	4.67±0.06	55.16	3.20±0.17	37.83	1.00±1.73	11.82
PSC7	4.88±0.03	59.78	4.67±0.06	57.12	3.42±0.10	41.84	2.00±1.73	24.48
PSC8	5.27±0.01	60.66	5.14±0.02	59.10	3.98±0.03	45.85	3.53±0.21	40.67
PSC9	5.18±0.03	62.17	5.08±0.00	60.90	3.94±0.03	47.21	3.53±0.21	42.37
PSD1	5.03±0.05	60.12	4.84±0.10	57.86	3.56±0.07	42.59	3.20±0.17	38.29

PSD 2	5.07±0.02	61.79	4.97±0.03	60.60	3.67±0.06	44.72	3.36±0.10	40.97
PSD 3	5.17±0.02	62.77	5.14±0.02	62.40	3.92±0.03	47.63	3.63±0.06	44.16
PSD 5	5.18±0.03	59.73	5.17±0.02	59.52	4.05±0.05	46.69	3.86±0.03	44.52
PSD 7	5.03±0.05	58.11	4.94±0.03	57.08	3.67±0.06	42.39	2.10±1.83	24.28
PSP 1	4.94±0.03	57.01	4.82±0.11	55.61	3.53±0.21	40.81	2.00±1.73	23.09
PSQ 21	5.03±0.05	59.13	4.63±0.06	54.52	1.00±1.73	11.76	0.00±0.00	0.00
PSY 12	4.67±0.06	55.10	2.67±2.31	31.48	0.00±0.00	0.00	0.00±0.00	0.00
PSX 5	4.43±0.38	51.86	1.33±2.31	15.59	0.00±0.00	0.00	0.00±0.00	0.00
PSZ 1	5.04±0.04	59.58	4.88±0.09	57.67	2.20±1.91	26.01	0.00±0.00	0.00
PSZ 12	5.29±0.03	60.98	5.10±0.04	58.77	3.63±0.06	41.87	2.00±1.73	23.04
PSZ 25	5.01±0.02	59.19	4.92±0.03	58.09	3.55±0.13	41.93	1.00±1.73	11.81
PSZ 26	5.16±0.02	60.95	5.04±0.07	59.55	3.90±0.05	46.11	3.10±0.17	36.65
PSZ 27	5.10±0.04	59.81	4.97±0.06	58.24	3.50±0.17	41.05	2.10±1.83	24.62

Values are average of three replicates.

3. Autoaggregation and coaggregation

The results of the autoaggregation of the 28 isolates are depicted in Fig. (2). The percentage autoaggregation of the isolates increased over time. The isolate PSC2 exhibited the highest autoaggregation values, measuring 82.82±0.12, followed by PSB2 with a value of 78.63±0.74 after 24h. The isolate PSB1 demonstrated the lowest value of autoaggregation after 24h of incubation, measuring 33.01±0.57. Fig. (3) displays the outcomes of the coaggregation capacity of the 28 examined isolates. The coaggregation varied between 90.53±0.0% and 32.33±0.01% with *A. hydrophila* at 24th h. The isolate PSC2 exhibited the highest coaggregation value with *A. hydrophila*, measuring 90.53% at the 24th h. Similarly, the coaggregation value for PSB2 was 87.94% at the same time point.

The isolates PSC8 exhibited the lowest coaggregation values with *A. hydrophila*, measuring 32.33±0.01% at the 24th h.

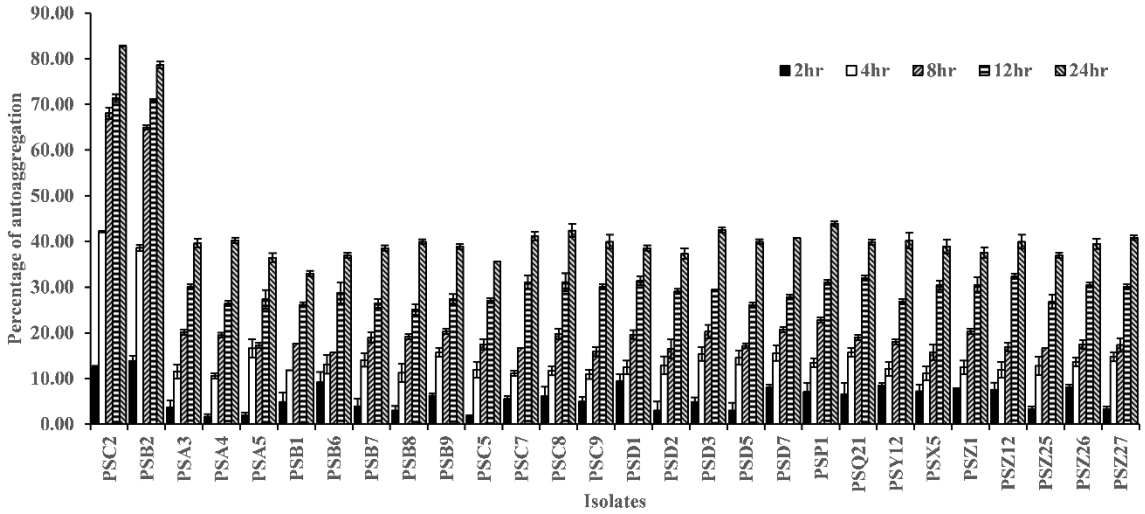

Fig. 2. Autoaggregation of isolates. Each bar represents mean±standard deviation. $P<0.05$ indicates a significant difference in autoaggregation between isolates. There is no significant difference between isolate PSC2 and PSB2

