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Characterisation and evaluation of novel bacterial consortium as a biofloc inoculum for nursery rearing of *Labeo rohita* and *Oreochromis mossambicus*

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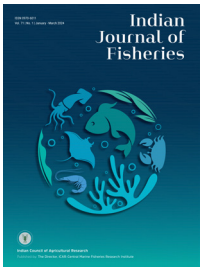

Abstract

The present study explored isolation, characterisation and formulation of a probiotic bacterial consortium for application as a biofloc inoculum to enhance growth and survivability during seed rearing of rohu (*Labeo rohita*) and tilapia (*Oreochromis mossambicus*). Six potential probiotic bacterial strains *viz., Priesta aryabhattai, Bacillus cereus, Exiguobacterium indicum, Acinetobacter indicus, Mixta calida*, and *Bacillus altitudinus,* were isolated from autochthonous sources and identified using 16s rDNA PCR, sequencing and phylogenetic analysis. The probiotic potential of these bacterial isolates was assessed through comprehensive *in vitro* assays along with safety analyses. All the isolates and their combination exhibited antimicrobial potency against important fish pathogens; tolerance to a wide range of pH and bile concentrations and strong adhesion properties including auto-aggregation, co-aggregation, biofilm formation capacity, and cell surface hydrophobicity. All the isolates were non-haemolytic, non-pathogenic to fish when at high doses ($5x10^9$ cells) and demonstrated significant free radical scavenging activity. Further, all the isolates showed tolerance to ammonia, nitrite and nitrate and ability to grow on fish mucus. The efficacy of the consortium was further validated through fry to fingerling rearing trials in biofloc systems for rohu (*L. rohita*) and tilapia (*O. mossambicus*), with jaggery as a carbon source. Fish fry were stocked at one fish per litre and reared for 90 days without water exchange and fed with feed containing 30% crude protein. On termination of the experimental trial, higher survival rates of 80% for rohu and 100% for tilapia, were observed in the treatment groups, compared to control groups (71% for rohu and 92% for tilapia. The present study highlights the effective utilisation of a bacterial consortium as biofloc inoculum for seed rearing of rohu and tilapia, along with improved growth performance. The study demonstrated effectiveness of the bacterial consortium as a biofloc inoculum for enhancing seed rearing efficiency in rohu and tilapia.

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Introduction

Among various food producing sectors, aquaculture is the most rapidly growing sector with more than 10% annual growth that provides a sustainable opportunity for food availability worldwide with the intensification of culture practices (Najdegerami and Tukmechi, 2022). On the other hand, intensification also brings many problems related to environmental deterioration and frequent disease outbreaks. Hence, researchers worldwide are in search of environmentally sustainable intensive culture practices and among them recirculating aquaculture system (RAS) is the most renowned but found to be associated with certain intrinsic challenges regarding operation (Ajamhasani *et al*., 2023). New generation eco-friendly aquaculture technologies *viz*. biofloc-based culture of fishes have gained major momentum in the last decade. Biofloc offers high-density culture of fish which is an alternative to traditional semi-intensive culture systems that require large area of land and huge volume of water. In biofloc system, beneficial microbial community is produced under a controlled environment with the addition of

reliable and sustainable carbon sources to reduce water consumption and production of autochthonous protein source for the culture system (Bakhshi *et al*., 2018).

In biofloc culture system, heterotrophic bacteria and other beneficial microorganisms decompose fish excreta and other organic matters available in the system to produce the microbial biomass and reduce the detrimental nitrogenous metabolites (NO_2-N and NH_4-N) (Avnimelech, 2009). The biofloc culture system has multifaceted beneficial effects on fish and shellfish growth (Kumar *et al*., 2017; Bakhshi *et al*., 2018), immune system (Long *et al*., 2015), gut microbial community (Bakhshi *et al*., 2018; Li *et al*., 2018), antioxidant capacity (Banuelos-Vargas *et al*., 2021), as well as in improving water quality (Ebrahimi *et al*., 2019). Although there are good numbers of literatures available for the optimisation and utilisation of biofloc technology using different C/N ratios or carbon sources, there are very little information available on microbial characteristics and combinations for the effective development of efficient microbial floc to be used in biofloc systems in general and freshwater aquaculture in particular.

Probiotic bacteria are the best candidates for the development of microbial consortia for production of bioflocs. Probiotic bacteria are beneficial microbes that got much attention in the recent past due to their effectiveness in reducing disease incidences and improving the physiological and immunological status of fish (Banerjee and Ray, 2017; Khan *et al*., 2021). Good probiotics should possess some inherent attributes *i.e.* non-pathogenicity, natural availability, capability to colonise and proliferation, and acceptance from the host (Verschuere *et al*., 2000; Kesarcodi-Watson *et al*., 2008). The probiotic bacteria should be initially screened thoroughly for desired criteria such as tolerance to low pH as well as high bile salt concentrations to tolerate gut enzymes, production of antimicrobial and extracellular enzymes, good adherence capacity to mucus and other hydrophobic surfaces and need to be non-pathogenic to host organisms (Papadimitriou *et al*., 2015; Nandi *et al*., 2017).

To date, several probiotic bacteria have been isolated and studied for their probiotic potential and among them most widely studied group is LAB (lactic acid-producing bacteria) and *Bacillus* spp. *Bacillus* group of bacteria demonstrated greater potential in maintaining water quality and act as potent substitute for antibiotics and antimicrobial agents in aquaculture (Hlordzi *et al*., 2020; El-Saadony *et al*., 2021). Several species of *Bacillus* have been identified to have beneficial effects on fish when used as probiotics and have been extensively studied. Another Gram-positive bacterial group closely related to *Bacillus* is *Priestia* and recently proven to have good probiotic potential in different fish species (Melo-Bolívar *et al*., 2022). In addition to *Bacillus,* several other bacterial species have been identified with significant probiotic potential, contributing to enhanced fish growth and improved immunity (Diaz *et al*., 2017; El-Saadony *et al*., 2021; Teja *et al*., 2021; Abdellatif and Arafat, 2024).

L. rohita is the most widley cultured Indian major carp (IMC) species in India while *O. mossambicus* is a globally important species widely farmed to meet protein demand and ensure food security. Nursery rearing is critical stage in the production cycle of both species (Gjerde *et al*., 2019; Oliveira *et al*., 2025). Although biofloc technology has been successfully applied for the rearing of several fish species, including tilapia, there is a scarcity of efficient and region-specific biofloc inocula.

