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Probiotic Potential Characterization and Unsupervised Algorithmic Cluster Analysis of Lactic Acid Bacteria Isolated from Gut of *Channa gachua* Fish

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AABSTRACT

This is the first effort in Northeast India to isolate lactic acid bacteria from the gut of *Channa gachua* and to evaluate their probiotic potential through clustering analysis. The study examined the effectiveness of these bacteria against freshwater pathogens. A variety of tests were conducted, including morphological differentiation, catalase activity, IMViC tests, acid and bile tolerance, auto aggregation and coaggregation, hydrophobicity, hemolytic and biosafety assays, 16S rRNA sequencing for molecular identification, pathogen antagonism, antibiotic susceptibility, growth performance, coexistence tests between the isolated probiotic strains, and antagonism of the consortia against pathogens. Clustering analysis was performed using heat maps and principal component analysis, focusing on the probiotic attributes of the isolates. Among all the isolates, two strains with the most promising probiotic characteristics were identified through Sanger's dideoxy sequencing of the 16S rRNA gene: *Staphylococcus hominis* strain BICG1 and *Streptococcus equinus* strain BICG2. These strains exhibited a high degree of auto aggregation, coaggregation, and hydrophobicity, with their growth unaffected by varying levels of acid and bile. When tested against pathogens *Aeromonas hydrophila* and *Aeromonas jandaei*, both *Staph. hominis* and *Strep. equinus* strains showed effectiveness. All strains, except for *Strep. equinus*, were found to be sensitive to four antibiotics. Both strains were compatible, and their consortium displayed enhanced *in vitro* inhibition against aquatic pathogens. This investigation led to the screening of two potential probiotic strains, *Staph. hominis* strain BICG1 and *Strep. equinus* strain BICG2, for use in the aquaculture sector.

INTRODUCTION

The rapid advancement of aquaculture has been hindered by disease outbreaks, presenting substantial obstacles to the industry. Over the past few decades, chemical drugs, particularly antibiotics, have been used to manage diseases in aquaculture. However, the use of antibiotics poses a significant risk due to the long-term presence of

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their residues in animal tissues and the rise in antimicrobial resistance **(Cooke, 1976; McPhearson *et al*., 1991; Balcazar *et al*., 2006)**. Consequently, antibiotic-resistant bacteria have emerged, posing challenges in the treatment of infectious diseases **(Penders & Stobberingh, 2008; Berglund, 2015)**. Probiotics have emerged as highly suitable substitutes for antibiotics in this context **(Fjellheim *et al*., 2010)**. Probiotics are live microorganisms that, when administered in sufficient quantities, improve host health **(Kesarcodi-Watson *et al*., 2008)**. Probiotics can improve fish health and water quality by inhibiting pathogens and by improving feed utilization **(Sarmah & Sarma, 2023)**. The various major probiotic bacteria belonging to the genera *Lactobacillus*, *Lactococcus*, and *Bacillus* **(Ringo *et al*., 1998; Irianto *et al*., 2002; Balcázar *et al*., 2007)** have been effectively isolated from the intestines of healthy fish. Recent studies have identified more possible isolates, including bacteria from the genera *Streptococcus* **(Giri *et al*., 2013; Mutamed *et al*., 2018)**, *Pediococcus* **(Xing *et al*., 2013; Jaafar *et al*., 2019)**, *Staphylococcus* **(Rajeswari *et al*., 2016; Kanjan *et al*., 2020)**, and *Enterococcus* **(Dias *et al*., 2019)**. Improvements in our understanding of profitable fish species have led to the identification of several strains that show promising characteristics as probiotics. Thus, bacteria are screened for their probiotic potential using microbiological isolation techniques, Gram staining, morphology, catalase, antagonism, low pH tolerance, bile salt tolerance, auto aggregation, coaggregation, hydrophobicity, and haemolytic tests **(Nikoskelainen *et al*., 2001; Balouiri *et al*., 2016)**.

These techniques are important for characterizing the strains present in the desired host and for identifying new microorganisms that can be used as probiotic in economically important fish species. Assam being one of the states of North East India, is a hotspot for biodiversity and is the richest in terms of freshwater aquatic resources among all North eastern states **(Goswami *et al*., 2002; Kashyap *et al*., 2012)**. The Northeast region of India is recognized as a biodiversity hotspot for its diverse range of plant and animal species, especially economically important microorganisms that have yet to be studied **(Banerjee *et al*., 2015)**. Isolating bacteria from fish of such a heterogeneous environment provides an opportunity to obtain a novel strain with probiotic potential.

The gut of aquatic animals in Northeastern India reflects the undiscovered microbes in this region, as the digestive tracts of these animals are packed with bacteria from the water and food they consume **(Muthukumar *et al*., 2015)**. The composition of the intestinal microbiota is influenced by various physicochemical factors, such as intestinal motility, pH levels, redox potential, nutrient availability, and host secretions such as digestive enzymes, hydrochloric acid, bile, and mucus **(Booijink *et al*., 2007)**. Therefore, the gastrointestinal (GI) tract contains numerous distinctive environments, each hosting a diverse microbial ecosystem that becomes more diverse as it progresses along the GI tract **(Gerritsen *et al*., 2011)**. In addition to aiding digestion, indigenous microbes also play a crucial role in the immune system by preventing the colonization of pathogenic

microorganisms (**Dethlefsen *et al*., 2006; Gerritsen *et al*., 2011**). As indigenous probiotics are already accustomed to the fish intestinal environment, they are more significant as potential probiotics (**Kotzent *et al*., 2020**). Since the positive impacts of probiotic bacteria are primarily focused on the GI tract, it is important for probiotics to possess strong surface hydrophobicity and aggregation properties to effectively adhere to and establish colonies in the GI tract (**Del Re *et al*., 2000; Collado *et al*., 2009**). Additionally, it has been established that probiotic formulations including multiple strains or species may enhance their efficacy by causing synergistic positive effects on the host's health, such as an extension or improvement of the desired effects (**Timmerman *et al*., 2004**). The freshwater fish *Channa gachua* are cultivated by fish farmers and used both as food and as a raw material for medicines. It also has pharmaceutical effects that may prevent diabetes, skin infections, heart problems, and other conditions (**Mustafa *et al*., 2012; Shillewar, 2021**).

However, studies on the gut microbial flora of *Channa gachua* for the development of probiotics are limited. With this in mind, the present study was designed with the primary objective of identifying and characterizing a novel probiotic strain from the gut of *Channa gachua* and assessing its probiotic potential for use in the aquaculture industry. The strain underwent comprehensive characterization using various microbiological techniques, including catalase activity, acid and bile tolerance, hydrophobicity, antagonism, hemolytic and safety assays, antibiotic susceptibility, molecular identification, coexistence tests, and the antagonistic effects of the consortia. Out of 70 preliminary selected isolates, 30 were chosen for further study based on their morphology and Gram staining. These isolates were selected for their potential to provide health benefits to the host, as reported by **Kotzent *et al.* (2020)**.

MATERIALS AND METHODS

1. Sample collection

Healthy freshwater fish, *Channa gachua* (N=150) were collected from different parts of Assam. Geographical distribution of sampling sites is shown in Fig. (1). The fish were immediately transported to the Fish Molecular Biology Laboratory, Gauhati University, ensuring adequate aeration, for further research.

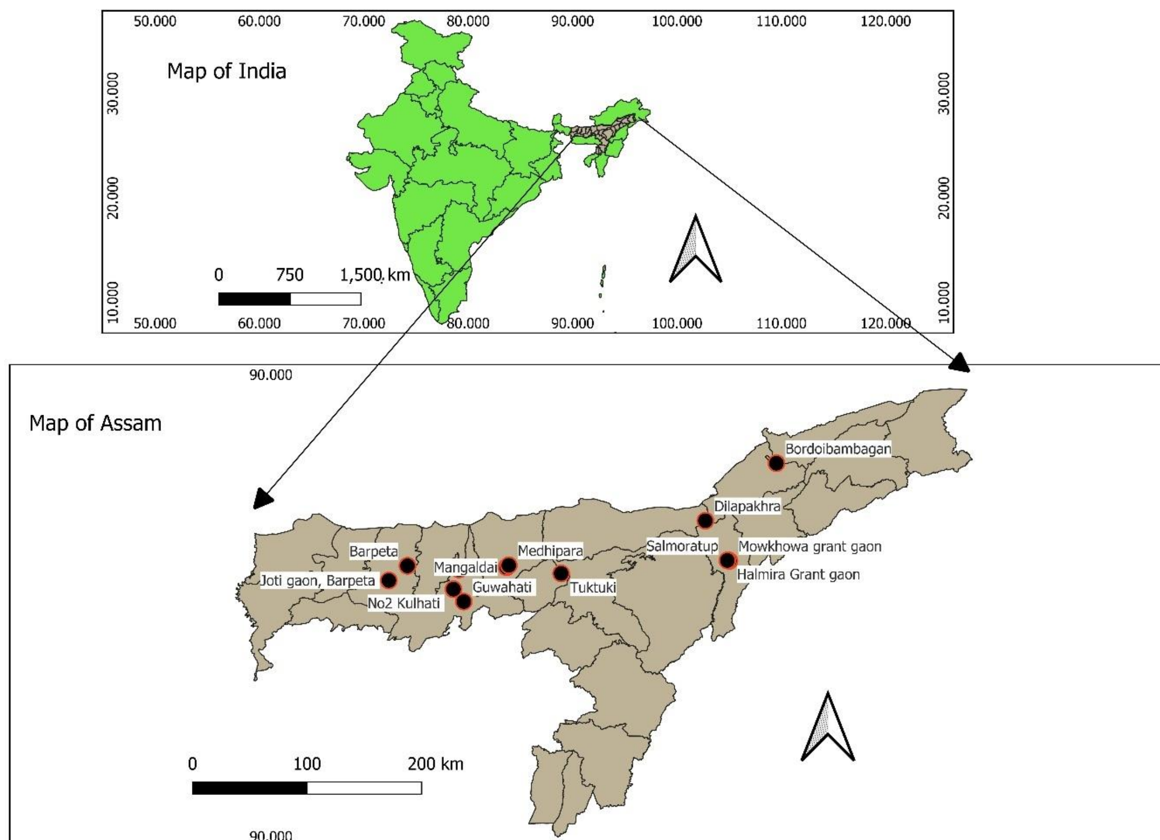

Fig. 1. Geographical locations of sample collection sites shown in the map. Dilapakhra (Lat 26.845806° Long 93.729679°), Tuktuki (Lat 26.394006° Long 92.491187°), Medhipara (Lat 26.463612° Long 92.041519°) No2 Kulhati (Lat 26.25874° Long 91.566737°), Mangaldai (Lat 26.447179° Long 92.023178°), Guwahati (Lat 26.152517° Long 91.654968°), Joti gaon, Barpeta (Lat 26.333343° Long 91.011658°), Barpeta (Lat 26.459341° Long 91.171723°), Halmira Grant gaon (Lat 26.507637° Long 93.925143°), Mowkhowa grant gaon (Lat 26.502307° Long 93.936047°), Salmoratup (Long 26.504996° Long 93.918528°), Bordoibambagan (Lat 27.338317° Long 94.339875°) of Assam state, India