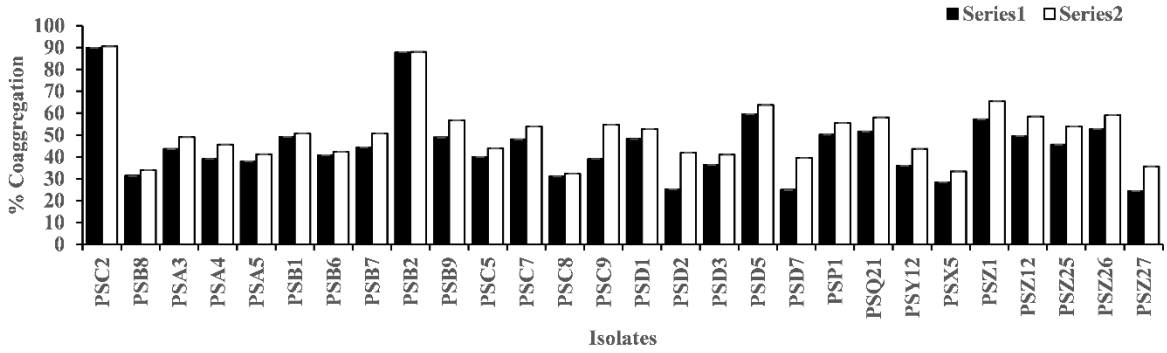

Fig. 3. Coaggregation of isolates. Each bar represents mean±standard deviation. $P<0.05$ indicates a significant difference in coaggregation between isolates. No significant difference is found between isolates PSC2 and PSB2.

4. Hydrophobicity

The isolates exhibit a pronounced affinity for xylene, as shown in Fig. (4). The highest level of hydrophobicity was observed with xylene for PSC2 (79.63%) and PSB2 (75.39±0.01%). These isolates demonstrate a higher affinity to chloroform, which is an electron acceptor and an acidic solvent. However, they demonstrate a reduced affinity to ethyl acetate, an electron donor, and basic solvent.

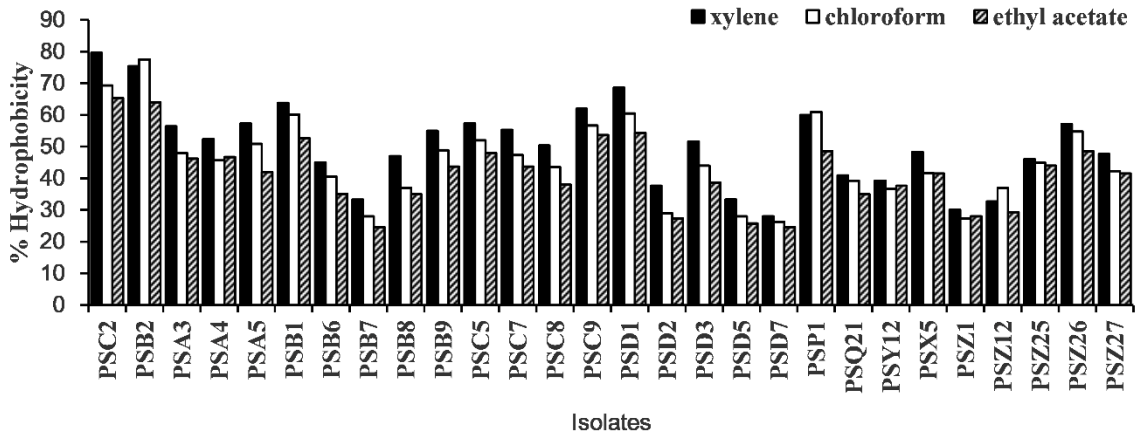

Fig. 4. Hydrophobicity of the isolates with different solvents. Each bar represents values as mean±standard deviations. $P<0.05$ indicates a significant difference in coaggregation between isolates. No significant difference is found between isolates PSC2 and PSB2.

5. Antagonistic test

The ZOI by the CFS of 28 isolates are shown in Table (4). Highest ZOI was shown by isolate PSB2 (22.00±1.00mm) and PSC2 (22.33±1.15mm) against *A.hydrophila* and 21.67±0.58mm, 18.67±0.58mm against *A. jandaei* (Fig. 5A, B, C, D).

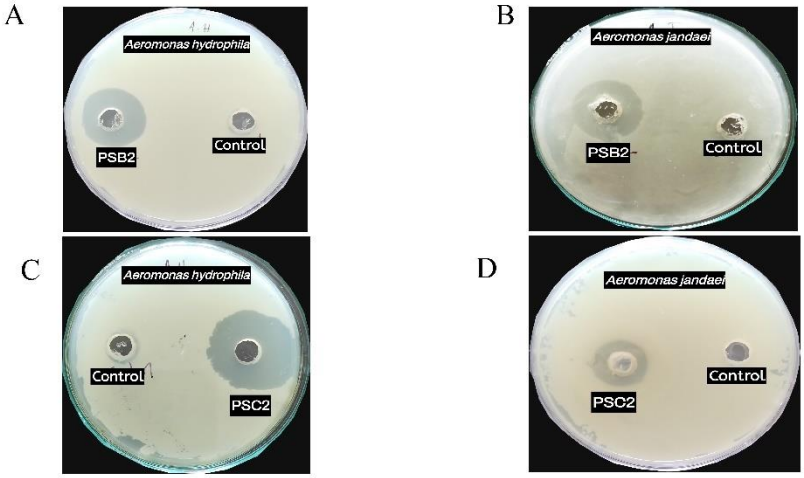

Fig. 5. Antagonistic effect of the supernatants of two isolates PSB2 and PSC2 against A) *Aeromonas hydrophila* B) *Aeromonas jandaei* and C) *Aeromonas hydrophila* D) *Aeromonas jandaei*

Table 4. Antagonistic activity of the isolates against pathogen *A. hydrophila* and *A. jandaei*

Isolates	ZOI (in mm) against *Aeromonas hydrophila*	ZOI (in mm) against *Aeromonas jandaei*
PSB2	22.00±1.00[a]	18.67±0.58[a]
PSC2	22.33±1.15[a]	21.67±0.58[b]
PSA3	0.33±0.58 [f,g]	0.33±0.58[j]