The present study aimed to isolate and characterise freshwater probiotic bacteria and develop a consortium for use as a biofloc inoculum during the nursery phase of seed rearing. Further, the efficacy of the developed biofloc system was evaluated through nursery rearing trial of two commercially important fish species *viz.* rohu (*L. rohita*) and and tilapia (*O. mossambicus*). The study provides comprehensive insight on the beneficial bacterial isolates and their use in biofloc technology, offering a foundation for the sustainable development of biofloc based nursery rearing systems for these two species.

Materials and methods

Bacterial isolation

During the study, six bacterial isolates were obtained from autochthonous sources (polyculture fish farm) at ICAR-Central Institute of Freshwater Aquaculture (ICAR-CIFA) after screening a larger pool of isolates based on selected probiotic properties. The isolates were preserved in glycerol stocks at -80^{o}C for subsequent use. Each bacterial isolates was cultured on tryptone soy agar (HiMedia, Mumbai, India) and single well isolated colonies were obtained using the streak plate method. Pure cultures were than grown on tryptic soy broth (TSB) at 30°C for 24 h, prior to further identification and characterisation.

Molecular identification of the bacterial isolates

Pure cultures of all six bacterial isolates were pelleted down and added with one ml of lysis buffer (50 mM Tris/HCl, 100 mM NaCl, 100 mM EDTA, 1% [w/v] SDS, pH 8.0) to extract DNA following phenol-chloroform extraction method (Sambrook and Russell, 2001). Subsequently, phenol/chloroform/isoamyl alcohol (25:24:1) was added to the solution to assist DNA extraction, followed by precipitation with ethanol. The DNA pellet was dried and diluted in 1xTE (50 mM Tris/HCl, 1 mM EDTA, pH 7.5). The quality and quantity of the extracted DNA were assessed using NanoDrop® spectrophotometer (ND-1000, V3.7.0, Thermo Fisher Scientific, USA).

The DNA extracted from the individual bacterial strain was subjected to 16s rDNA polymerase chain reaction (PCR) based amplification using universal primer set 16S-F 5' AGAGTTTGATCATGGCTCAG 3' and 16S-R 5' GGTTACCTTGTTACGACTT 3" (Pattanayak *et al*., 2018). Briefly, 1 µl bacterial template DNA (approximately 100 ng) was amplified using 1 µl (10 pmol) of each primer, 2.5 µl of 10× *Taq* buffer A, 0.5 µl of dNTPs (2 mM), 0.25 µl of *Taq* DNA polymerase (5 U µl^{-1}) with the final volume of 25 µl (final adjusted with ddH_2O). The PCR conditions followed were: 95°C for 3 min followed by 45 cycles of denaturation for 30 s at 95°C, at 47°C for 1 min for annealing, and at 72°C for 1 min extension, and final extension for 10 min at 72°C. The amplified product was visualised by 1% (w/v) agarose gel electrophoresis.

The PCR products were purified using gel purification kit (Qiagen, Netherlands) and sequenced at AgriGenome Labs Pvt. Ltd., Kochi, India. The sequences obtained for all the six isolates were analysed for their sequence homology using the Basic Local Alignment Search Tool (BLAST) of NCBI (http://www.ncbi.nlm.nih.gov/blast). Multiple sequence alignments were performed using the obtained

sequences and along with previously published sequences of the same bacterial species in MEGA 11, using the ClustalW algorithm. Phylogenetic relationships among the bacterial species were inferred using the Maximum Likelihood method in MEGA 11 and phylogenetic tree was constructed.

Antimicrobial susceptibility test

Antimicrobial susceptibility assay for each of the six bacterial isolates were performed using the disc diffusion method. The antibiogram study was done on Mueller-Hinton agar (Cruickshank *et al*., 1975) and the resistance was determined by measuring the zone of inhibition. Briefly, individual bacterial cultures were inoculated into TSB and incubated at 28°C for 24 h. Subsequently, the individual inoculum (1.5×10^8 cfu ml^{-1}) was spread on Muller-Hinton agar media. Subsequently. antibiotic discs of standard concentrations were placed on the agar surface and incubated at 37°C for 24 h. The zone of inhibition was measured and compared according to the manufacturer's instructions (Hi Media, India).

In vitro antimicrobial activity

The antimicrobial efficacy of the six bacterial isolates individually and and as a pooled consortium, was assessed using the agar well-diffusion method (Schillinger and Lucke, 1989). Three most important freshwater bacterial pathogens *viz. Aeromonas hydrophila*, *Edwardseilla tarda* and *Flavobacterium columnare* were sourced from the National Referral Laboratory for Freshwater Fish Diseases of ICAR-CIFA, Bhubaneswar, for analysing the antimicrobial activity of all the six selected probiotic bacteria individually and as pooled culture. Briefly, 100 µl of each pathogenic bacterial culture (~10^5 CFU ml^{-1}) was spread uniformly onto Mueller-Hinton agar plates. Subsequently wells of 6 mm diameter were prepared and 10^6 CFU ml^{-1} of individual probiotic bacterial isolates and their pooled consortium were added to the respective wells. The plates were incubated at 37°C for 24 h, and the zones of inhibition were measured.

pH and bile tolerance

The pH and bile tolerance of all six probiotic bacterial isolates were measured using the method described by Khan *et al.* (2021). Briefly, for pH tolerance, 10^6 CFU ml^{-1} of each bacterial isolate was inoculated into TSB with variable pH in different tubes ranging from 2 to 9 in triplicate (pH adjusted with 1 N HCl and 1 N NaOH). The growth of the bacteria in the broth at 28°C for 24 h was measured by observing the change in optical density (OD) in a spectrophotometer (Bio-Rad, USA) at a wavelength of 600 nm. Further to determine the bile tolerance of all six bacterial isolates, a cell concentration of 10^7 CFU ml^{-1} was obtained from the overnight bacterial culture and 1 ml each of the culture was centrifuged at 10,000 *g* for 10 min, and washed three times with phosphate-buffered saline (PBS, pH 7.4). Subsequently, all the bacterial pellets were dissolved in PBS containing different concentrations [0% (control), 2.5%, 5% and 10%] of bile salts (HiMedia, India), incubated at 30°C for 1.5 h, and absorbance was measured at 600 nm using a spectrophotometer.

Haemolytic assay

Haemolytic assay was carried out for all six isolates by streaking each isolate on an individual plate containing blood agar base (HiMedia, India) enriched with 5% chicken blood and incubated at 28°C for 24 h (Joseph *et al*., 1982). The streaked plates were observed for haemolytic zones and classified as α (incomplete/partial haemolysis), β (complete haemolysis), or γ (no-haemolysis) haemolysis.