2. Isolation and culture of gut microbes

All of the collected fish were kept in starved conditions for 48hrs to remove the allochthonous bacteria. Following starvation, fish were anesthetized by providing hypothermia condition and disinfected using 1% iodine immediately **(Trust *et al.,* 1974)**. The fish were dissected, and their intestines were aseptically extracted and homogenized using normal saline solution (NSS; 1:10 volume) **(Das *et al.,* 1991)**. The homogenized mixture was serially diluted in NSS for each fish individually. 0.25ml of each dilution was evenly spread on a pre-dried MRS (Man, Rogosa, and Sharpe) agar plate (Himedia®, India). The plates were incubated at 34°C with carbon dioxide tension for 48hrs. The milky white colonies were then streaked on MRS agar for isolation and purification. The colonies were selected based on the characteristics identified through the Gram staining technique. Only bacteria belonging to the Gram-positive group were chosen for further examinations. Pathogenic bacteria, *Aeromonas hydrophila* (GenBank Accession

no. MN097841) and *Aeromonas jandaei* (GenBank Accession no. MN204041) were already available in Fish Molecular Biology Laboratory, Gauhati University.

3. Morphological and biochemical characterization

The investigation involved the examination of colony morphology, Gram staining, and biochemical characteristics: catalase production test, IMViC test (Methyl red test, Indole test, Voges-proskauer, Citrate utilization test), following the recommendations provided in Bergey's manual of Determinative Bacteriology (**Holt *et al.,* 1994**). Carbohydrate utilization tests were performed using KB009A-5KT HiCarbo™ Kit (KB009A, KB009B1) (HiMedia, India) following the manufacturer's instructions.

4. Acid and bile tolerance test

The ability to survive across various intestinal environments, including low pH and bile salts, are essential requirement for a probiotic (**Sánchez *et al.,* 2013**). The acidic pH and bile salt tolerance were assessed using the methodology given by **Tan *et al.* (2013)**. The isolates were cultured in MRS broth at a concentration of 10^8 CFU/ml. They were then centrifuged at 2822×g for 10min, washed, and resuspended in MRS broth. The pH of the MRS broth was adjusted using sterile 1.0 N HCl (Labsynth in Diadema, Brazil) to 1, 2, 3, 4, 5, 6, 7, and a control group was left without pH adjustment. Subsequently, the samples were placed in an incubator at 34°C. After 4hrs, 100µl aliquots were taken out from the samples for the counting of colony-forming units (CFUs) on MRS 1.5% (w/v) agar plate.

To assess the effect of bile salts, lactic acid bacteria (LAB) were cultured in MRS broth with various concentrations of bile salts (1, 2, 3, 4, 5%, and a control without any addition) at 34°C. Aliquots (100µl) were collected after 4hrs of incubation for CFU counting on MRS 1.5% (w/v) agar plates. Survival rates were determined by following the equation (**Govindaraj *et al.,* 2021**):

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

5. Auto aggregation and coaggregation assay

The ability of the selected isolates to autoaggregate was examined using protocol of **Angmo *et al.* (2016)**. The isolates were cultured in MRS broth, and the cells were collected by centrifugation at 2822 ×g for 10min. The collected cells were then washed and suspended in phosphate buffered saline (PBS, contains NaCl, KCl, Na_2HPO_4, and KH_2PO_4.) at pH of 7.4. The suspension was adjusted to optical density (OD) of 1.0 and then incubated at 34°C. The absorbance was taken at time intervals of 2, 4, 8, 12, and 24hrs, at a wavelength of 600nm. The following formula was used to determine the auto aggregation percentage:

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

Where, A_0 denotes absorbance at 0hr, and A_t denotes absorbance at different time points.

Coaggregation test was performed following the protocol of **Zuo *et al.* (2015)**. Equal volume (1×10^8 CFU/ml) of selected isolates and suspension of pathogenic bacteria *A. hydrophila* (GenBank Accession no MN097841) were mixed and incubated for 12 and 24hrs. O.D was measured at 600nm at 0, 12, and 24hrs. The coaggregation was calculated by following the formula of **Nagaoka *et al.* (2008)**:

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

Where, A_0 denotes O.D at 0hr, and A_t denotes O.D at different time points.

6. Hydrophobicity assay

According to **Li *et al.* (2014)**, hydrophobicity was evaluated using xylene, chloroform, and ethyl acetate. Exactly, 1.0ml of sample of bacterial suspension (1×10^8 CFU/ml) was mixed with an equal volume of xylene, chloroform, and ethyl acetate individually. The two-phase system was thoroughly mixed using a vortex mixer for 60 seconds. The suspension was left at room temperature for 2, 4, and 8hrs, and the absorbance was measured in an aqueous phase at a wavelength of 600nm. A reduction in the absorbance of the aqueous phase is considered as a measure of cell surface hydrophobicity. Percentage of hydrophobicity was expressed following the formula as follows:

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

Where; A_t denotes OD at different time points and A_o denotes initial OD of the mixtures.

7. Antagonistic assay

The well diffusion method **(Magaldi *et al.,* 2004; Valgas *et al.,* 2007)** was used to assess the antimicrobial activity of cell-free supernatant (CFS) produced by selected isolates against pathogen *A. hydrophila* and A. *jandaei*. The supernatant of the selected isolates was collected by centrifugation at 2822×g for 10min and filtered by a membrane filter (0.2μ) (Millipore, Bedford, MA, USA) to get CFS. The 80μL of pathogenic bacterial culture i.e. *A. hydrophila* and *A. jandaei* (1×10^8 cfu/ml) were spread separately on MHA plate and CFS of selected isolates were poured into the respected holes, punched in the plates and one kept as control. The plates were incubated at 34°C for 48hrs and observed for formation of zone of inhibition (ZOI). The isolates which have shown the most effectiveness against the pathogens along with most promising probiotic characteristics were selected for further evaluation.

8. Hemolytic activity and biosafety assessment

The haemolytic activity was analyzed by adopting the method described by **Gerhardt *et al.* (1982)** and **Buxton (2005)**. An overnight culture of both potential probiotic isolates was streaked onto blood agar supplemented with 5% sheep blood and incubated at 34°C for 48 hours. The presence or absence of clearing zones around the colonies was observed to interpret the result. The *in vivo* safety assessment of the potent probiotic isolates was done in *Labeo rohita* and *Cirrhinus mrigala.* Each fish species (n = 12 each) was housed in two 25-liter tanks with constant water flow and aeration. They were fed commercial food (Cargill, India) until they reached satiation. The animals were randomly divided into three treatments groups (each containing two duplicates): control group (PBS), group 1 (*Staph. hominis*), and group 2 (*Strep. equinus*). A standard bacterial calibration curve was prepared using OD and CFU/ml to prepare the inoculum of desired concentration. The two strains were grown for 24hrs at 34ºC in MRS broth. After adjusting the optical density of the cultured broth at 600nm to get a concentration of 10^7 CFU/ml, the strains were centrifuged, washed, and resuspended in sterile PBS. The inoculum was injected intraperitoneally at 0.1ml per 10g of fish.

9. Molecular identification

9.1 Genomic DNA extraction

The isolates that exhibited significant antagonistic activity against *Aeromonas hydrophila* and *Aeromonas jandaei*, as well as other probiotic characteristics, were grown in MRS broth at 30°C for 48hrs under carbon dioxide tension condition. Bacterial pure cultures (1.5ml) were subjected to centrifugation at 7840×g for 5min at 4°C. The resulting pellets were collected, and the supernatant, which contained the broth media, was discarded in preparation for DNA extraction. The genomic DNA was extracted using the QIAamp® DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions and stored at –20°C until further use. The obtained DNA was quantified using a Nanodrop Lite Spectrophotometer (Thermo Scientific, USA) in ng/µl. Additionally, a qualitative assessment was conducted by running the DNA to 2% (w/v) agarose gel electrophoresis.

9.2 Amplification of 16S rRNA gene

The amplification of the 16S rRNA gene of the isolates was carried out using a thermal cycler T100™ Thermal Cycler (Bio-Rad, Berkeley) by using a pair of primers (5$^{/}$ AGAGTTTGATCCTGGCTCAG-$^{/}$3, 5$^{/}$TACGGTTACCTTGTTACGACTT 3$^{/}$) **(Weisburg *et al*., 1991)**. The PCR reaction mixture consisted of 25µL of a ready-to-use PCR master mix (R2523-100RXN, Sigma, USA), 2.5µL each of forward and reverse primers, 5µL of DNA template (100ng), and 15µL of sterile nuclease-free water, resulting in a total volume of 50µl. Additionally, a negative control (without DNA template) was included. The PCR conditions were set as follows: an initial denaturation

at 95°C for 3min, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 45 seconds, and a final extension at 72°C for 3min. The qualities of the PCR amplified product were assessed using 2% (w/v) agarose gel electrophoresis with ethidium bromide stain. The PCR-amplified product was subsequently sent to Mediomix Diagnosis and Bioresearch in Bengaluru, India for Sanger's dideoxy sequencing prior to which, the PCR product was purified through gel extraction. The same PCR primers were used as sequencing primers.