PSA4	4.33±0.58 ^{c,d}	2.67±0.58^{f,g,h}
PSA5	10.00±1.00^{b}	6.67±0.58 ^{c,d}
PSB1	-	0.67±0.58 ^{i,j}
PSB6	4.33±1.15 ^{c,d}	2.33±0.58 ^{f,g,h,i}
PSB7	-	0.33±0.58^{j}
PSB8	10.00±0.00^{b}	5.67±0.58^{d,e}
PSB9	-	0.67±0.58 ^{i,j}
PSC5	9.67±0.58^{b}	7.33±0.58 ^{c,d}
PSC7	0.67±0.58 ^{f,g}	1.00±1.00 ^{h,I,j}
PSC8	3.67±1.15^{c,d,e}	1.33±0.58 ^{h,I,j}
PSC9	10.67±1.15^{b}	7.67±0.58^{c}
PSD1	10.00±1.00^{b}	7.33±1.15^{c,d}
PSD2	10.67±1.15^{b}	6.67±0.58 ^{c,d}
PSD3	5.33±0.58^{c}	3.33±0.58^{f,g}
PSD5	5.67±0.58^{c}	4.00±0.00^{e,f}
PSD7	0.67±0.58 ^{f,g}	0.00±0.00^{j}
PSP1	2.67±1.15 ^{d,e,f}	0.67±0.58 ^{i,j}
PSQ21	0.67±0.58 ^{f,g}	0.33±0.58^{j}
PSY12	4.33±0.58 ^{c,d}	2.33±0.58^{f,g,h,i}
PSX5	2.67±0.58^{d,e,f}	0.67±0.58 ^{i,j}
PSZ1	1.00±1.00^{f,g}	1.67±0.58 ^{h,I,j}
PSZ12	0.33±0.58 ^{f,g}	0.33±0.58^{j}
PSZ25	1.33±0.58^{e,f,g}	0.67±0.58 ^{i,j}
PSZ26	4.67±0.58^{c,d}	0.67±0.58^{i,j}
PSZ27	0.67±0.58 ^{f,g}	1.67±0.58 ^{h,I,j}

The values are average of three replicates. - represent no inhibition. $^{a–j}$ Values followed by the same letters are not significantly different (P> 0.001).

6. Hemolytic activity and biosafety assessment

No clear halo zone was observed for both the isolates, indicating no hemolytic activity, suggesting safety of the isolates **(FAO & WHO, 2002)**. Both strains were considered safe for *L.rohita* and *C.mrigala,* as they demonstrated 100% survival rates without clinical signs or behavioral changes.

7. Molecular identification and phylogenetic analysis

Both the isolates were identified at molecular level by PCR amplifications of genomic DNA using bacterial universal primer targeting 16S rRNA gene and subsequently analyzed by 2% agarose gel electrophoresis using 100bp DNA ladder as reference (Fig. 6). BLAST analysis of 16S rRNA partial sequence from the isolate PSB2 showed 100% sequence similarity with *Streptococcus equinus.* Similarly BLAST analysis of 16S rRNA partial sequence from the isolate 'PSC2' showed 100% sequence similarity with *Leuconostoc pseudomesenteroides*. The 16S rRNA partial sequence of both the isolates has been deposited in NCBI GenBank data base and their respective GenBank Accession No. are shown in Table (5).

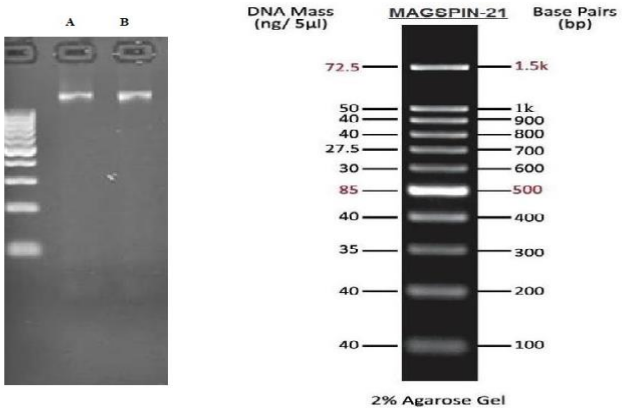

Fig. 6. Quality and size of 16s rRNA gene A) Isolate PSB2 B) Isolate PSC2 amplified by 16s rRNA primer, 100bp ladder was used.

Table 5. Identified potential probiotic isolates by 16S rRNA gene sequencing and their Genbank accession numbers

Isolate	Species	GenBank Accession no
PSB2	*Streptococcus equinus* strain BICP2	PP094633
PSC2	*Leuconostoc pseudomesenteroides* strain BICP3	PP094658

Phylogenetic tree constructed by neighbor-joining method in MEGA 11 for isolate PSB2, PSC2 (Figs. 7, 8) that further corroborates the accuracy of their identification.