Cell surface hydrophobicity

To measure cell surface hydrophobicity, all six bacterial isolates along with *A. hydrophila* (as pathogen control) were incubated for 48 h at 28°C in TSB, centrifuged at 10,000 *g* for 3 min, followed by washing with PBS twice to obtain pellets. The pellets were suspended in PBS (pH 7.4), and OD was measured at 600 nm (OD_1). Subsequently, the same bacterial culture was added with an equal volume of xylene and chloroform separately and vortexed thoroughly. The combination was left undisturbed to separate into two phases for 30 min, and the OD of the aqueous phase was again measured at 600 nm (OD_2). Hydrophobicity was calculated using the following formula (Khan *et al*., 2021):

$$\text{Hydrophobicity (\%)} = (1-OD_2/OD_1)\times100$$

Growth on mucus

The adhesion capability of all six probiotic bacterial isolates and *A. hydrophila* on the skin mucus of rohu and tilapia was evaluated as per Midhun *et al*. (2017). Anaesthetised fish were placed individually in sterile beakers containing 5 ml of 100 mM ammonium bicarbonate buffer (pH 7.8) for 10 min, to collect skin mucus. The fish were then removed and rinsed with an equal volume of the same buffer. The mucus-buffer mixtures were then transferred to sterile 15 ml tubes and centrifuged at 12,000 *g* for 15 min at 4°C. The supernatant was collected and filtered through a 0.22 µm syringe filter (HiMedia, India), to obtain sterile mucus samples. These sterile mucus preparation from rohu and tilapia were inoculated separately with each of the six probiotic bacterial isolates and *A. hydrophila* (10^6 CFU ml^{-1}), followed by incubation at 30°C for 24 h, After incubation, bacterial adhesion was assessed by measuring OD at 600 nm.

Autoaggregation and coaggregation capacity

To determine the auto-aggregation capacity of all six bacterial isolates, these were cultured in TSB at 28°C for 18 h. The cultures were then centrifuged at 5,000 *g* for 15 min, washed in PBS three times, and suspended in 2 ml PBS to an OD600 of 0.25±0.05. All the bacterial isolates and control (*A. hydrophila*) were then incubated at room temperature and OD was measured at 0, 2, 4, 6, 10, and 24 h intervals. At each time point, 100 µl of the upper bacterial suspension was collected and OD was measured at 600 nm (Collado *et al*., 2007). Autoaggregation was then calculated as:

$$\text{Autoaggregation (\%)} = [(OD_x - ODy)/ODx]\times100$$

where OD_x denotes the absorbance at time (t) = 0, OD_y denotes the absorbance at t = 2, 4, 6, 10, or 24 h.

For estimation of coaggregation of all the probiotic bacterial isolates, bacterial cultures were prepared as mentioned above. Subsequently, a 2 ml culture of each isolate was co-cultured with *A. hydrophila* (pathogenic bacterial control) and incubated at room temperature. At time points 0, 2, 4, 6, 10, and 24 h, the OD600 of

mixed culture suspension was recorded (Valeriano *et al*., 2014) and coaggregation was calculated as:

Coaggregation (%) = [(ODpro+ODpat)-ODmix]/(ODpro+ODpat)×100

where ODpro+ODpat represents the combined absorbance of individual probiotic bacterial isolate and *A. hydrophila* at 0 h, and ODmix denotes the absorbance of their mixture at at subsequent time points.

Antioxidant activity

Radical scavenging activity was determined using the method described by Xing *et al*. (2015). Individual bacterial isolates were cultured in TSB for 24 h and centrifuged at 8000 *g* for 10 min at 4°C. The cell-free supernatant was collected in sterile tubes and used for scavenging assay. A 10% ascorbic acid solution was used as the antioxidant standard control for each isolate.

For determination of DPPH (1, 1-diphenyl-2-picrylhydrazyl) antioxidant activity, 100 µl of individual bacterial supernatant was mixed with absolute ethanol (3 ml) and 0.06 mM DPPH (2 ml) (HiMedia, India). The mixture was incubated for 30 min, and OD was recorded at 517 nm (Brand *et al*., 1995). A mixture of ethanol and sample was denoted as blank, whereas DPPH solution without sample served as control. DPPH scavenging rate was calculated using the formula:

DPPH scavenging rate (%) = 100–[ODsample–OD blank×100⁄OD control]

The hydrogen peroxide (H_2O_2) radicals scavenging activity was determined following the method described by Ruch *et al*. (1989). Briefly, 600 µl of 40 mM H_2O_2 (HiMedia) dissolved in PBS (pH 7.4) was mixed with 100 µl of the bacterial supernatant and incubated for 10 min. OD was measured at 230 nm against a blank (PBS without H_2O_2) and control (H_2O_2 solution without sample). H_2O_2 scavenging rate was then estimated as:

H_2O_2 scavenging rate (%) = [OD control–OD sample⁄OD control]×100

Growth curve

To determine the growth characteristics of all six bacterial isolates and their pooled consortium, cultures were inoculated into TSB in triplicate and incubated at 28°C 48 h. OD was measured at every 2 h interval at 640 nm using a spectrophotometer, to generate growth curves (Vine *et al*., 2004).

In vitro pH determination

To evaluate the changes in pH of the culture medium, each of the six bacterial isolates and their pooled consortium were cultured, separately in TSB in triplicate at 28°C for 48 h. The pH of each culture was measured at 2 h intervals to monitor change in pH over time.

In vitro ammonia, nitrate and nitrite tolerance assays

To evaluate ammonia tolerance, each of the six bacterial isolates was cultured separately in 10 ml of TSB supplemented with different concentrations of ammonium chloride (1.0, 2.5, 5.0 and 10.0 mg l^{-1}). For nitrite tolerance, the isolates were exposed to sodium nitrite at concentrations of 1.5, 10.0 and 20.0 mg l^{-1}, while nitrate tolerance was assessed using sodium nitrate at 1.5; 10.0 and 20.0 mg l^{-1} in TSB. All cultures were incubated at 28°C. for 24 h. TSB without any added chemicals served as control. The experiments were all performed in triplicate. following incubation, bacterial growth was quantified by measuring absorbance (de Mello Junior *et al*., 2021) in a microplate reader.

Challenge study

To evaluate the safety and non-pathogenicity of all the bacterial isolates and their consortium, a challenge study was conducted following Das *et al*. (2013). Rohu and tilapia (Average size: 65 mm and 55 mm respectively) were kept in FRP tanks (200 l capacity) in triplicate with 6 fish per tank. All the fishes were intraperitoneally injected with 0.1 ml of freshly prepared probiotic bacterial culture at $5x10^9$ CFU ml^{-1}, control groups received 0.1 ml sterile PBS. Fish were monitored over a 2 weeks period for any signs mortality or clinical abnormalities.