10. Species identification and phylogenetic analysis

The 16S rRNA partial sequences obtained from sequencing were modified by aligning forward and reverse reads using BIOEDIT ver7.0.5.3 software alignment editor **(Hall, 1999)**. Similarity of the modified sequence was searched by using Basic Local Alignment Search Tool (BLAST) in the National Centre for Biotechnology Information (NCBI) in Rockville Pike, Bethesda, USA (NCBI) database to determine the closest neighboring individual(s). The 16S rRNA partial sequences of the isolates were compared to deposited partial 16S rRNA sequences. The redesigned sequences of the two isolates were submitted to the GenBank database (NCBI).

For phylogenetic tree construction, sequences were aligned using the CLUSTAL W algorithm **(Thompson *et al.,* 1994)** with default settings within the Molecular Evolutionary Genetic Analysis 11 (MEGA Ver 11) software **(Kumar *et al.,* 2016)**. The phylogenetic tree was constructed using the neighbor-joining method **(Saitou *et al.,* 1987)** in MEGA Version 11, based on evolutionary distances. The bootstrap test (1000 replicates) reveals the percentage of replicate trees where the associated taxa clustered together, displayed next to the branches **(Felsenstein, 1985)**. The tree is accurately depicted, with branch lengths measured in the same units as the evolutionary distances used to construct the phylogenetic tree. The evolutionary distances were calculated using the Maximum Composite Likelihood method **(Tamura *et al.,* 2016)** and are expressed in terms of the number of base substitutions per site. All positions with ambiguous information were eliminated for each pair of sequences (using the pairwise deletion option).

11. Determination of antibiotic susceptibility

The two selected isolates were examined for their antibiotic susceptibility by Kirby-Baurer disc diffusion method **(Bauer *et al.,* 1966)**. The antibiotic discs (Himedia®) used for this study were gentamicin (10µg), streptomycin (10µg), tetracycline (30µg), and ampicillin (10µg). Results were analyzed according to Clinical and Laboratory Standards Institute **(Wayne *et al.,* 2010)**.

12. Growth performance

Growth performance was analyzed by inoculating the pure bacterial isolates (1ml, 1×10^8 CFU/ml) in MRS broth (Himedia®, India) and was incubated under carbon dioxide tension condition at 34^0C. OD was measured (n=3) at 600nm after each 2hrs up to 24hrs.

13. Coexistence test

This test examines the feasibility of co-cultivating the two probiotic bacteria that are being evaluated. The tests were conducted following the methodology outlined by **Guo *et al.* (2009)**. The bacteria were cultivated under their specific growth conditions for 48hrs. Afterward, samples were streaked in a perpendicular manner on the surface of 1.5% MRS (w/v) agar plates. Following a 24-hour incubation period, the plates were inspected for potential antagonistic effects **(James *et al.,* 2017; Al-Hussini *et al.,* 2018)**.

14. Preparation and antagonistic activity of the consortia

Consortia of the probiotic isolates were made by following Direct Mixing method **(Kapoore *et al.,* 2021)**. Direct mixing makes it more effective than monoculture in achieving its targets **(Brenner *et al.,* 2008)**. The antagonistic activity of the consortia against pathogen *A. hydrophila* and *A. jandaei* were tested by well diffusion method **(Magaldi *et al.,* 2004; Valgas *et al.,* 2007)**. The CFS of the consortia was collected by centrifugation at 2822 ×g for 10min, followed by filtration through 0.22 micrometer filter (Millipore, Bedford, MA, USA). 0.25µl of the suspensions of *A. hydrophila* and *A. jandaei* were spread separately on MHA plate, and supernatant of the bacterial consortia was added in one hole pincered on each MHA plate, one hole kept as a control. Plates were incubated for 24hrs at 34°C and observed for formation of ZOI.

15. Unsupervised clustering and statistical analysis

The heat map of all the bacterial isolates for the probiotic characteristics i.e. auto aggregation (12hrs, 24hrs), coaggregation (12hrs, 24hrs), hydrophobicity (with xylene, chloroform, ethyl acetate), survival in bile salts (1, 2, 3, and 4% concentration), survival in acidic condition (pH3, pH4, pH5, pH6, pH6, and pH7) was generated using Graph Pad prism 10.1.0 (316). For clustering using unsupervised algorithm of the isolates considering the probiotics attributes, principal component analysis (PCA) was done that reduce dimensionality, forming unbiased clustering using Origin Pro (2019b) software **(Farhadian *et al.,* 2021)**. To determine statistically significant difference among the parameters of isolates ANOVA was done following Tukey test in SPSS (IBM SPSS Version 29.0.2.0(20)) (**Sola *et al.,* 2022**). Additionally, Holm-Sidak **(Avican *et al.,* 2021)** and Dunnett tests were performed in Graph pad prism to find out if there was any

significant difference between each isolate (**Govindaraj *et al.,* 2021**). All the experiments were carried out thrice, and results were presented in Mean value ± S.D.

RESULTS

1. Isolation, morphological, and biochemical characterization of bacterial isolates

A total of 70 isolates having round, milky white colonies were initially selected among 150 isolates. Thirty Gram-positive isolates among 70 isolates were selected for further analysis. These isolates were named as PS1A, PS1B, PS1C, PS1D, PS1E, PS5A, PS5B, PS5C, PS5D, PS5E, PS5F, PS6A, PS6B, PS7A, PS8A.PS8B, PS9A, PS30A, PS30B, PS30C, PS66A, PS70A, PS90A, PS110A, PS120A.PS120B, PS120C, PS140A, PS140B, and PS140C. Result of carbohydrate utilization are mentioned in supplementary materials (Table A).

Table 1. Biochemical characterization of all the 30 isolates from gut of *Channa gachua* from different locations of Assam, North East India

Isolates	Shape	MR test	VP Test	Indole production	Citrate utilization test	Catalase Test
PS110A	Round	+	-	-	-	+
PS120A	Round	+	-	-	-	+
PS120B	Round	+	-	-	-	-
PS120C	Round	+	-	-	-	-
PS140A	Round	+	-	-	-	-
PS140B	Round	-	+	-	-	-
PS140C	Round	-	+	-	-	-
PS1B	Round	+	-	-	-	-
PS1C	Round	+	-	-	-	-
PS1D	Round	+	-	-	-	+
PS1E	Round	+	-	-	-	-
PS30A	Round	+	-	-	-	+
PS30B	Round	-	+	+	-	-
PS30C	Round	-	+	+	-	-
PS5A	Round	-	+	-	-	+
PS5b	Round	+	-	-	-	+
PS5C	Round	-	+	-	-	-
PS5D	Round	-	+	-	-	-
PS5E	Round	-	+	-	-	-
PS5F	Round	+	+	-	-	-

PS66A	Rod	-	+	-	-	-
PS6A	Round	+	-	-	-	-
PS6B	Round	+	-	-	-	-
PS70A	Rod	+	-	-	-	-
PS7A	Round	+	-	-	-	-
PS7B	Round	+	-	-	-	-
PS8B	Round	+	-	-	-	-
PS90A	Round	+	-	+	-	-
PS9A	Round	+	+	+	-	+
PSA2	Round	+	-	-	-	-

+ Positive; – negative.

2. Acid and bile test

Table (2) displays the survival rates of 30 round milky white isolates following a 4hrs period of incubation at pH levels ranging from 1-7. The isolates PS5b and PSA2 demonstrated the highest level of resistance to low pH conditions, with survival rates of 85.28 and 86.55%, respectively, at pH3. In contrast, isolates PS140B and PS140A exhibited the lowest viability after 4hrs, with survival rates of 23.04 and 36.65%, respectively. No growth was observed at pH1 and 2.

Table 2. Survivability of the screened 30 isolates at different pH values

Isolates	pH3		pH4		pH5		pH6		pH7	
	Log CFU/ ml	Survival (%)	Log CFU/ ml	Survival (%)	Log CFU/ ml	Survival (%)	Log CFU/ ml	Survival (%)	Log CFU/ ml	Survival (%)
PS110A	3.57± 0.23	41.18	3.69± 0.09	42.65	5.06± 0.08	58.46	5.23± 0.03	60.39	6.36± 0.05	73.44
PS120A	3.16± 0.28	37.17	3.53± 0.21	41.58	5.06± 0.10	59.53	5.24± 0.03	61.63	6.33± 0.01	74.50
PS120B	3.59± 0.11	42.42	3.82± 0.04	45.13	4.98± 0.05	58.83	5.20± 0.07	61.40	6.39± 0.01	75.43
PS120C	3.59± 0.11	42.02	3.84± 0.06	44.94	4.98± 0.03	58.30	5.18± 0.03	60.64	6.33± 0.01	74.06
PS140A	3.10± 0.17	36.65	3.46± 0.15	40.90	4.81± 0.13	56.86	5.20± 0.03	61.51	6.33± 0.01	74.85
PS140B	2.00± 1.73	23.04	3.20± 0.17	36.87	4.73± 0.05	54.44	5.12± 0.07	59.00	6.19± 0.02	71.31
PS140C	3.00± 0.00	37.74	2.20± 1.91	27.68	4.73± 0.05	59.44	4.98± 0.03	62.70	6.06± 0.03	76.23