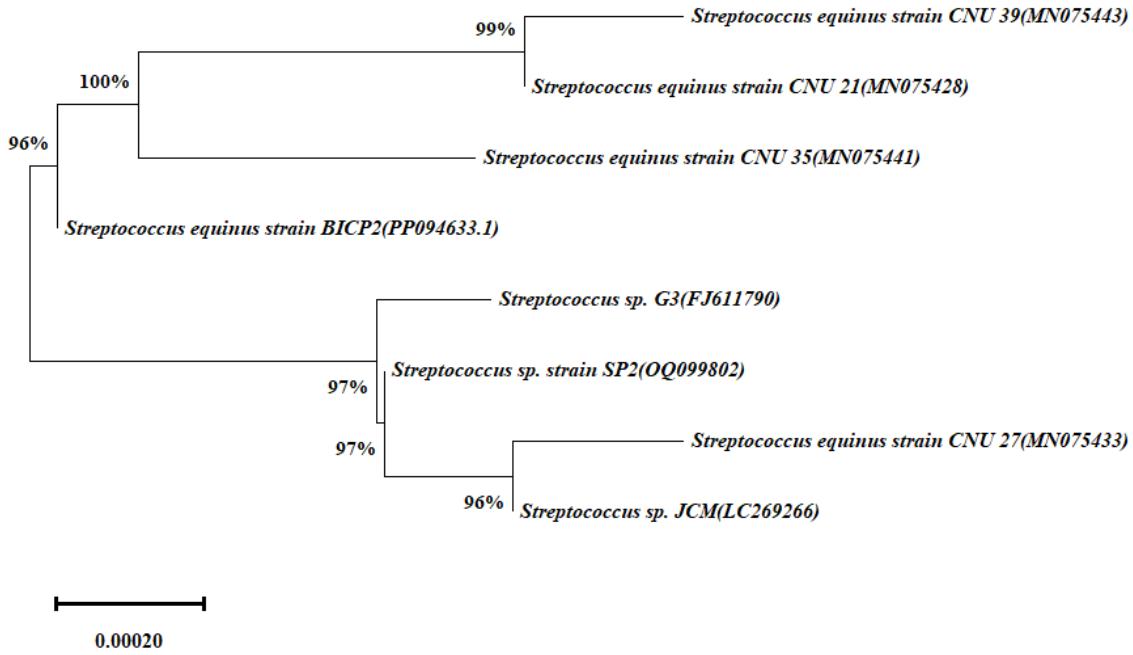

Fig. 7. Phylogenetic tree of *Streptococcus equinus* strain BICP2 with 7 other closely related strain based on partial 16S rRNA sequencing. Bar 0.00020 nucleotide substitution, values in bracket denotes GenBank accession no. Bootstrap values (1000 replications) are represented at branch point.

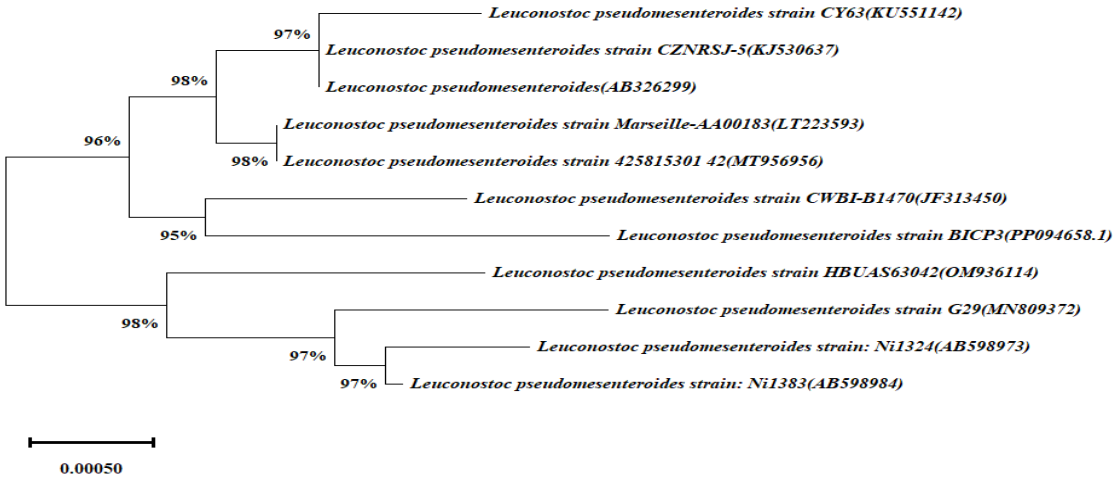

Fig. 8. Phylogenetic tree of *Leuconostoc pseudomesenteroides* strain BICP3 with 10 other closely related strain based on 16S rRNA partial sequence. Bar 0.00050 nucleotide substitution, values in bracket denotes GenBank accession no. Bootstrap values (1000 replications) are represented at branch point.

8. Antibiotic susceptibility assay

The two isolates exhibited different sensitivity profiles, determined by ZOI when exposed to various antibiotics. *Strep.equinus* strain BICP2 and *Leuconostoc pseudomesenteroides* strain BICP3 have exhibited resistance to streptomycin, and sensitivity to gentamicin, tetracycline, and ampicillin (Fig. 9). The ZOI are mentioned in Table (6).

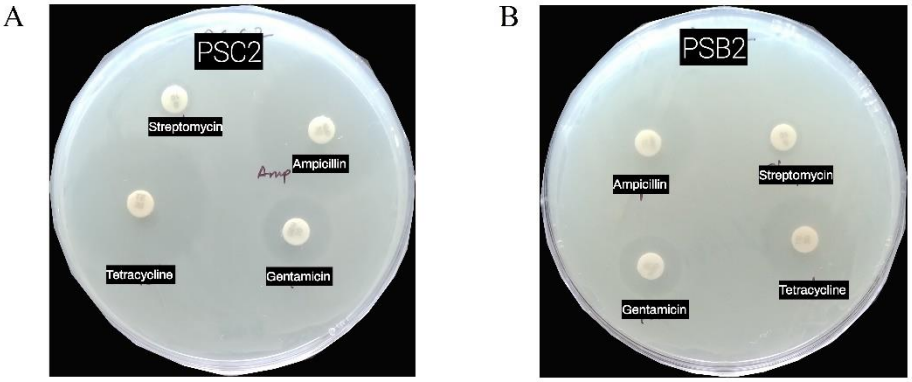

Fig. 9. Antibiotic susceptibility of A) *Leuconostoc pseudomesenteroides* strain BICP3and B) *Streptococcus equinus* strain BICP2

Table 6. Susceptibility test of the probiotic strains against four commercial antibiotics

antibiotics	ZOI in mm (*Strep. equinus* strain BICP2)	ZOI in mm (*Leuconostoc pseudomesenteroides* strain *BICP3*)
Gentamicin	14.00±0.00	13.33±0.58
Streptomycin	9.33±0.58	10.00±1.00
Tetracycline	19.67±0.58	32.67±0.58
Ampicillin	18.67±0.58	33.67±0.58

Values represent average of three replicates.

9. Growth performance

The two isolates have been analyzed for their growth performance by measuring the O.D at 600nm at an interval of 2h interval up to 24hr (Fig. 10).