Mixed bacterial inoculum for biofloc production

All six bacterial isolates were co-cultured in a single conical flask at 28°C for 24 h, achieving a maximum cell density of $2.4x10^9$ CFU ml^{-1}. This culture served as the primary inoculum for biofloc development. A container was filled with 100 l of water and inoculated with 100 ml of the mixed bacterial inoculum. Jaggery was added to the culture as a carbon source, and the system was maintained with continuous aeration in a shaded place to facilitate desirable biofloc formation over 48 h. Subsequently, the resulting biofloc-rich consortium was used as a reservoir for inoculum for further experimental applications.

Nursery rearing of rohu and tilapia fry

For nursery rearing of rohu and tilapia, twelve numbers of 1000 l FRP tanks were filled with water. Six tanks were added with 10 l of the prepared mixed bacterial consortium (treatment), while the remaining six tanks received a commercial biofloc preparation prepared in a similar manner, which served as control. In each tank, jaggery was added as a carbon source. In six tanks (three treatment and three control), 1000 nos. of rohu fry (length: 20-25 mm) were stocked at a density of one fish per litre. Similarly, tilapia fry (length: 30-35 mm) were stocked in the remaining six tanks under identical conditions. The rearing period lasted for 90 days. No water exchange was carried out during the experimental period. Fish were fed *ad libitum* with a diet containing 30% crude protein. During the experimental period, water quality parameters were recorded at 15 days intervals. Alkalinity was maintained above 120 mg $CaCO_3$ l^{-1} by adding calcium hydroxide when required. Freshwater was added only to compensate for the evaporation loss.

Results

Molecular identification of the bacterial isolates and phylogenetic analysis

The six bacterial isolates were identified as *Priestia aryabhattai* (Gram +ve) (S-1), *Bacillus cereus* (Gram +ve) (S-3), *Exiguobacterium*

indicum (Gram +ve) (S-4), *Bacillus aerophilus* (Gram +ve) (S-14), *Mixta calida* (Gram -ve) (S-13) and *Acinetobacter indicus* (Gram -ve) (S-6) using 16s rDNA sequence analysis. The 16s rDNA gene sequences for all six bacteria were submitted to NCBI GenBank and accession numbers were obtained (OR512030, OR512053, OR512056, OR512113, OR512191 and OR512194 for *B. cereus*, *E. indicum*, *A. indicus, B. aerophilus*, *M. calida* and *P. aryabhattai* respectively). Phylogenetic analysis of revealed that all isolates clustered closely with previously reported sequences of the same bacterial species (Fig. 1).

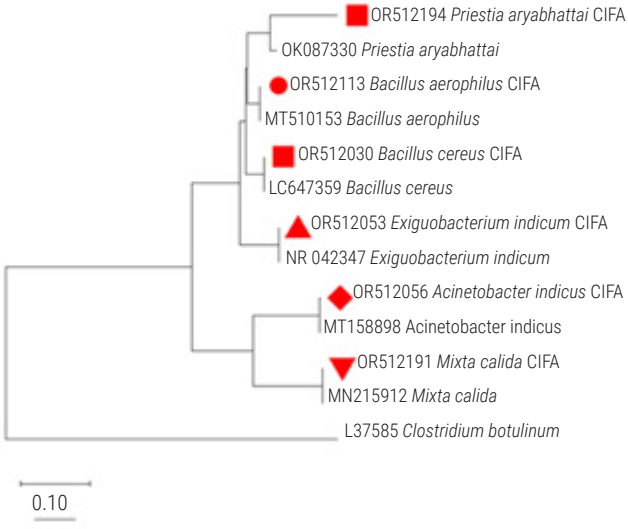

Fig. 1. Phylogenetic tree based on 16s rDNA sequences the of *B. cereus*, *E. indicum*, *A. indicus, B. aerophilus*, *M. calida* and *P. aryabhattai* and their closest relatives reported in previous studies

Antibiogram profile of the selected bacterial isolates

All the six bacterial isolates (S-1, 3, 4, 6, 13 and 14) were susceptible to 16 antibiotic drugs tested belonging to five groups *viz.* aminoglycocides (amikacin, gentamicin, tobramycin), carbapenems (imipenem), cephalosporins (ceftazidime, cefuroxime, cefotaxime, cefoperazone), fluoroquinolones (ciprofloxacin, levofloxacin, norfloxacin) and tetracyclines (tetracycline, doxycycline hydrochloride, oxytetracycline); while found resistant only to streptomycin (Table 1). Further, intermediate resistance/susceptibility was observed for norfloxacin and cefoperazone by *P. aryabhattai,* for imipenem by *A. indicus* and for streptomycin by *E. indicum*.

Antimicrobial activity

All six bacterial isolates, individually and as a mixed consortium were evaluated for antimicrobial activity against *A. hydrophila*, *E. tarda* and *F. columnare* and the results are depicted in Table 2. The mixed bacterial consortium exhibited strong antimicrobial activity with inhibition zone diameters of 20±0.7, 25±1.4 and 28±0.7 mm for *A. hydrophila*, *E. tarda* and *F. columnare*, respectively.

pH and bile tolerance assay

All six bacterial isolates exhibited a broad tolerance to pH ranging from highly acidic to highly alkaline pH (2 to 9). However, optimal

Table 1. Antibiotic resistance/susceptibility pattern of the six probiotic bacterial isolates

Antimicrobial agents used	Disc Concentration (µg)	Probiotic bacteria					
		P. aryabhattai	*A. indicus*	*M. calida*	*B. aerophilus*	*E. indicum*	*B. cereus*
Aminoglycosides							
Amikacin (Ak)	30	S	S	S	S	S	S
Gentamicin (Gen)	10	S	S	S	S	S	S
Tobramycin (Tob)	10	S	S	S	S	S	S
Streptomycin	10	R	R	R	R	I	R
Carbapenems							
Imipenem (Ipm)	10	S	I	S	S	S	S
Cephalosporins							
Ceftazidime (Caz)	30	S	S	S	S	S	S
Cefuroxime (Cxm)	30	S	S	S	S	S	S
Cefotaxime (Ctx)	30	S	S	S	S	S	S
Cefoperazone (Cfs)	75	I	S	S	S	S	S
Fluoroquinolones							
Ciprofloxacin (Cip)	5	S	S	S	S	S	S
Levofloxacin (Le)	5	S	S	S	S	S	S
Norfloxacin (Nx)	10	I	S	S	S	S	S
Tetracyclines							
Tetracycline (Te)	25	S	S	S	S	S	S
Doxycycline hydrochloride (Do)	30	S	S	S	S	S	S
Oxytetracycline (O)	30	S	S	S	S	S	S