PS1 B	3.87± 0.15	42.80	4.15± 0.03	45.86	5.19± 0.04	57.45	5.20± 0.05	57.54	6.23± 0.04	68.91
PS1 C	3.77± 0.07	44.56	3.83± 0.13	45.25	4.82± 0.11	56.86	4.91± 0.12	57.98	6.09± 0.07	71.95
PS1 D	4.12± 0.04	51.23	4.19± 0.04	52.09	5.15± 0.03	63.92	5.33± 0.04	66.27	6.31± 0.01	78.41
PS1E	4.04± 0.07	50.79	4.08± 0.04	51.30	5.20± 0.03	65.45	5.31± 0.03	66.85	6.31± 0.01	79.39
PS30 A	3.72± 0.10	44.60	3.74± 0.13	44.87	5.04± 0.04	60.43	5.10± 0.07	61.14	6.24± 0.05	74.84
PS30 B	3.67± 0.06	43.86	3.86± 0.09	46.16	5.12± 0.07	61.26	5.19± 0.03	62.13	6.28± 0.00	75.10
PS30 C	3.30± 0.30	40.26	3.66± 0.10	44.64	4.82± 0.11	58.73	5.02± 0.06	61.28	6.18± 0.04	75.31
PS5 A	3.53± 0.21	38.12	3.73± 0.05	40.19	4.98± 0.07	53.74	5.20± 0.07	56.10	6.30± 0.03	67.97
PS5b	7.38± 0.05	85.28	7.67± 0.03	88.71	7.71± 0.03	89.17	7.79± 0.03	90.10	8.63± 0.04	99.77
PS5 C	3.77± 0.07	45.75	3.90± 0.05	47.28	5.06± 0.08	61.36	5.05± 0.02	61.26	6.29± 0.02	76.24
PS5 D	4.01± 0.05	49.90	4.16± 0.05	51.80	5.28± 0.05	65.64	5.30± 0.02	65.93	6.41± 0.01	79.68
PS5E	3.86± 0.07	44.85	3.92± 0.03	45.53	5.01± 0.05	58.22	5.24± 0.04	60.83	6.29± 0.02	73.05
PS5F	4.13± 0.05	49.57	4.19± 0.08	50.24	5.19± 0.04	62.27	5.32± 0.02	63.81	6.37± 0.04	76.38
PS66 A	4.04± 0.04	49.09	4.19± 0.04	50.95	5.25± 0.07	63.81	5.32± 0.02	64.66	6.39± 0.01	77.63
PS6 A	3.53± 0.21	41.77	3.65± 0.16	43.16	4.92± 0.08	58.11	5.03± 0.02	59.43	6.19± 0.02	73.17
PS6 B	3.59± 0.11	43.97	3.72± 0.12	45.48	4.77± 0.07	58.43	5.09± 0.09	62.24	6.31± 0.01	77.25
PS70 A	3.36± 0.10	38.71	3.57± 0.23	41.09	5.03± 0.02	57.92	5.18± 0.03	59.62	6.29± 0.02	72.46
PS7 A	4.12± 0.04	51.29	4.18± 0.03	51.93	5.34± 0.04	66.43	5.36± 0.05	66.66	6.40± 0.02	79.57
PS7 B	4.08± 0.07	45.09	4.19± 0.02	46.40	5.32± 0.02	58.87	5.39± 0.03	59.58	6.43± 0.05	71.13
PS8 B	3.92± 0.06	45.08	3.94± 0.03	45.31	5.08± 0.07	58.40	5.12± 0.04	58.96	6.28± 0.00	72.25

PS90A	3.80± 0.04	43.94	3.91± 0.12	45.21	5.18± 0.03	59.83	5.33± 0.04	61.67	6.41± 0.01	74.06
PS9A	3.76± 0.14	45.08	4.01± 0.06	48.04	5.18± 0.03	61.98	5.24± 0.04	62.73	6.31± 0.01	75.59
PSA2	7.36± 0.06	86.55	7.47± 0.06	87.84	7.72± 0.03	90.82	7.86± 0.02	92.47	8.48± 0.01	99.76

Values are average of three replicates.

Table (3) displays the survival rates of these isolates at various bile concentrations. All 30 isolates exhibited no growth hypersensitivity to a 5% bile salt condition, although there were differences in their level of viability. Isolate PSA2 showed the highest tolerance, with a survival rate of 80.21% at 1% bile concentration and 24.68% at 4% bile concentration. Isolate PS5b had a slightly lower tolerance, with survival rates of 78.18% at 1% bile concentration and 24.93% at 4% bile concentration.

Table 3. Survivability of the selected 30 isolates at different bile concentrations

Isolates	1% bile		2% bile		3% bile		4% bile	
	Log CFU/ml	Survival %	Log CFU/ml	Survival %	Log CFU/ml	Survival %	Log CFU/ml	Survival %
PS1B	4.50±0.03	49.83	4.26±0.04	47.15	3.21±0.04	35.53	2.16±0.28	23.88
PS1C	4.45±0.01	52.56	4.26±0.04	50.33	3.00±0.04	35.40	1.33±1.15	15.74
PS1D	4.54±0.01	56.45	4.18±0.03	51.88	2.98±0.03	37.08	1.33±1.15	16.56
PS1E	4.34±0.02	54.53	4.32±0.05	54.35	2.73±0.05	34.28	1.33±1.15	16.77
PS5b	6.77±0.02	78.18	6.61±0.59	76.35	3.85±0.59	44.48	2.16±0.28	24.93
PS5A	4.48±0.01	48.30	4.36±0.04	47.04	3.12±0.04	33.70	2.16±0.28	23.29
PS5C	4.41±0.02	53.51	4.05±0.05	49.14	2.73±0.05	33.03	2.52±0.07	30.53
PS5D	4.30±0.02	53.49	4.16±0.04	51.69	2.80±0.04	34.83	2.26±0.24	28.10
PS5E	4.41±0.01	51.21	4.12±0.04	47.90	2.82±0.04	32.78	2.20±0.17	25.56
PS5F	4.27±0.03	51.21	3.82±0.06	45.84	2.63±0.06	31.59	1.33±1.15	15.99
PS6A	4.41±0.02	52.12	4.01±0.07	47.44	2.52±0.07	29.77	1.33±1.15	15.76
PS6B	4.16±0.	50.87	3.88±1.	47.54	1.33±1.	16.32	0.00±0.	0.00

	02		15		15		00	
PS7A	4.45±0.02	55.31	4.26±0.04	53.02	2.82±0.04	35.11	2.20±0.17	27.37
PS7B	4.17±0.02	46.09	3.56±1.15	39.39	1.33±1.15	14.75	0.00±0.00	0.00
PS8B	3.94±0.03	45.31	4.00±0.06	46.01	2.67±0.06	30.69	2.10±0.17	24.17
PS9A	4.17±0.03	49.89	3.67±1.15	43.91	1.33±1.15	15.97	0.00±0.00	0.00
PS30A	4.26±0.01	51.12	4.16±0.03	49.83	2.94±0.03	35.22	2.67±0.06	31.97
PS30B	3.56±0.07	42.59	3.00±0.00	35.89	0.00±0.00	0.00	0.00±0.00	0.00
PS30C	4.33±0.01	52.79	4.19±0.04	51.16	2.82±0.04	34.42	2.20±0.17	26.84
PS66A	4.15±0.00	50.38	3.80±0.28	46.13	2.32±0.28	28.17	2.10±0.17	25.52
PSA2	6.83±0.02	80.21	6.46±0.06	75.94	4.06±0.06	47.76	2.10±0.17	24.68
PS70A	4.68±0.02	53.93	4.32±0.14	49.79	2.68±0.14	30.91	1.33±1.15	15.36
PS90A	4.48±0.01	51.76	4.13±0.03	47.79	2.86±0.03	33.11	2.10±0.17	24.28
PS110A	3.63±0.06	41.97	3.36±1.15	38.80	1.33±1.15	15.40	0.00±0.00	0.00
PS120A	4.64±0.01	54.55	4.46±0.03	52.44	3.29±0.03	38.66	2.40±0.17	28.25
PS120B	4.03±0.05	47.54	3.67±0.17	43.29	2.10±0.17	24.80	0.00±0.00	0.00
PS120C	4.51±0.01	52.80	4.44±0.04	51.95	2.82±0.04	33.01	2.10±0.17	24.57
PS140A	3.42±0.10	40.41	2.00±1.15	23.64	0.67±1.15	7.88	0.00±0.00	0.00
PS140B	4.66±0.01	53.68	4.43±0.12	51.05	2.72±0.12	31.28	1.33±1.15	15.36
PS140C	4.00±0.04	50.30	3.90±0.17	49.07	2.20±0.17	27.68	0.00±0.00	0.00

Values are average of three replicates.

Survival rate equals to or greater than 75% in simulated gastric juicem, and bile salt as the cut-off level of tolerance (**Suwannaphan *et al.*, 2021**) of a bacterium is considered to be a probiotic.

3. Auto aggregation and coaggregation

The results of the auto aggregation of the 30 isolates are depicted in Fig. (2). The percentage of auto aggregation in the isolates increased over time. The isolate PS5b exhibited the highest auto aggregation values, measuring 78.27±0.32, followed by PSA2 with a value of 77.83±0.05 after 24hrs. The isolate PS1E demonstrated the lowest value of auto aggregation after 24hrs of incubation, measuring 32.90±0.38.

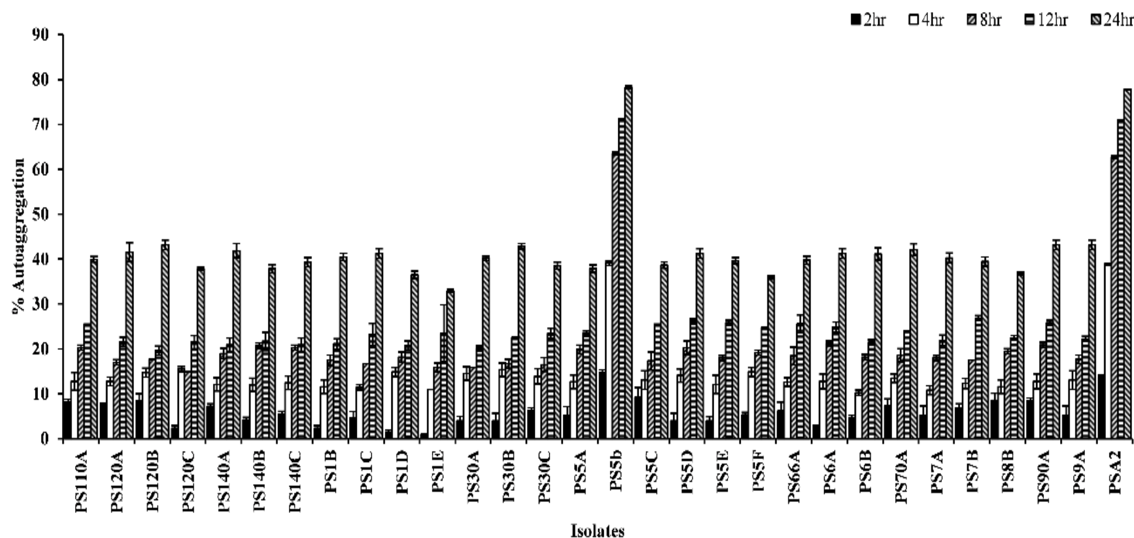

Fig. 2. Auto aggregation of isolates. Each bar represents mean ± standard deviation. *P*<0.05 indicates a significant difference in auto aggregation between isolates. There is a significant difference between PS5b, PSA2 with all other isolates, while there is no significant difference between PSA2 and PS5b.