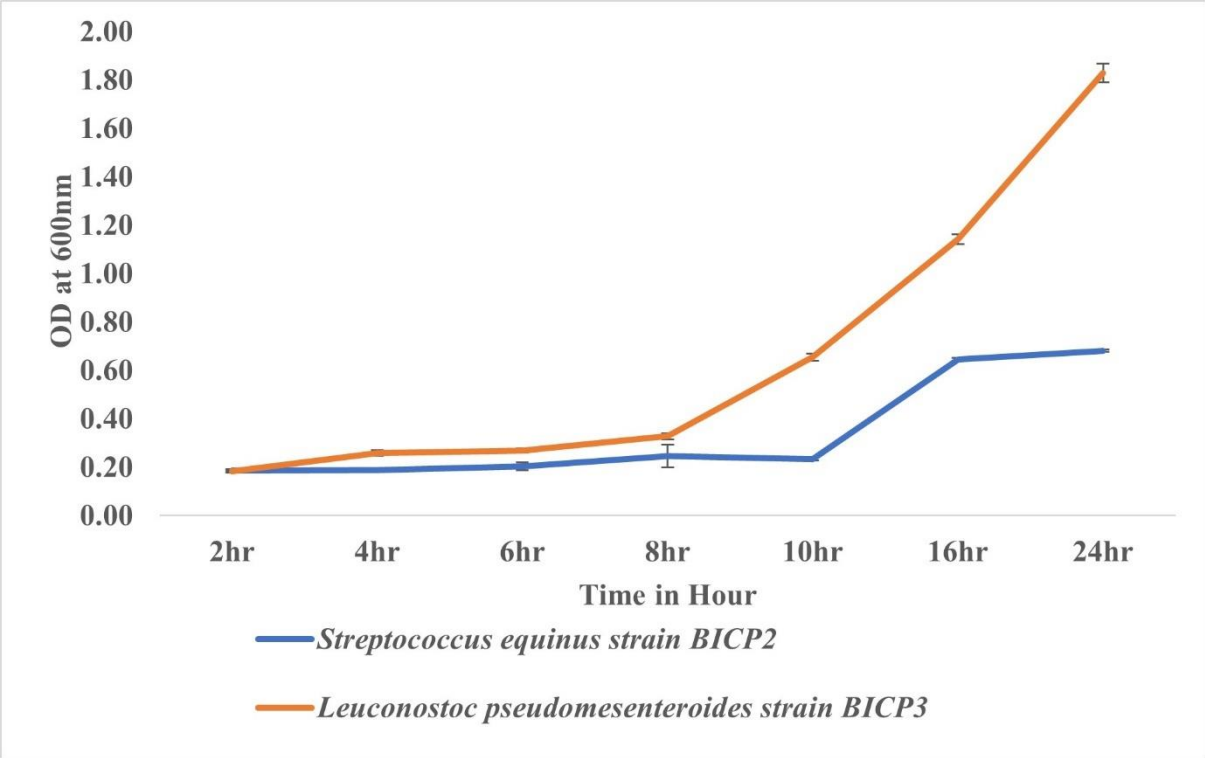

Fig. 10. Growth performance of the two isolates *Strep. equinus* strain BICP2 and *Leuconostoc pseudomesenteroides* strain *BICP3*

10. Compatibility and antagonistic test of the consortia

Following streaking the intersecting lines with both isolates, the plates were incubated for 48h at 34°C. Both the isolates exhibited significant growth, with no antagonistic interactions detected. The consortium of the two isolates showed ZOI of 27.67 ± 0.58mm and 25.33 ± 0.58mm against *Aeromonas hydrophila* and *Aeromonas jandaei*, respectively (Fig. 11B, C).

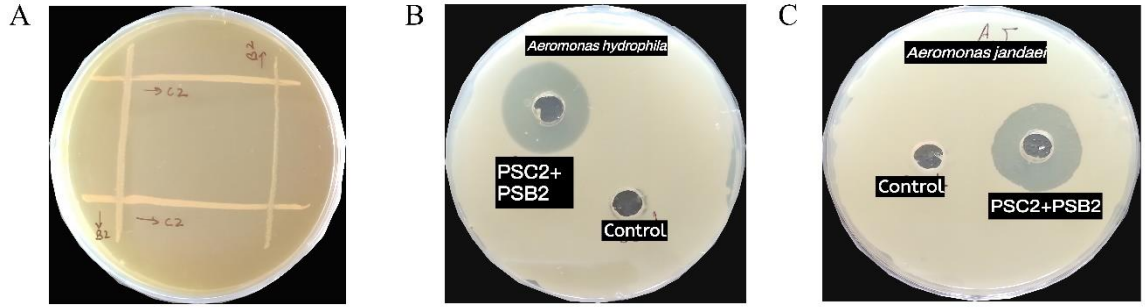

Fig. 11. A) Coexistence test between isolates *Strep. equinus* strain BICP2 and *Leuconostoc pseudomesenteroides* strain *BICP3*; B) Antagonistic activity of the consortia of PSC2 and PSB2 against *Aeromonas hydrophila*; C) Antagonistic activity of the consortia against *Aeromonas jandaei*

11. Clustering analysis

The heat map based on all the essential characteristics of a probiotic of the selected bacterial isolates clearly indicates that isolate PSB2 and PSC2 are potential

probiotics to be used in aquaculture. The Scores plot generated from PCA analysis, considering probiotic attributes and antagonistic effects against fresh water pathogens, positioned PSB2 and PSC2 as outliers, away from the main cluster. This positioning along with the heat map analysis underscores their unique probiotic characteristics.