R: Resistant; S: Sensitive; I: Intermediate

Table 2. Antibacterial activity of the probiotic bacterial isolates against selected fish pathogens

Probiotic bacteria	Zone of inhibition (mm)		
	A. hydrophila	*E. tarda*	*F. columnare*
A. indicus	22 ± 1.2	10 ± 0.7	13 ± 0.5
P. aryabhattai	11 ± 0.1	-	29 ± 1.2
M. calida	12 ± 0.12	08 ± 0.2	-
B. aerophilus	-	28 ± 1.1	-
E. indicum	20 ± 0.7	-	22 ± 0.8
B. cereus	18 ± 1.3	22 ± 1.2	30 ± 0.6
Mixed probiotic bacterial consortium	20 ± 0.7	25 ± 1.4	28 ± 0.7

growth was observed between pH 5 and 9 (Fig. 2a). Similarly, in the bile tolerance test, all the isolates exhibited tolerance to bile concentrations up to 10% concentration (Fig. 2b).

Haemolytic activity

All six bacterial isolates were identified non-haemolytic in nature (γ-haemolysis), with no visible haemolysis on blood agar plates.

Cell surface hydrophobicity of bacterial isolates

All six probiotic bacterial isolates exhibited higher levels of cell surface hydrophobicity against chloroform and xylene, compared to the fish pathogen *A. hydrophila,* which was used as control. The isolate S-4 (2.25%) showed the lowest cell surface hydrophobicity against chloroform. Further, isolate S-14 (64.73%) exhibited highest cell surface hydrophobicity against xylene, whereas S-13 (6.80%) showed the lowest (Fig. 3).

Growth on mucus

To ascertain the *in vitro* adhesion capacity of isolated bacteria, these were grown with rohu and tilapia mucus. All the isolates were grown to significantly higher level in both fish mucus as compared to the pathogenic bacterial control (Fig. 4a, b).

Autoaggregation and coaggregation

The autoaggregation and coaggregation capacity for all six isolates is represented in Fig. 5. All the probiotic bacteria showed better autoaggregation capacity and the percentage of autoaggregation

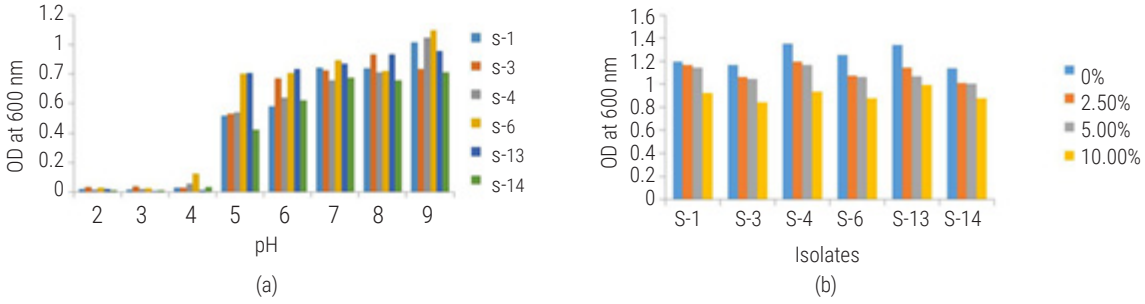

Fig. 2. Tolerance of bacterial isolates (*B. cereus*, *E. indicum*, *A. indicus, B. aerophilus*, *M. calida* and *P. aryabhattai*) to varying (a) pH levels and (b) bile concentrations

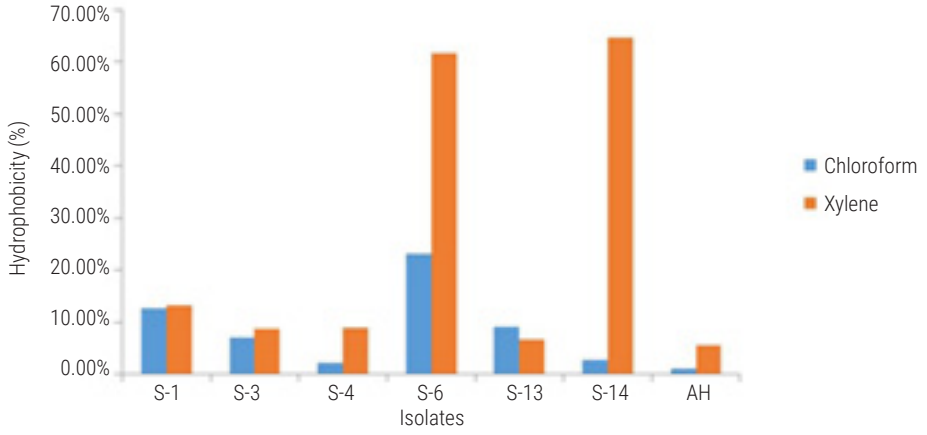

Fig. 3. Cell surface hydrophobicity of probiotic isolates against chloroform and xylene compared to *A. hydrophila*

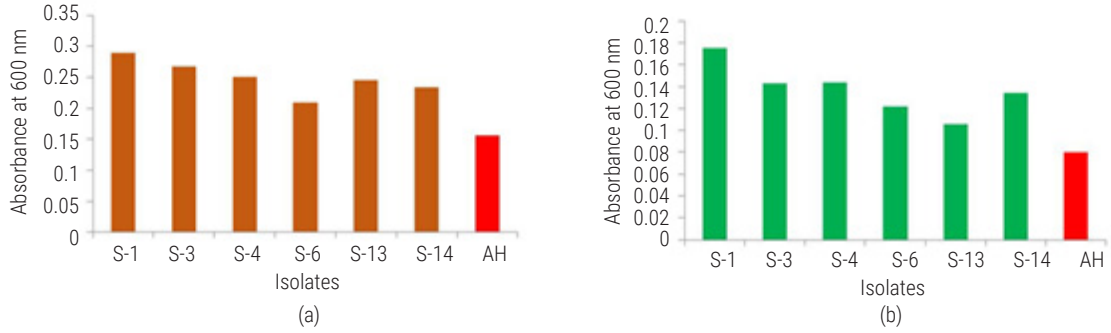

Fig. 4. Probiotic bacterial growth on (a) rohu mucus and (b) tilapia mucus, in comparison to *A. hydrophila* (Ah)

increased over time as compared to *A. hydrophila* (pathogenic bacteria control). Among all the isolates studied, S-13 showed the highest autoaggregation percentage of 80% at 24 h (Fig. 5a). The coaggregation percentage of all the isolates with *A. hydrophila* increased with time and the highest coaggregation capacity was observed for S-6 (72.80%) (Fig. 5b).