Fig. (3) displays the outcomes of the coaggregation capacity of the 30 examined isolates. The coaggregation percentages varied between 89.17±0.22% and 10.76±0.11% with *A. hydrophila* at 24hrs. The isolate PSA2 exhibited the highest coaggregation value with *A. hydrophila*, measuring 89.17±0.22% at the 24th hour. Similarly, the coaggregation value for PS5b was 88.21±0.01% at the same time point. The isolates PS66A exhibited the lowest coaggregation values with *A. hydrophila*, measuring 10.76±0.11% at the 24th hour.

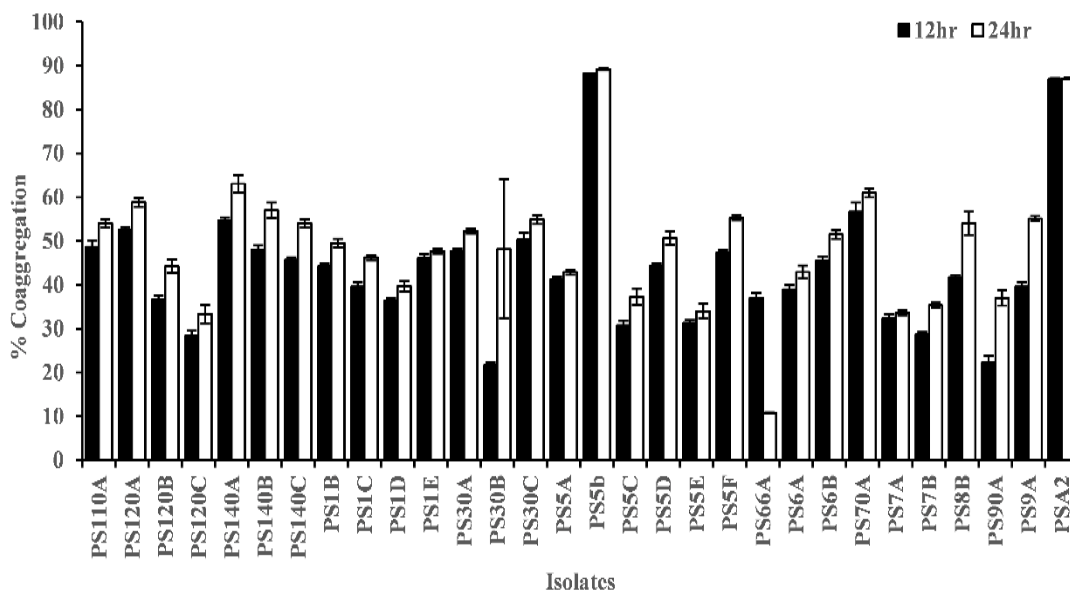

Fig. 3. Coaggregation of isolates. Each bar represents mean ± standard deviation. $P<0.05$ indicates a significant difference in coaggregation between isolates. There is a significant difference between PS5b and PSA2 with all other isolates, while there is no significant difference between PSA2 and PS5b

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 PSA2 (74.07±0.77%) and PS5b (73.38±0.53%). 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.

5. Antagonistic test

The zone of inhibition (ZOI) by CFS for 30 isolates is shown in Table (4). The highest ZOI was observed for isolates PS5b (20.33±0.58mm) and PSA2 (20.67±0.58mm) against *A. hydrophila*, and 12.33±0.58mm and 14.33±1.15mm against *A. jandaei* (Fig. 5A, B).

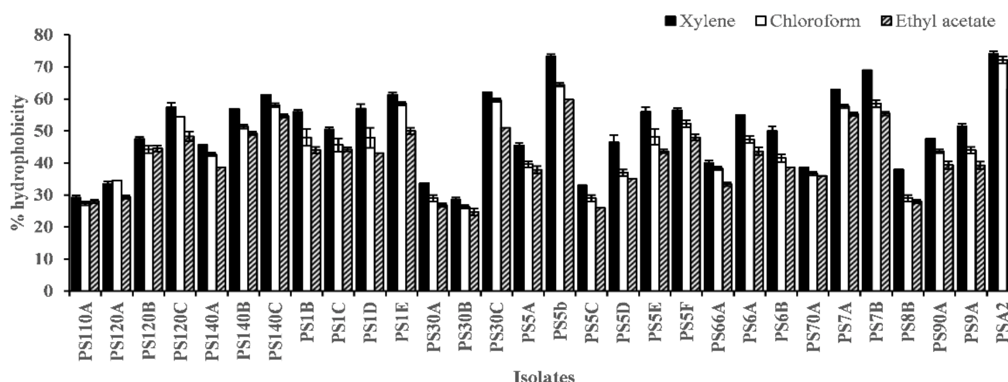

Fig. 4. Hydrophobicity of the isolates with different solvents. Each bar represents values as mean ± standard deviations. $P<0.05$ indicates a significant difference in percentage hydrophobicity between isolates. Isolate PS5b has no significant difference with PSA2, PS120C, PS140C, PS1E, PS30C, PS7A, and PS7B; isolate PSA2 have no significant differences with PS140C, PS7A, and PS7B

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

Isolates	ZOI (in mm) against *Aeromonas hydrophila*	ZOI (in mm) against *Aeromonas jandaei*
PS1B	0.33 ± 0.58^{g}	$1.67\pm0.58^{i,j,k,l}$
PS1C	$4.33\pm0.58^{c,d,e}$	$2.67\pm0.58^{h,I,j,k}$
PS1D	10.00 ± 1.00^{b}	$7.33\pm1.15^{b,c,d}$
PS1E	-	$2.33\pm0.58^{h,I,j,k,l}$
PS5A	$4.33\pm1.15^{c,d,e}$	$3.67\pm0.58^{f,g,h,I,j}$
PS5C	-	$2.67\pm1.15^{h,I,j,k}$
PS5D	10.00 ± 0.00^{b}	$6.33\pm0.58^{c,d,e}$
PS5E	-	$2.33\pm0.58^{h,I,j,k,l}$
PS5F	9.67 ± 0.58^{b}	9.33 ± 0.58^{b}
PS5b	20.33 ± 0.58^{a}	13.33 ± 1.15^{a}
PS6A	0.67 ± 0.58^{g}	$1.67\pm1.15^{i,j,k,l}$

PS6B	3.67±1.15 ^{c,d,e,f}	3.33±1.15^{f,g,h,I,j}
PS7A	10.67±1.15^{b}	8.33±0.58 $^{b, c}$
PS7B	10.00±1.00^{b}	5.67±0.58^{d,e,f}
PS8B	10.67±1.15^{b}	7.33±0.58^{b,c,d}
PS9A	5.33±0.58^{c,d}	5.33±0.58^{d,e,f,g}
PS30A	5.67±0.58^{c}	4.00±1.00^{e,f,g,h,i}
PS30B	0.67±0.58 ^{g}	-
PS30C	2.67±1.15 ^{d,e,f,g}	1.67±1.15^{i,j,k,l}
PS66A	0.67±0.58^{g}	1.33±0.58^{j,k,l}
PS70A	4.33±0.58^{c,d,e}	4.33±1.15^{e,f,g,h}
PS90A	2.67±0.58 ^{d,e,f,g}	0.67±0.58^{k,l}
PS110A	1.00±1.00 ^{f,g}	3.00±1.00^{g,h,I,j,k}
PS120A	0.33±0.58^{g}	2.33±0.58^{h,I,j,k,l}
PS120B	1.33±0.58 ^{f,g}	1.33±0.58^{j,k,l}
PS120C	4.67±0.58^{c,d,e}	2.33±0.58^{h,I,j,k,l}
PS140A	0.67±0.58 ^{g}	1.67±1.15^{i,j,k,l}
PS140B	0.50±0.71^{g}	-
PS140C	2.50±1.91^{e,f,g}	1.33±0.58^{j,k,l}
PSA2	20.67±0.58 ^{a}	14.33±0.58^{a}

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

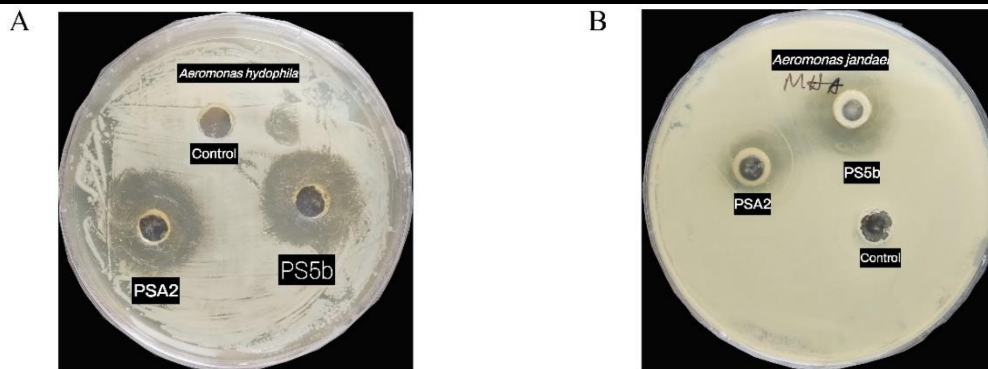

Fig. 5. Antagonistic effect of the supernatants of two isolates PS5b and PSA2 against **A)** *Aeromonas hydrophila* and **B)** *Aeromonas jandaei*

6. Haemolytic activity and biosafety assessment

Both isolates did not produce any clear halo zone that indicates no haemolytic activity, representing safety of the isolates **(FAO & WHO, 2002)**. The strains are considered safe for *L. rohita* and *C. mrigala,* as they exhibited 100% survival rates and showed no clinical signs or behavioral changes.