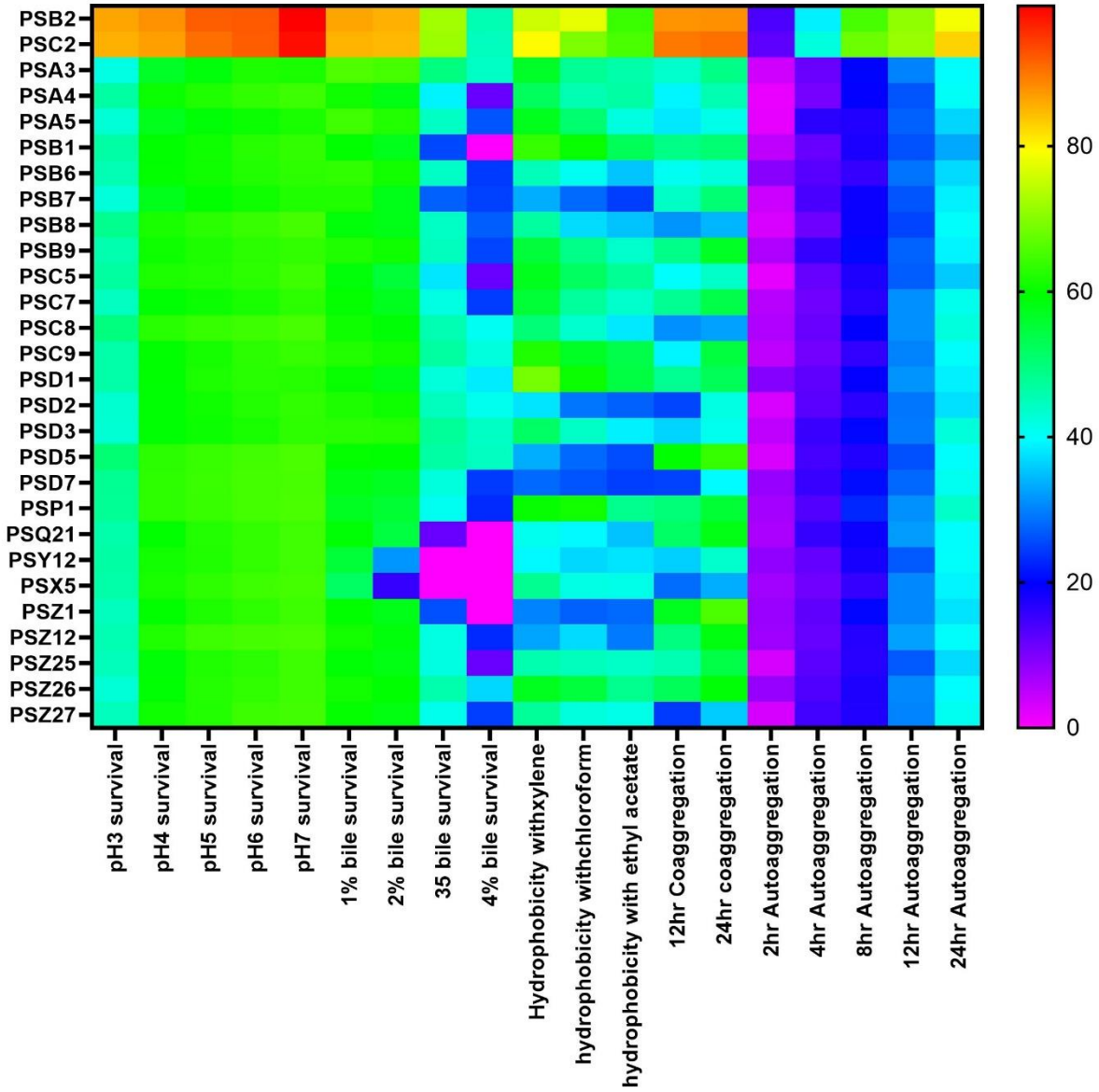

Fig. 12. Heat map of all the 28 isolates considering the probiotic properties of bacteria

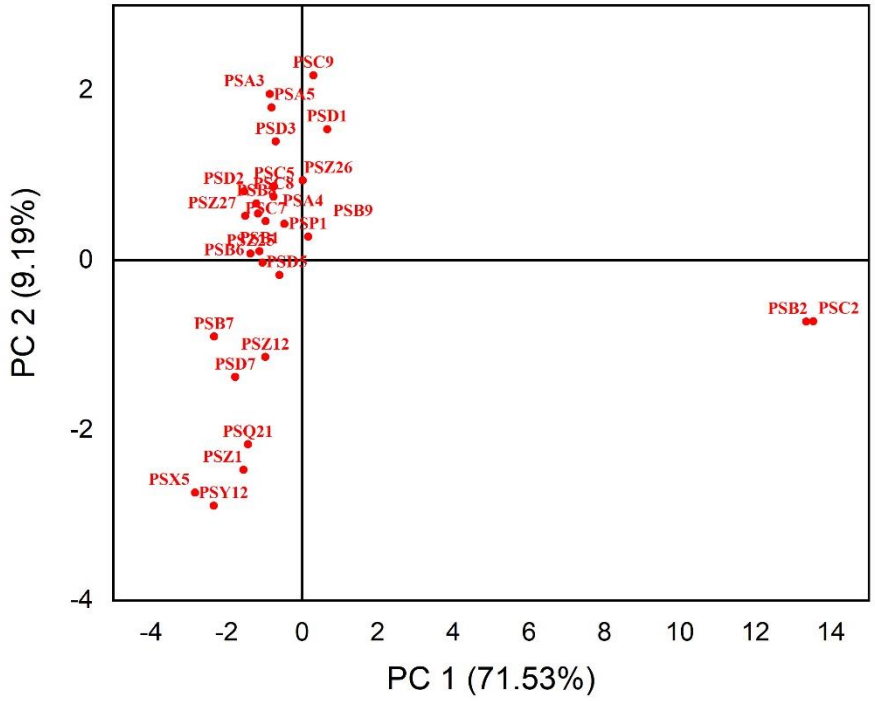

Fig. 13. Cluster analysis of bacterial isolates using PCA analysis

DISCUSSION

Research has shown that the gut microflora of fish species can be influenced by aquatic microorganisms **(Cahill *et al.*, 1990)**. However, the microbial communities in aquatic habitats in Northeastern India have not received extensive research attention **(Joshi *et al.*, 2015)**. It has been observed that probiotics that are naturally present in the digestive system tend to be more effective compared to those that are introduced from external sources **(Ghosh *et al.*, 2007; Ramesh *et al.*, 2015)**. Studies conducted by researchers have indicated that the incorporation of probiotics in one's diet can potentially reduce the reliance on antibiotics; a study conducted by **Selim *et al.* (2015)**, demonstrated this correlation. In order for bacteria to be classified as probiotics, they need to be capable of enduring the challenging conditions of a low pH in the stomach for a minimum of 4h **(Culligan *et al.*, 2012; Argyri *et al.*, 2013)**. In addition, it is important for individuals to possess the capacity to withstand bile salt **(Zavaglia *et al.*, 1998)**. In this study, *Leuconostoc pseudomesenteroides* strain BICP3 (PSC2) and *Strep. equinus* strain BICP2 (PSB2) showed logCFU/ml= 7.36±0.02 and 7.32±0.01, along with survival rates of 85.52 and 86.37% at pH3. Similarly, in a 1% bile solution, the logCFU/ml values were 7.33±0.01 and 7.32±0.01, with survival rates of 85.22 and 86.37%, respectively. These findings indicate that the two isolates have the ability to withstand both acidic conditions