Antioxidant activity

All the bacterial isolates exhibited varied potential to scavenge free radicals (Fig. 6). The maximum scavenging activity observed was 90.52% and 42.44% in S-13 for DPPH and H_2O_2, respectively.

Growth curve and in vitro pH determination

The 48 h growth curve of all bacterial isolates and their mixed consortium are presented in Fig. 7a. All isolates exhibited exponential growth between 4 and 6 h of culture. No inhibitory effects on growth was observed when all the isolates were cultured together, indicating compatibility within the consortium.

Further, the change of culture pH was measured during the culture of all probiotic bacterial isolates and their mixed culture (Fig. 7b). All bacterial isolates were found to maintain a pH above 5.2 at 48 h culture duration with the highest by S-4 (6.32). The mixed culture maintained a pH of 5.69 at the end of 48 h.

In vitro ammonia, nitrate and nitrite tolerance assays

There was no significant change in the growth of any of the probiotic bacteria incubated under different concentrations of ammonia, nitrite and nitrate in the culture media at different concentrations (Fig. 8).

Host safety

All probiotic bacterial isolates were found to be non-pathogenic to both the fish species even at a very high concentration of bacteria exposure. Further, there were no morbidity or clinical signs observed during the two weeks' post-challenge.

Nursery rearing of rohu and tilapia fry

The biofloc consortium comprising the six bacterial isolates were used for nursery rearing of rohu and tilapia fry. The water quality of culture tanks was not significantly altered during the entire culture period for both species. After 90 days of culture, fingerling survivability of 80% was observed for rohu and 100% for tilapia. There was a significant increase in the size of the fishes observed

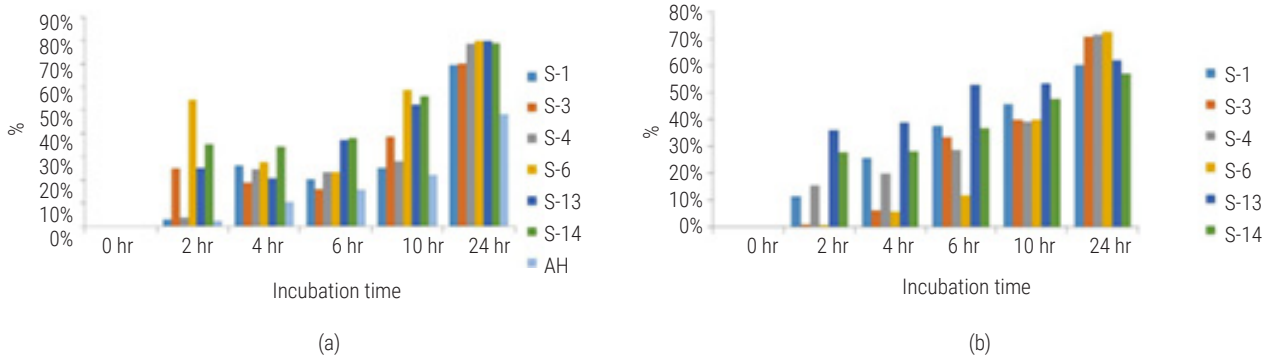

Fig. 5. Autoaggregation and coaggregation capacity for all six bacterial isolates. (a) Autoaggregation in comparison with *A. hydrophila* (Ah) and (b) Coaggregation

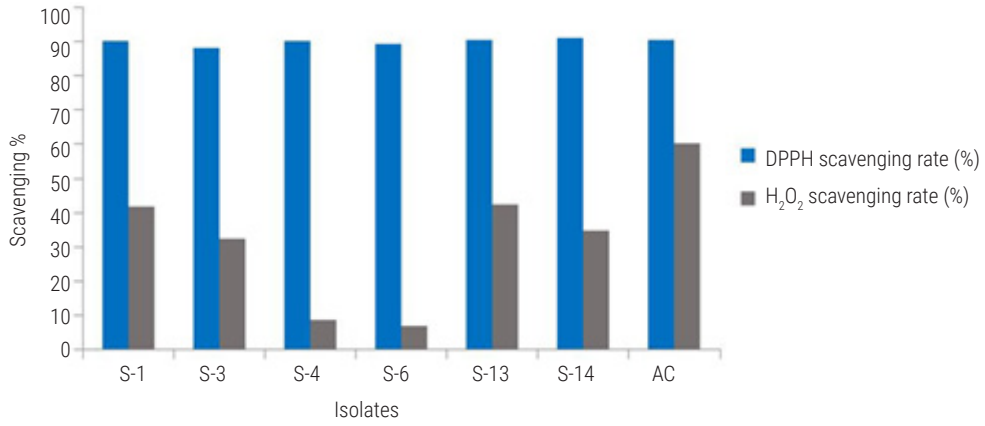

Fig. 6. DPPH and H_2O_2 scavenging activity in comparison to control ascorbic acid

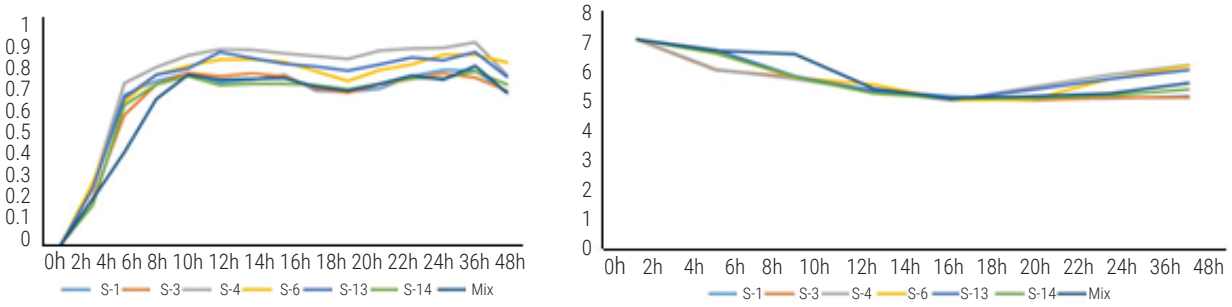

Fig. 7. Growth curve of the all six bacterial isolates and their consortium at 28^{0}C (7a). Change in pH of the medium during bacterial culture (7b)