7. Molecular identification and phylogenetic analysis

Molecular identification of both the isolate was done by PCR amplifications of genomic DNA with 16S rRNA bacterial universal primer and subjected to agarose gel electrophoresis for analysis using 100bp DNA ladder (Fig. 6). BLAST analysis of the obtained 16S rRNA partial sequence of isolate PS5b showed similarity with *Staphylococcus hominis.*

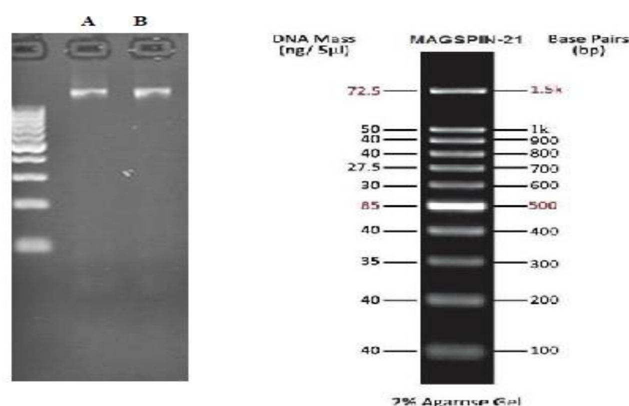

Fig. 6. Quality and size of 16s rRNA gene amplified by 16s rRNA primer, 100bp ladder was used

The 16S rRNA sequence of isolate 'PSA2' showed 100% identity with *Streptococcus equinus*. The 16S rRNA sequences of both isolates have been submitted to the NCBI GenBank database, and the corresponding GenBank accession numbers are shown in Table (5).

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

Isolate	Species	GenBank Accession No
PS5B	*Staphylococcus hominis*	PP094627
PSA2	*Streptococcus equinus*	PP094631

Phylogenetic tree constructed by neighbor joining method in MEGA 11 for isolate PS5b, PSA2 (Figs. 7, 8) that also confirms the identification is correct.

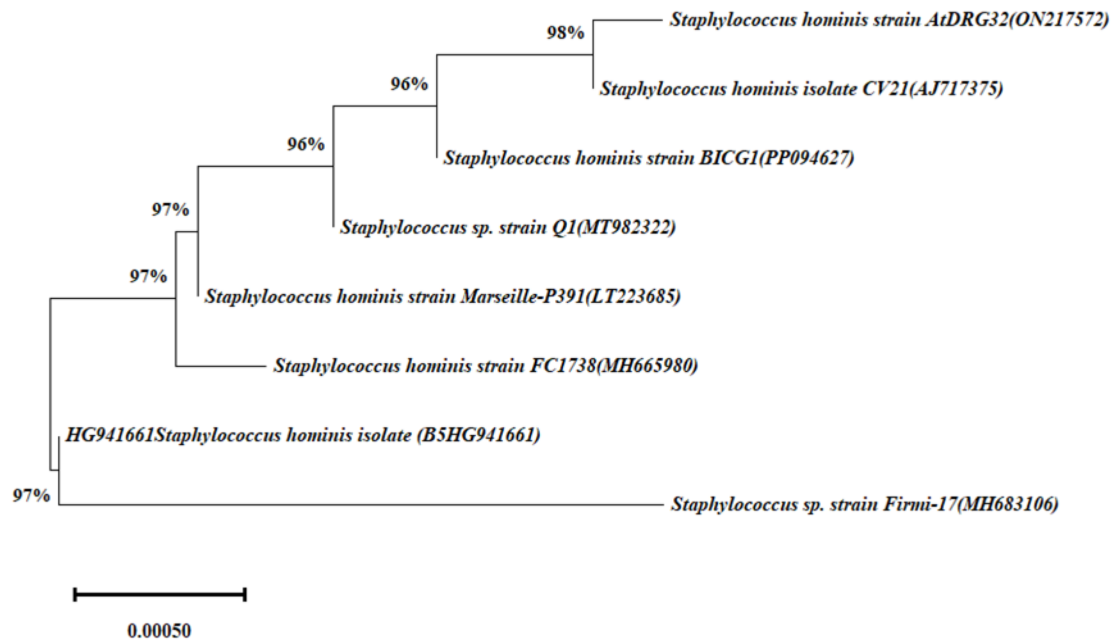

Fig. 7. Phylogenetic tree of *Staphylococcus hominis* strainBICG1 with 7 other closely related strains based on partial 16S rRNA sequencing. Bar 0.00050 nucleotide substitution, values in bracket denotes GenBank accession no. Bootstrap values (1000 replications) are represented at branch point

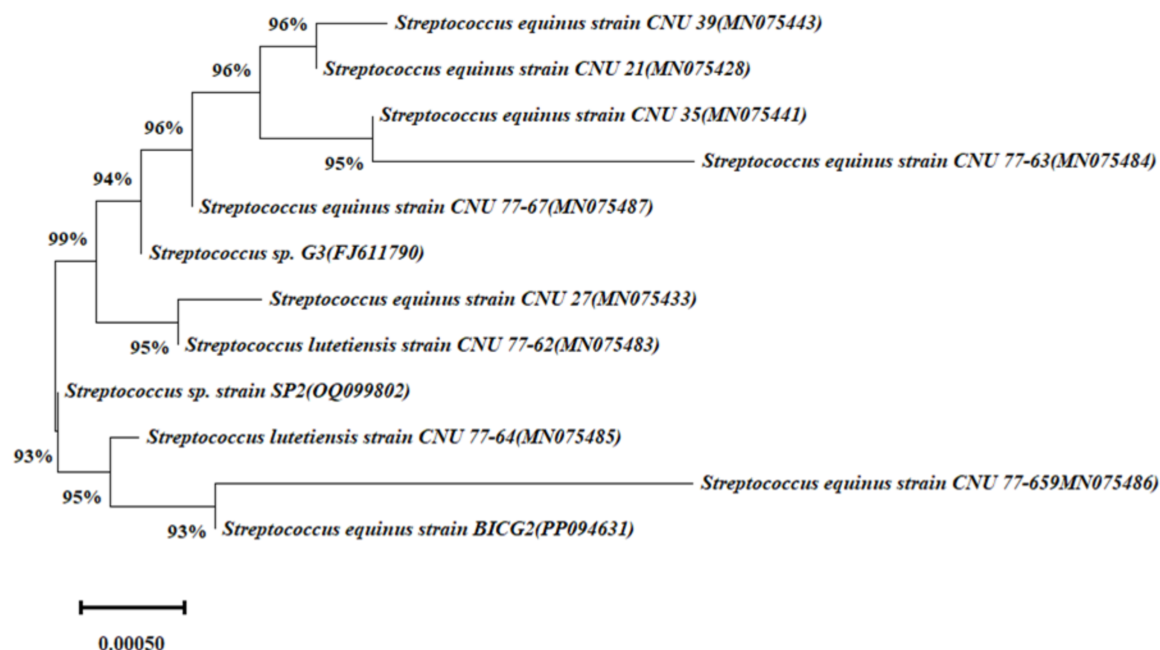

Fig. 8. Phylogenetic tree of *Streptococcus equinus* strain BICG2 with 11 other closely related strains based on 16S rRNA partial sequence. Bar 0.00050 nucleotide substitution, values in bracket denotes GenBank accession no. Bootstrap values (1000 replications) are represented at branch point

8. Antibiotic susceptibility assay

The two isolates exhibited different sensitivity profiles, determined by ZOI when exposed to various antibiotics. *Strep. equinus* BICG2 exhibited resistance to streptomycin, while *Staph. hominis* BICG1 demonstrated sensitivity to streptomycin, gentamicin, tetracycline, and ampicillin (Fig. 9). The ZOI are mentioned in Table (6).

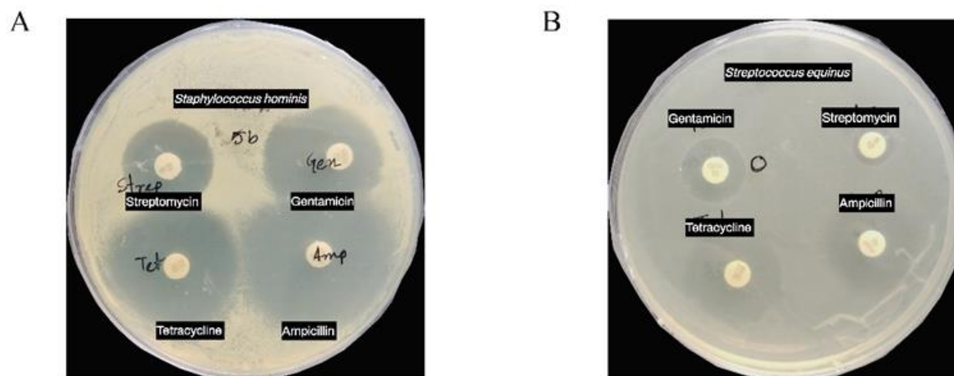

Fig. 9. Antibiotic susceptibility of **A)** *Staphylococcus hominis* BICG1 and **B)** *Streptococcus equinus* BICG2

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

antibiotics	ZOI in mm (*Strep. equinus*BICG2)	ZOI in mm (*Staph. hominis* BICG1)
Gentamicin	16.67±0.47	21.67±0.58
Streptomycin	10.33±0.47	21.00±0.00
Tetracycline	21.67±0.47	28.67±0.58
Ampicillin	18.33±0.47	37.67±0.58

Values represent an average of three replicates.

9. Growth performance

The two isolates were analyzed for their growth performance by measuring the OD at 600nm at two intervals up to 24hrs (Fig. 10).