and bile solutions in the intestine. This aligns with a previous study by **Wang *et al.* (2018)**, in which it was reported that *Leuconostoc pseudomesenteroides* can survive at pH 4, although its survival rate decreases below this threshold. The authors observed a survival rate of 60.43%–97.40% in environments with less than 0.3% bile salt concentration. Similarly, **Ayyash *et al*. (2018)** demonstrated logCFU/ml values of 9.2±0.00 and 7.5±0.05 in pH2 after 2h for *Strep. equinus*. In a study conducted by **Gómez *et al*. (2016)**, it was found that *Leuconostoc mesenteroides* had a survival rate of 74.98% in an acidic environment with a pH of 2.5 and 100% survival rate in 0.3% and 1% bile solution. A strain of *Leuconostoc mesenteroides* with exceptional acidifying abilities in milk, was obtained from fermented mare's milk **(Morandi *et al*., 2013)**. *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides* were found to be very closely related on the basis of the 16S rRNA sequence, with five nucleotide differences **(Martinez-Murcia *et al*., 1990)**. The survival percentage of these isolates in acidic and bile-concentrated environments is similar to that of other LAB probiotics, as demonstrated in studies by **Govindaraj *et al*. (2021)** and **Mazlumi *et al*. (2022)**. No significant growth was observed in pH2 and 5% bile solutions, which aligns with previous findings on LAB probiotics **(Sung *et al*., 2010; Allameh *et al*., 2013)**. It is possible that the variations in acid and bile tolerance could be attributed to variations in the source of isolation. Based on available information, this is the first report of isolation of *Strep. equinus* and *Leuconostoc pseudomesenteroides* from the gut of *Channa punctata* from the Northeastern region of India.

Furthermore, a bacterium that is deemed a successful probiotic must exhibit robust auto aggregation and hydrophobicity. Autoaggregation has been observed as a promising method for inhibiting the colonization of pathogenic bacteria in the intestinal gut, as demonstrated by studies conducted by **Collado *et al*. (2009)** and **Mazlumi *et al*. (2022)**. In the study conducted by **Nami *et al*. (2019)**, it was found that the hydrophobicity of bacteria is a key factor in their ability to stick to the intestinal wall. Assessing the ability of bacteria to adhere to the outer lining of intestinal cells is a crucial consideration **(Onifade *et al*., 1997)**. Research has shown the effectiveness of probiotics in helping to remove soluble organic matter from water bodies **(Sánchez-Ortiz *et al*., 2015)**. The potential for aggregation has important implications for both survival and persistence in the GI tract, as well as for cell adhesion properties. Autoaggregation is a critical factor in the promotion of biofilm production, which in turn enhances the colonization process **(Sorroche *et al*., 2012; Kragh *et al*., 2016)**. The study found that both isolates demonstrated strong autoaggregation and a hydrophobicity percentage (>67%) **(Reuben *et al*., 2020)**. A high level of autoaggregation, exceeding 45%, is necessary to qualify as an effective probiotic strain, as stated by **Roghmann *et al*. (2006)**. *Leuconostoc pseudomesenteroides* strain BICP3 and *Strep. equinus* strain BICP2 have demonstrated hydrophobicity percentages of 79.63±0.00, 69.29±0.02, 65.31±0.01 and 75.39±0.01, 77.57±0.02, 64.00±0.02 when exposed to Xylene, Chloroform, and ethyl acetate,

respectively. Additionally, they exhibited autoaggregation percentages of 82.82±0.12 and 78.63±0.74 after 24h, which surpass the previously reported values for *Leuconostoc mesenteroides* **(Nikolic *et al*., 2010)** and *Strep. equinus* **(*Mahadin et al.*, 2018)**.

Unlike autoaggregation, coaggregation refers to the capacity of bacteria to join forces with different types of bacteria, effectively thwarting the colonization of the gut by harmful bacteria. Coaggregation with bacteria is essential for eliminating pathogens from the GI tract, as demonstrated by **Tuo *et al*. (2013)**. Over time, the coaggregation ability of the two isolates with *A. hydrophila* showed an increase. **Nikolic *et al*. (2010)** found coaggregation of *Leuconostoc mesenteroides* ranges between 31.21% with *V. parahaemolyticus* and 10.74% with *E. coli* O157. Also, data from other reports of LAB probiotic **(Espeche *et al*., 2012; Kassaa *et al*., 2014; Puniya *et al*., 2016)** demonstrate that the coaggregation abilities of these two isolates PSB2 and PSC2 with aquatic pathogens are quite high, implying an important host defense mechanism against infection **(Rickard *et al*., 2003)**.

In the current investigation, the *Leuconostoc pseudomesenteroides* strain BICP3 and *Strep. equinus* strain BICP2 exhibited a ZOI measuring 22.33±1.15, 22.00±1.00, and 21.67±0.58, 18.67±0.58mm respectively, when tested against *A. hydrophila* and *A. jandaei*. In a study conducted by **Wang *et al*. (2018)**, it was found that *Leuconostoc pseudomesenteroides* exhibited a ZOI measuring between 15-20mm against *E. coli*, *S. aureus*, and *S. enteritidis*. Another report by **Govindaraj *et al*. (2021)**, demonstrated that LAB displayed a ZOI ranging from 16.67-20.67mm against *A. hydrophila*. Also, **Paray *et al*. (2018)** reported a ZOI of 15-20mm by *Leuconostoc mesenteroides* against *E. coli*, *S.enterica*, *L.monocytoenes*, *S.aureus* and *B.subtilis*. Based on current understanding, the remarkable inhibition of *A. hydrophila* by *Strep.equinus* has not been observed before. There is a scarcity of research on the inhibition of *A.jandaei* by *Leuconostoc pseudomesenteroides* and *Strep. equinus*. These two isolates have been found to be compatible with each other, and their consortia have demonstrated a ZOI measuring 27.67±0.58 and 25.33±0.58mm against *A hydrophila* and *A. jandaei*, respectively. This indicates a greater inhibitory activity than what each individual bacterium can exhibit. This suggests that the utilization of a combination of these two bacteria yields greater efficacy in combating pathogens compared to their individual use.