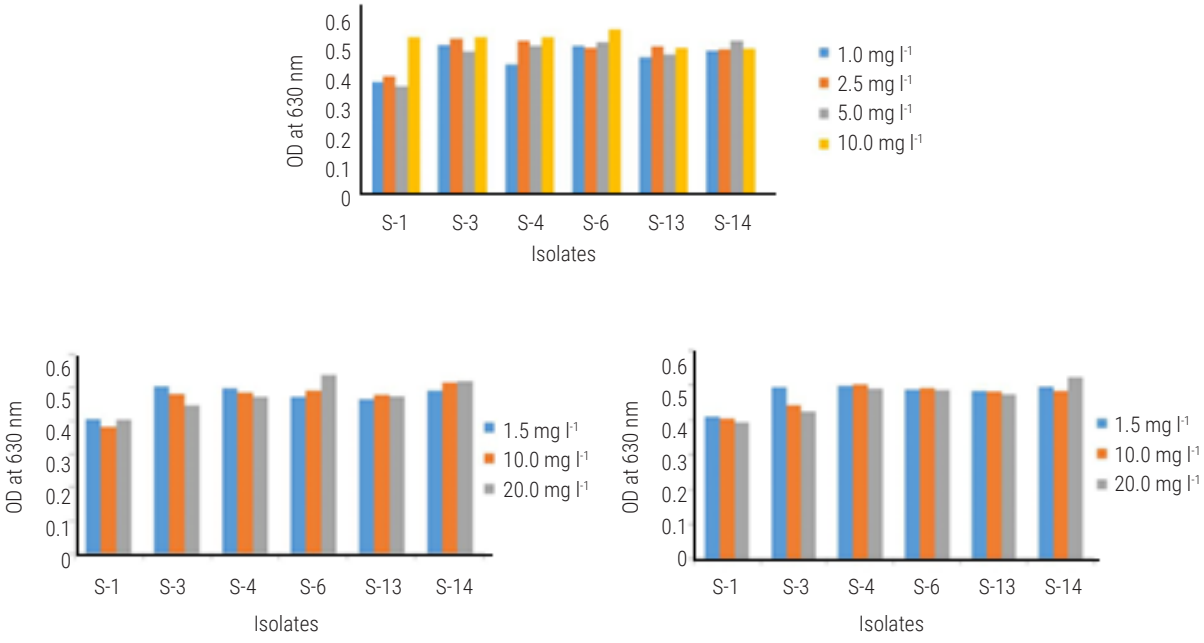

Fig. 8. *In vitro* ammonia, nitrite and nitrate tolerance of the six bacterial isolates

to 60±10 mm and 50±10 mm, for rohu and tilapia, respectively. Further, in comparison with the commercial biofloc group there was significant increase in growth and survivability observed for both the fish species (Table 3).

Table 3. Production indexes for the nursery rearing of rohu and tilapia with new biofloc consortium and control group (commercial biofloc inoculum). Data are presented as mean±standard deviation. Different letters indicate a significant difference ($p<0.05$) between the treatment and control

Growth indices	Rohu (Treatment)	Rohu (Control)	Tilapia (Treatment)	Tilapia (Control)
Initial length (mm)	26±2.0	26±2.0	15±2.0	15±2.0
Final length (mm)	$60±10^{b}$	$52±8^{a}$	$50±10^{b}$	$45±7^{a}$
Survivability (%)	80^{b}	71^{a}	100^{b}	92^{a}

Discussion

With the increasing demand for the supply of aquatic animals for food purposes, there is an increase in interest in the use of probiotics for various purposes including health promotion and growth performance. Different *in vitro* tests are advantageous for initial screening, understanding of the mechanism and degree of probiotic effect (Khan *et al*., 2021)**.** Recently probiotic bacteria have been used for the development of effective biofloc consortiums for use in intensive aquaculture (Kumar *et al*., 2023). However, for initial characterisation, the efficiency and safety of probiotic bacteria need to be studied in-depth. The present study aims at identifying probiotic bacteria and analysing their different beneficial characteristics to explore their potential for the development of a biofloc consortium.

Presently molecular based identification of bacterial pathogens has taken a major leap (Pattanayak *et al*., 2018). In the present study, we have taken six potential probiotic bacteria and identified them using 16s rDNA based amplification, sequencing and phylogenetic analysis. The isolates designated having biofloc potential *i.e.* S-1, S-3, S-4, S-6, S-13 and S-14 were identified as *Priestia aryabhattai* (OR512194), *Bacillus cereus* (OR512030), *Exiguobacterium indicum* (OR512053), *Acinetobacter indicus* (OR512056), *Mixta calida* (OR512191) and *Bacillus aerophilus* (OR512113), respectively. The phylogenetic study also revealed a close relation of each of the probiotic bacterial species under the present study with the same species of bacteria reported earlier. The antimicrobial activity of probiotic bacteria is considered to be one of the important decisive factors in the selection of bacteria for further analysis (Verschuere *et al*., 2000; Vine *et al*., 2006). In the present study, S-1, S-3, S-4, S-6, S-13 and S-14, and their mixed consortium demonstrated different degrees of antimicrobial efficacy against three important aquaculture pathogenic bacteria *i.e*. *A. hydrophila*, *E. tarda* and *F. columnare*. Hence, all these isolates were subjected to further analysis of probiotic potential.

The emergence of multi-drug resistance in different bacteria due to the wide use of antibiotics has caused havoc worldwide (Aich *et al*., 2018). It is always advisable to use probiotic strains having less resistance to different antibiotics to further contain the spread of antibiotic resistance. In this study, we have found that all isolates were susceptible to as many as 16 antibiotics belonging to 5 groups namely, aminoglycosides (amikacin, gentamicin, tobramycin), carbapenems (imipenem), cephalosporins (ceftazidime, cefuroxime, cefotaxime, cefoperazone), fluoroquinolones (ciprofloxacin, levofloxacin, norfloxacin) and tetracyclines (tetracycline, doxycycline hydrochloride, oxytetracycline) and resistant to only streptomycin. Intermediate resistance/susceptibility was observed for norfloxacin and cefoperazone by *P. aryabhattai,* for imipenem by *A. indicus* and for streptomycin by *E. indicum*. These results suggest all the isolates are susceptible to broad classes of antibiotics and hence pose no major immediate risk to spreading antibiotic resistance to other bacteria and have the potential for use in nonclinical or other environmental settings (Bunnoy *et al*., 2019).