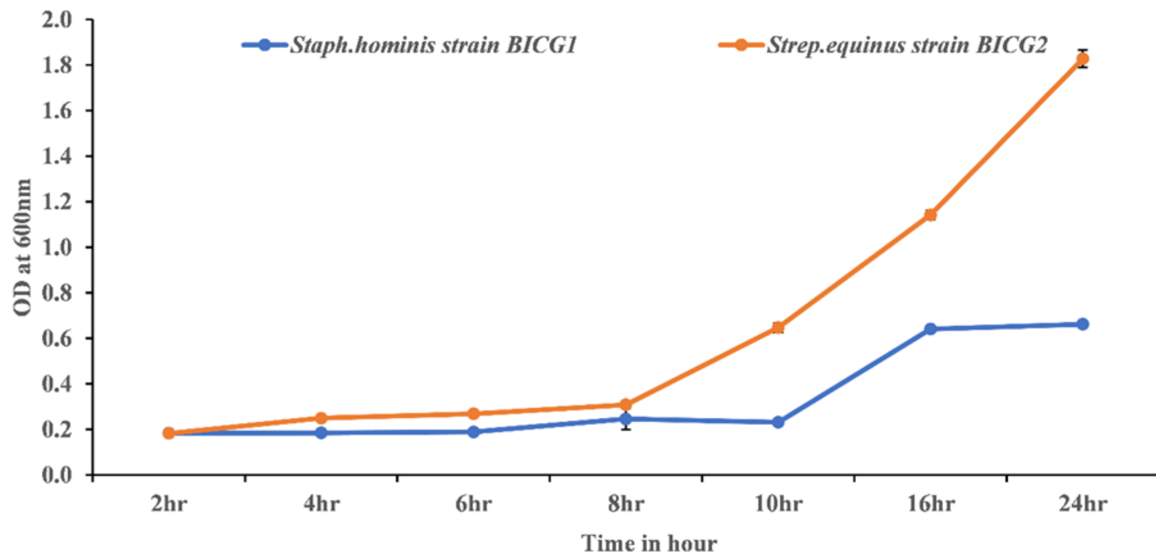

Fig. 10. Growth performance of the two isolates *Staph. hominis* strain BICG1 and *Strep. equinus* strain BICG2

10. Compatibility and antagonistic test of the consortium

After streaking both isolates into intersecting lines, the plates were incubated for a duration of 48hrs at 34°C. Upon completing the experiment, it was found that there was significant proliferation of all isolates examined, and no signs of antagonistic effects were seen (Fig. 11A). The consortia of the two isolates showed a ZOI of 27.33±0.58mm and 26.33±0.58mm against *A. hydrophila* and *A. jandaei* (Fig. 11B, C)

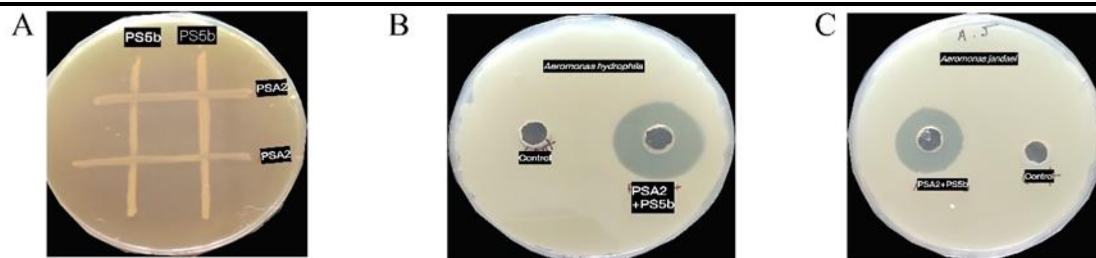

Fig. 11. A) Coexistence test between isolates *Staph. hominis* strain BICG1 and *Strep. equinus* strain BICG2. **B)** Antagonistic activity of the consortium of PSA2 and PS5b against *Aeromonas hydrophila*. **C)** Antagonistic activity of the consortium against *Aeromonas jandaei*

11. Clustering analysis

The heat map of the selected bacterial isolates, considering all the essential characteristics of a probiotic, clearly indicates that isolating PS5b and PSA2 are potential probiotics to be used in aquaculture. The Scores plot from PCA analysis, considering probiotic attributes and antagonistic effects against freshwater pathogens reveal PSA2 and PS5b as outliers, positioned away from the main cluster, indicating its unique probiotic characteristics.

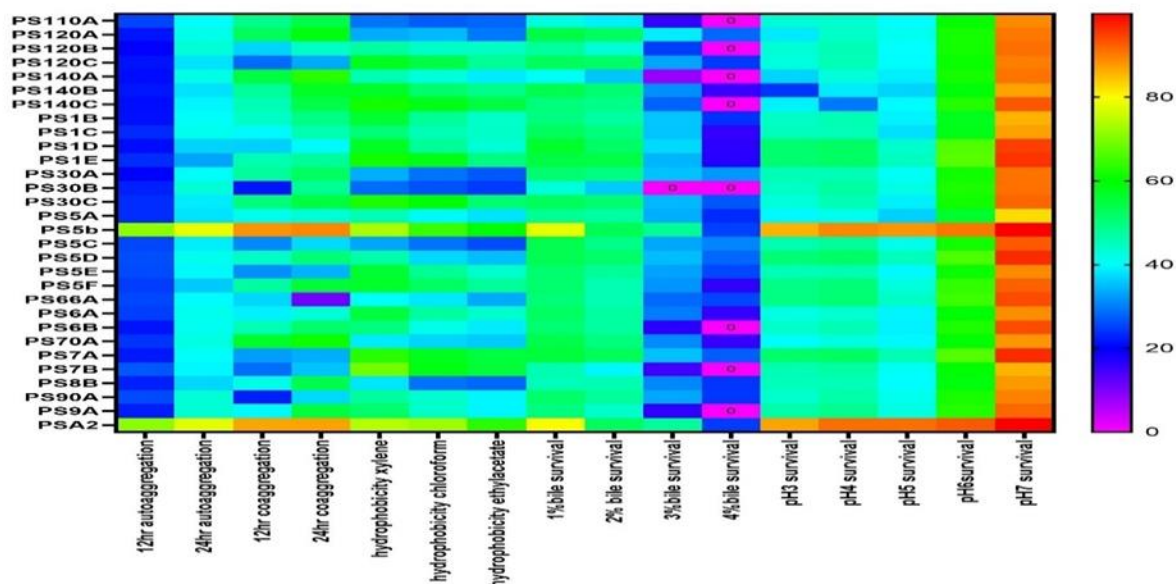

Fig. 12. Heat map of all the 30 isolates considering the probiotic properties of bacteria

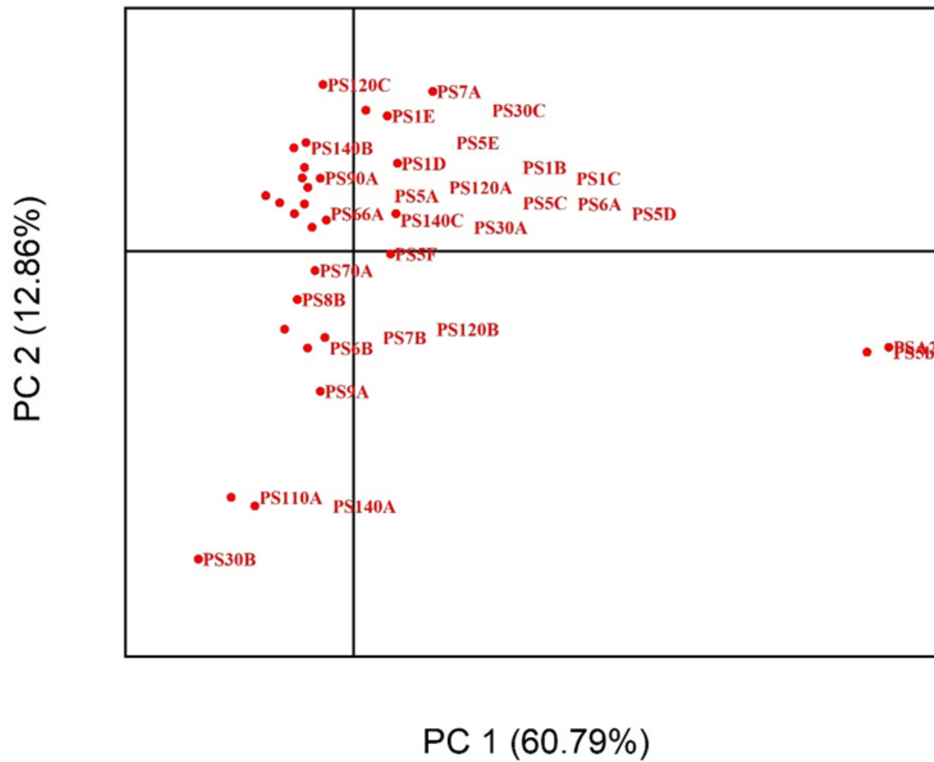

Fig. 13. Cluster analysis of bacterial isolates Using PCA analysis

Supplementary materials

Table A. Result of carbohydrate utilization test

Isolates	Lactose	Maltose	Fructose	Dextrose	Galactose	Sucrose	Sorbitol
PS110A	+	+	+	-	-	+	-
PS120A	+	+	+	+	+	-	-
PS120B	+	+	+	+	-	-	-
PS120C	+	+	+	+	+	-	-
PS140A	+	+	+	+	+	-	-
PS140B	+	-	-	-	-	-	-

PS140C	+	+	+	+	-	-	-
PS1B	+	+	+	-	-	-	-
PS1C	+	+	+	-	-	-	-
PS1D	+	+	+	+	-	-	-
PS1E	+	+	+	+	+	-	-
PS30A	+	=	-	-	-	-	-
PS30B	+	+	+	+	+	=	-
PS30C	+	+	+	+	+	+	-
PS5A	+	+	+	+	+	+	-
PS5b	+	+	+	+	+	w	w
PS5C	+	+	+	+	+	+	+
PS5D	+	+	+	+	+	+	-
PS5E	+	+	+	+	w	+	+
PS5F	+	+	+	+	+	+	+
PS66A	+	+	+	+	+	+	-
PS6A	+	+	+	+	+	+	+
PS6B	+	+	-	-	-	-	-
PS70A	+	-	-	-	-	-	-
PS7A	+	+	+	-	-	-	-
PS7B	+	+	+	-	-	-	-
PS8B	w	W	-	-	+	+	+
PS90A	+	+	+	w	-	-	-