Based on the FAO/WHO report, it is worth noting that while Lactobacillus is generally regarded as safe, but there have been reports of potential side effects **(Food Agriculture Organization/World Health Organisation FAO/WHO, 2002)**. Thus, it is essential to utilize antibiotic resistance and toxicity studies for safety testing purposes. Moreover, certain probiotics have been found to potentially cause hemolysis because hemolysin is regarded as a virulence factor (**Foulquié Moreno *et al*., 2006; Spinosa, 2009)**. No hemolytic activity was detected in either of the potent probiotic isolates. In a study conducted by **Wang *et al*. (2018)**, it was discovered that *Leuconostoc*

pseudomesenteroides does not exhibit hemolytic properties. Additionally, our probiotic strains have been proven safe through *in vivo* safety testing.

Two different categories of antibiotics were utilized to choose effective LAB probiotics. The first category consists of inhibitors that target cell wall synthesis, like ampicillin. The second category includes inhibitors that affect protein synthesis, such as tetracycline, gentamicin, and streptomycin (Additives, E. P. O. & Feed, P. O. S. U. I. A. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance EFSA J. 10, 2740 2012). In addition, it is important for probiotics to be responsive to commonly used antibiotics in order to minimize the risk of transferring antibiotic resistance genes to the host, which can be potentially fatal **(Reuben *et al*., 2020)**. It is also crucial to prevent the horizontal transfer of antibiotic resistance genes to pathogens **(Doyle *et al*., 2012)**. Although it is widely recognized that *Leuconostoc* strains possess a natural resistance to glycopeptides such as vancomycin, however, there is limited research available on their resistance to other antibiotics **(Hemme & Foucaud-Scheunemann, 2004; Hummel *et al*., 2007; Cardamone *et al*., 2011)**. The susceptibility of *Leuconostoc pseudomesenteroides* strain BICP3 to Ampicillin, Tetracycline, and Gentamicin was observed, while it showed resistance to Streptomycin, which aligns with the findings of **Morandi *et al*. (2013)** and **Wang *et al*. (2018)**. Similar observations were reported by **Rodríguez *et al*. (2009)**. In addition, *Strep. equinus* BICG2 exhibited resistance to Streptomycin. According to previous studies, it has been found that lactic acid bacteria (LAB) generally show sensitivity to Tetracycline and Ampicillin, while they tend to be resistant to Streptomycin and Gentamicin **(Katla *et al*., 2001; Zhou *et al*., 2010)**. On the other hand, our research results differ when it comes to the sensitivity of Gentamicin. This could be due to differences in the origin of the strains and their geographical locations **(Anandharaj *et al*., 2014; Kassaa *et al*., 2014)**.

This discovery is in line with the research conducted by **Gómez *et al*. (2016)**, which identified *Leuconostoc mesenteroides*, a species closely related to *Leuconostoc pseudomesenteroides*, as a probiotic. Further corroboration came from **Chen *et al*. (2017)**, who identified antibacterial activity of *Leuconostoc* against several Gram-positive bacteria. **Paray *et al*. (2018)** also demonstrated antibacterial activity of *Leuconostoc mesenteroides*. In a recent study by **Pan *et al*. (2020)**, it was found that certain exopolysaccharides produced by *Leuconostoc pseudomesenteroides* have the ability to selectively stimulate the gut microbiota. These exopolysaccharides act as prebiotics. **Morandi *et al*. (2013)**, also highlighted the antibacterial activity of *Leuconostoc* against various *enterococci* species and Gram-negative bacteria. All these finding suggests that *Leuconostoc pseudomesenteroides* has the potential to be used as a probiotic. Similarly, **Ayyash *et al*. (2017)** highlighted the beneficial properties of *Strep. equinus*, specifically its probiotic qualities. In a recent study by **Christophers *et al*. (2023)**, reported the production of the antibacterial peptide NISIN E. by *Strep. equinus* MDC1. This finding has significant implications for the field of antibacterial research. The antibacterial

substances produced by *Strep. equinus* have demonstrated significant efficacy in inhibiting the growth of a wide range of bacteria, such as *Bacillus cereus* ATCC 14579, *Enterococcus faecalis*, *Klebsiella* sp., and *Pseudomonas* sp. **(Sabino *et al.*, 2018)**.

CONCLUSION

This investigation focused on isolating lactic acid bacteria from the gut of *Channa punctata* to evaluate their probiotic potential for aquaculture applications. Two potential probiotic isolates were identified: *Leuconostoc pseudomesenteroides* strain BICP3 (Isolate PSC2) and *Streptococcus equinus* strain BICP2 (Isolate PSB2). Both strains demonstrated significant probiotic properties, including the formation of larger inhibition zones against pathogens compared to previously reported cases. Additionally, the two compatible isolates showed a strong synergistic effect against fish pathogens when used together, surpassing the efficacy of individual applications. These findings suggest that *Leuconostoc pseudomesenteroides* BICP3 and *Streptococcus equinus* BICP2, whether used separately or in combination, hold substantial potential for developing effective probiotics tailored for aquaculture, offering a promising strategy for combating aquatic pathogens.

ETHICAL STATEMENT

The protocols of the present study were duly reviewed and approved by Institutional Animal Ethical Committee, Gauhati University (IAEC) Gauhati University (Permit No-IAEC/2024/ETHICAL-Per/2024-5). All the experiments have been carried out in accordance with the IAEC guidelines and regulations.

DATA AVAILABILITY

All the obtained 16S rRNA partial sequences have been deposited in GenBank as *Streptococcus equinus* strain BICP2 (GenBank Accession no. PP094633) and *Leuconostoc pseudomesenteroides* strain BICP3 (GenBank Accession no PP094658).

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CONFLICT OF INTEREST

No conflict of interest.

DECLERATION OF COMPETING INTEREST

None to declare

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