In fish, bile is produced in the liver and stored in the gall bladder, and released into the intestine during lipid digestion and fat-soluble vitamin assimilation (Solovyev *et al*., 2023). Further, the pH of fish intestines is also low in favour of digesting foods. Hence, tolerance of a wide variety of bile and pH is a prerequisite characteristic of probiotic bacteria. In the present study, all bacterial isolates survived and grown in different pH conditions and bile concentrations designating these as potential probiotic bacteria. Several other studies have also reported the high pH and bile tolerance of probiotic bacteria (De Smet *et al*., 1995; Giri *et al*., 2012; Khan *et al*., 2021). The cell surface hydrophobicity is the measurement of colonising capacity of probiotic bacteria (Kos *et al*., 2003). In this study, we analysed the hydrophobicity of all probiotic bacteria selected, with the pathogenic *A. hydrophila*. All probiotic bacteria were found to pose higher hydrophobicity percentages in comparison to the pathogenic control. The higher hydrophobicity may be the function of higher electron donation and acceptance by the targeted bacteria (Kuebutornye *et al*., 2019). Colonisation and resistance to fish gut and body mucus are also important to designate bacteria as probiotics. The mucus colonisation capability must be higher for the probiotic bacteria as compared to pathogenic bacteria in order to facilitate exclusion of pathogenic bacteria and render protection to the host (Van Zyl *et al*., 2020). Hence, all six isolates were observed to grow in rohu and tilapia mucus significantly more in comparison to the pathogenic control (*A. hydrophila*). This suggests the capability of present isolates to adhere and grow in fish mucus. There are also many mucus adhesion proteins being identified in different probiotic strains which helps probiotic isolates to adhere better to mucus surfaces and exclude pathogenic ones (Van Zyl *et al*., 2019).

For preventing the colonisation of unwanted and pathogenic bacteria, the autoaggregation and coaggregation capacity of probiotics plays an important role (Sharma *et al*., 2019). Autoaggregation refers to the aggregation of the individual bacterial isolate to form a group which helps them to colonise and dominate in the intestinal environment and coaggregation denotes the aggregation of different bacterial groups including the pathogenic one (Lukic *et al*., 2014). During coaggregation, a close proximity among probiotic bacteria and non-probiotic/pathogenic bacteria occurs and the production of antimicrobial substances can inhibit pathogens from causing diseases (Arena *et al*., 2018). In the present study, all six probiotic bacteria exhibited better autoaggregation capacity in comparison to *A. hydrophila* (pathogenic bacteria control) which shows their capability to colonise in the gut environment better. Further, coaggregation capacity of all the isolates along with *A. hydrophila* showed an increasing trend with time and the highest percentage of coaggregation of 72.80% was observed

for S-6, which further strengthened the information about the effectiveness of the probiotic bacteria in eliminating pathogenic counterpart. Moreover, different probiotic bacteria expressing certain aggregation promoting factors could promote the elimination of different pathogens *via* microbial balance, competitive exclusion and the coaggregation mechanism (Li *et al*., 2020).

It is opined that a good probiotic can help reducing the oxidative stress through producing different biomolecules which modulate redox status of the host through different regulating signalling pathway (Wang *et al*., 2017). Several authors have reported successful reduction in the free radical production by the probiotic bacteria (Pieniz *et al*., 2014; Khan *et al*., 2021). In the present study, varied levels of scavenging activity were observed for all the bacterial isolates ranging from 91.14% to 88.28% and 42.44% to 6.97% for DPPH and H_2O_2, respectively. Interestingly, S-13 (90.52%) and S-14 (91.14) showed higher DPPH scavenging activity compared to ascorbic acid control (90.34). This indicates the good antioxidant capacity of the bacterial isolates under the study. Further, the growth curve of all the probiotic isolates and their mixed culture showed similar growth patterns. This indicates their compatibility in the culture medium and growth potential together, and this can be beneficial for the development of biofloc consortia. Further, the culture of individual isolate and their mixture have not greatly modulated the pH of the medium which will also be an added benefit for the bacteria-based culture of fishes as in aquaculture pH plays a major role in maintaining the physiological balance of fish.

Non-pathogenicity to the host is the prerequisite safety characteristic of any probiotic strain. To evaluate the non-pathogenicity of all six probiotic bacterial isolates, haemolytic assay was conducted and all isolates were found to be non-haemolytic (γ-haemolysis). In addition to this, an *in vivo* challenge test was also conducted on two different fish species *i*.e. rohu and tilapia to assess the non-pathogenicity of the isolates. As expected at a very high bacterial dose *i.e.* $5x10^9$ CFU ml^{-1}, there was no clinical sign or mortality observed in any of the fishes under experimentation. Growth in extreme environmental conditions is also very much necessary for potential biofloc bacteria. In a biofloc setup, the microbial community needs to recycle organic matter accumulated during the culture period continuously, improving water quality through the assimilation of different nitrogen compounds, and converting them into microbial biomass (Samocha, 2019; Ebeling *et al*., 2006). In the present study, we estimated the *in vitro* tolerance of all the probiotic bacteria under study for their ability to withstand high ammonia, nitrite and nitrate concentrations. It was observed that all the isolates were tolerant and able to grow at 10, 20 and 20 mg l^{-1} concentrations of ammonia, nitrite and nitrate, respectively. The biofloc system runs in a principle of using less amount of water for the production of fish with higher efficiency by continuously converting the harmful element produced in the tank to microbial biomass and reducing the risk of fish mortality by an excess of ammonia or any other nitrogenous wastes (Emerenciano *et al*., 2013; Irani *et al*., 2023; Tayyab *et al*., 2023).

In the present study, we have used the indigenously developed biofloc consortium for rearing of rohu and tilapia fry to fingerling during 90 days of culture period and commercially available biofloc inoculum was used as control. Water quality was monitored throughout the experimental period. Biofloc based farming has shown good potential in growing and culturing different fish species including carps and tilapia (Bakhshi *et al*., 2018; Adineh *et al*., 2019). After 90 days of culture in biofloc, the rohu and tilapia survivability observed were 80% and 100% respectively, at a very high stocking density of 1 fish per litre of water. There was a significant increase in the size of the fish observed to 60±10 mm and 50±10 mm, respectively for rohu and tilapia in comparison to the control group. There are several reports on the improvement of the survivability of the different carp species and tilapia under biofloc by using good carbon sources (Nath *et al*., 2017; Adineh *et al*., 2019; Azimi *et al*., 2022). The improvement in the survivability of fishes may be the result of maintaining good water quality, continuous presence of immunostimulants in the form of biofloc bacteria and their ingestion by fish (Romano *et al*., 2018; Khanjani *et al*., 2023). Furthermore, continuous consumption of biofloc by fish helps in the continuous harvest of probiotic bacteria, which helps in maintaining the floc density and improving fish wellbeing (Fauji *et al*., 2018; Khanjani *et al*., 2023). These observations suggest that the probiotic inoculum developed in this study has the potential to function as biofloc inoculum for rearing of rohu and tilapia fry to fingerling with high efficiency. This study will definitely pave way for the development of biofloc-based aquaculture and nursery seed rearing in future with high efficiency.

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