PS9A	+	+	+	+	-	-	-
PSA2	+	+	+	+	+	+	+

+ Positive, –negative, w weakly positive

DISCUSSION

Microbes in aquatic environments affect the gut microflora of fishes **(Cahill *et al.,* 1990)**. These aquatic microorganisms from the Northeastern region of India have not received much research attention **(Joshi *et al.,* 2015)**. Since autochthonous probiotics are more effective than allochthonous as autochthonous bacteria are already familiar with the digestive system of the host, therefore this study was conducted to isolate prospective probiotics from the gut of *Channa gachua* **(Ghosh *et al.,* 2007; Ramesh *et al.,* 2015)**. Application of probiotics reduces antibiotic usage **(Selim *et al.,* 2015)**. Bacteria need to be able to endure at least 4hrs in a stomach with a low pH to qualify as a probiotic **(Culligan *et al.,* 2012; Argyri *et al.,* 2013)**. Moreover, it should also have the capability to resist bile salt **(Zavaglia *et al.,* 1998)**. In this study, the isolate 'PS5b' *Staph. hominis* BICG1 and the isolate 'PSA2' *Strep. equinus* BICG2 showed log CFU/ml=7.38±0.05, 7.36±0.06, and survival rate of 85.28% and 86.55% at pH3 and log CFU/ml=6.77±0.02, 6.83±0.02, with survival rates of 78.18% and 80.21%, respectively, in 1% bile solution. This shows that these two isolates can survive in both acidic condition and bile solution of the intestine which is comparable with the report of **Sung *et al.* (2010)** that showed log CFU/ml=5.69 of *Staph. hominis* at pH2.5 and log CFU/ml=9.2±0.00 and 7.5 ± 0.05 in pH2 at 2hrs for *Strep. equinus* by **Ayyash *et al.* (2018)**. The survival percentage of these isolates in acidic and bile-concentrated environments is also comparable to that of other LAB probiotics **(Govindaraj *et al.,* 2021; Mazlumi *et al.,* 2022)**. No growth was observed in pH 2 and 5% bile solutions, which is consistent with other reports on LAB probiotics **(Sung *et al.,* 2010; Allameh *et al.,* 2013)**. These differences in acid and bile tolerance may be attributed to variations in the source of isolation. To the best of our knowledge, *Staphylococcus hominis* and *Streptococcus equinus* have not been previously isolated from the gut of *Channa gachua* in the northeastern region of India.

Moreover, a bacterium that is a good probiotic should exhibit high auto aggregation and hydrophobicity. Auto aggregation can prevent pathogenic bacteria from colonizing the intestinal gut **(Collado *et al.,* 2008; Mazlumi *et al.,* 2022)** and hydrophobicity is the ability to adhere to the intestinal wall **(Nami *et al.,* 2019)**. Hydrophobicity is the assessment of the ability of bacteria to adhere to the outer lining of intestinal cells **(Onifade *et al.,* 1997)**. This ability of probiotics can aid in the bioremediation of the soluble organic matter present in water bodies **(Sánchez-Ortiz *et al.,* 2015)**. The potential for aggregation affects both survival and persistence in the GI

tract as well as cell adhesion properties. Increased colonization is also supported by auto aggregation, which in turn supports biofilm production, thereby increasing colonization **(Sorroche *et al.,* 2012; Kragh *et al.,* 2016)**. In this study, both isolates showed good auto aggregation and hydrophobicity percentage (>67%) **(Reuben *et al.,* 2020)**. Auto aggregation higher than 45% is required to be a good probiotic strain **(Roghmann *et al.,* 2006)**. *Staphylococcus hominis* strain BICG1 and *Streptococcus equinus* strain BICG2 showed hydrophobicity percentages of 73.38± 0.53, 64.41± 0.55, and 59.80± 0.00, and 74.07± 0.77, 72.24± 1.09, and 63.15± 1.68, respectively, when tested with xylene, chloroform, and ethyl acetate. The percentage of auto aggregation at the 24th hour was 78.27± 0.32 for BICG1 and 77.83±0.05 for BICG2. These values are notably higher than those previously reported for *Streptococcus equinus* **(Mahadin *et al.,* 2018)**.

In contrast to auto aggregation, coaggregation is the ability of bacteria to combine with other types of bacteria, thereby preventing colonization of the gut by pathogenic bacteria. The ability to coaggregate with bacteria may be crucial for the removal of pathogens from the GI tract **(Tuo *et al.,* 2013)**. The coaggregation ability of the two isolates with *Aeromonas hydrophila* increased with time. The coaggregation abilities of these two isolates with pathogens are quite high compared to those previously reported for LAB probiotics **(Espeche *et al.,* 2012; Kassaa *et al.,* 2014; Puniya *et al.,* 2016)**.

In the present study, *Staphylococcus hominis* strain BICG1 and *Streptococcus equinus* strain BICG2 showed zones of inhibition (ZOI) of 20.33± 0.58 and 20.67± 0.58mm against *Aeromonas hydrophila*, and 12.33± 0.58 and 14.33± 1.15mm against *Aeromonas jandaei*, respectively. **Kotzent *et al.* (2020)** reported a ZOI of 6mm for *Staphylococcus hominis* against *A. hydrophila*, while another study indicated that the ZOI by LAB strains against *A. hydrophila* ranges from 16.67 to 20.67mm **(Govindaraj *et al.,* 2021)**. To the best of our knowledge, such a significant inhibition of *A. hydrophila* by *Staphylococcus hominis* has not been previously reported. Few studies have examined the inhibition of *A. jandaei* by *Staphylococcus hominis* and *Streptococcus equinus*. In addition to *A. hydrophila*, *Staphylococcus hominis* has shown antagonistic activity against the foodborne pathogen *Clostridium botulinum* **(Hwang *et al.,* 2020)**. **Sung *et al.* (2010)** reported that *Staphylococcus hominis* exhibited the highest level of antagonism among all isolated bacteria against human pathogens. It has also been found to secrete proteins with anti-tubercular activity **(Ismail *et al.,* 2024)**. Furthermore, these two isolates were found to be compatible with each other, and their consortia demonstrated a ZOI of 27.33± 0.58 and 26.33± 0.58mm against *A. hydrophila* and *A. jandaei*, respectively. This enhanced inhibitory activity suggests that using the consortia of these two bacteria is more effective against pathogens than using them individually. Another important criterion for selecting safe probiotics is the evaluation of the absence of haemolytic activity, as haemolysins are considered virulence factors (**Moreno *et al.,* 2006; Oh & Jung, 2015**). Neither of the potent probiotic isolates showed haemolytic activity. **Hwang**

***et al.* (2020)** and **Kotzent *et al.* (2020)** also found *Staphylococcus hominis* to be non-haemolytic. Additionally, *in vivo* safety tests confirmed that our probiotic strains are safe for use.

Two groups of antibiotics, the first group comprising cell wall synthesis inhibitors such as ampicillin, and the second group comprising protein synthesis inhibitors such as tetracycline, gentamicin, and streptomycin, were used to select functional LAB probiotics (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). Moreover, it is desirable that probiotics should be sensitive to commonly used antibiotics to have less or no chance of transferring antibiotic resistance genes to the host, which could be lethal and could prevent horizontal transfer of antibiotic resistance genes to pathogens **(Doyle *et al.,* 2012; Reuben *et al.,* 2020)**. *Staph. hominis* BICG1 has been found to be sensitive against ampicillin, tetracycline, gentamicin, streptomycin that align with the report of **Hwang *et al.* 2020** and *Strep. equinus* BICG2 was resistant to streptomycin. Although according to previous studies LAB should be sensitive to tetracycline, ampicillin, and resistant to streptomycin and gentamicin **(Katla *et al.,* 2001; Zhou *et al.,* 2005)**, but our result of sensitivity for streptomycin and gentamicin deviated from some studies which could be due to difference in source and geographical location **(Anandharaj *et al.,* 2014; Kassaa *et al.,* 2014)**.

Purkhayastha *et al.* (2013) demonstrated the inhibitory effects of *Staph. hominis* on Gram-negative pathogens and proposed its probiotic potential, which was initially reported by **Sung *et al.* (2010)** and corroborated by **Hanidah *et al.* (2019)**, with **Saeed *et al.* (2024)** further proposing *Staph. hominis* isolated from human milk as a probiotic. **Kotzent *et al.* (2020)** also revealed *Staph. hominis* as a probiotic isolated from *Colossoma macropomum*. **Ayyash *et al.* (2017)** reported *Strep. equinus* as a probiotic having essential qualities, while **Christophers *et al.* (2023)** documented the production of antibacterial NISIN E by *Strep. equinus* MDC1. Antibacterial substances produced by *Strep. equinus* have been shown to inhibit *Bacillus cereus* ATCC 14579, *Enterococcus faecalis*, *Klebsiella* sp., and *Pseudomonas* sp. **(Sabino *et al.,* 2018)**.

CONCLUSION

The gut of *Channa gachua* was examined for the isolation of potential probiotics for use in aquaculture. In the present study, two isolates *Staph. hominis* strain BICG1 (isolate PS5b) and *Strep. equinus* strain BICG2 (Isolate PSA2) showed the potent probiotic properties, with greater ZOI against pathogens than earlier reports. In addition, the consortia of these two isolates were more effective against fish pathogens than those used alone. Hence, these two bacteria alone or in combination for greater effectiveness

against aquatic pathogen can be the good candidates for formulation of probiotics for use in aquaculture.

Ethical statement

The protocols of the present study were duly reviewed and approved by Institutional Animal Ethical Committee, Gauhati University (IAEC; 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 *Staphylococcus hominis* strain BICG1 (GenBank Accession no. PP094627) and *Streptococcus equinus* strain BICG2 (GenBank Accession no PP094631).

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Conflict of interest

No conflict of interest.

Declaration of competing interest

None to declare.

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