



Original Research Paper

**Isolation and characterization of a novel probiotic bacteria
Staphylococcus species from indigenous sea bass, *Lates calcarifer*
and evaluation of its probiotic potential in pacific white shrimp,
*Litopenaeus vannamei***

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ABSTRACT

In the present investigation, bacterial strains were isolated from the intestinal tract of the Asian sea bass (*Lates calcarifer*) and screened for potential probiotic characteristics. *L. calcarifer* was collected from the river Penna, Nellore, Andhra Pradesh, Nellore. Bacteria was extracted from fish intestine using standard protocols and characterized by using gram staining, molecular and biochemical analysis. Among the 27 isolates exhibiting γ -hemolytic activity, only two strains demonstrated high cell surface hydrophobicity, a key attribute associated with probiotic adhesion and colonization ability. Among them, strain LCF15 (the 15th isolate) displayed superior hydrophobicity and was therefore selected for subsequent evaluation of probiotic efficacy. Molecular identification based on the sequence analysis and BLAST comparison, these isolates shared close similarity to *Staphylococcus* spp. The immunomodulatory capacity of LCF15 strains was investigated in white shrimp (*Litopenaeus vannamei*) following experimental challenge with *Vibrio harveyi*. To accomplish this task, two probiotic feed concentrations (1×10^4 and 1×10^6 CFU g⁻¹ feed) were prepared using LCF15 and were administered to control and infected shrimps. Compared with infected control groups, shrimp receiving probiotic supplementation exhibited markedly lower mortality rates. Furthermore, probiotic treatment resulted in significant improvements in several innate immune parameters, including total hemocyte count, phenoloxidase activity, phagocytic activity, and lysosomal activity. These immunological responses showed a clear dose-dependent pattern, with shrimp receiving the higher probiotic concentration exhibiting stronger immune stimulation. Taken together, the results suggest that LCF15 possesses promising probiotic properties capable of enhancing immune function and increasing resistance to pathogenic infection in shrimp and might be used as a tool for promoting health and sustainability in shrimp aquaculture systems.

Key words: Aquaculture, *Lates calcarifer*, probiotics, shrimp

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Introduction:

The significance of crustacean aquaculture has increased considerably in recent years and now parallels the growth observed in capture fisheries. The demand for edible crustaceans, particularly shrimp, is rising rapidly due to their high economic value and nutritional importance as aquatic food resources. Global shrimp production reached approximately four million tons in 2018, with a substantial proportion originating from Asian countries [1]. Among these producers, India ranks as one of the leading shrimp-producing nations, making shrimp aquaculture a major revenue-generating sector for the country [2].

Despite this growth, shrimp farming is frequently challenged by disease outbreaks that lead to significant economic losses for aquaculture farmers. For instance, viral diseases such as white spot syndrome have severely affected *Penaeus monodon*, while both bacterial and viral infections have been reported in *Macrobrachium rosenbergii* [3,4]. Antibiotics are widely used to control bacterial infections in aquaculture; however, their extensive and often indiscriminate use has resulted in the emergence of antibiotic-resistant microorganisms [5]. Continuous exposure of microbial populations to antimicrobial agents creates strong selective pressure, thereby facilitating the development and spread of antimicrobial resistance. Consequently, effective disease management remains one of the major challenges faced by the shrimp aquaculture industry [6]. Therefore alternative disease control strategies is the need of the hour [7].

Among the alternative approaches, probiotics have gained considerable attention as a potential substitute for antibiotics in shrimp and prawn aquaculture as they can able to improve disease resistance [8]. According to the World Health Organization, probiotics are defined as live microorganisms that, when administered in adequate amounts, confer health benefits to the host [9]. In aquaculture systems, probiotics exert multiple beneficial effects, including enhancement of growth performance, stimulation of host immune responses, improvement of nutrient utilization, and maintenance of water quality [10]. Probiotic microorganisms may act through several mechanisms such as competition with pathogenic bacteria for nutrients and ecological niches, production of antagonistic substances, and synthesis of antimicrobial compounds [11]. These interactions can directly or indirectly enhance the immune competence of the host and improve resistance against pathogenic infections [9]. Consequently, probiotic application is widely regarded as an environmentally friendly and sustainable strategy for disease management in aquaculture [12]. Nevertheless, the identification of effective probiotic

strains and their successful application in shrimp aquaculture remain active and expanding areas of research [13].

Fishes are one of the important sources of probiotics. The Asian sea bass, *Lates calcarifer* is popularly known as sea perch or barramundi. It is endemic to Indo-Pacific region and is one of the economically important aquaculture fishes. Studies have shown that bacteria isolated from intestine of sea bass exhibits probiotic properties. Reports have shown that lactic acid bacteria isolated from the intestine of wild sea bass (*Lates calcarifer*) exhibited antibacterial activity against *Aeromonas hydrophila* infected juvenile sea bass [14]. In addition, bacterial strains capable of degrading short- and long-chain N-acyl homoserine lactones—molecules associated with quorum sensing in several pathogenic *Vibrio* species including *Vibrio harveyi*—have also been isolated from the intestinal microbiota of sea bass [15]. These findings suggested that bacteria derived from sea bass intestinal microbiota could potentially serve as probiotic candidates for controlling bacterial infections in shrimp aquaculture systems.

Based on the aforementioned data, the present study was aimed to isolate bacterial strains from the intestine of wild sea bass collected from the Nellore coastal region and evaluated their probiotic potential in shrimp, *Litopenaeus vannamei*, under *Vibrio harveyi* challenge conditions. Furthermore, understanding the mechanisms employed by pathogens to evade host defenses requires a comprehensive understanding of shrimp immune responses [16]. Therefore, this study also investigated aspects of shrimp immunity associated with probiotic-mediated protection against pathogenic infection.

Materials & Methods:

Chemicals

All the chemicals, consumables and microbiological media used in this study were purchased from Merck Pvt. Ltd. (India), and HiMedia Pvt. Ltd. (India). Gel purification kit and high-fidelity polymerase enzyme were purchased from Fermentas (USA) and Thermo Scientific, respectively.

Collection of fishes, isolation of bacteria

Fish samples, *L. calcarifer* (Fig. 1) weighing 2.52 ± 0.53 Kg collected from various regions of Nellore coastal area. The specific sampling areas were the river mouth of Penna (Penna Estuary), Iskapalli, Utukuru, Maipadu and Ramudupalem. The collected fish samples were transported in sterilized polythene bags containing habitat water to the laboratory and then transferred on ice. The surface of the fishes was disinfected with 70 % ethanol. The intestines of fishes were dissected under aseptic conditions and any food remains were evacuated. After evacuation step, the gut samples were cut into pieces followed by the washing

step (thrice) using normal saline (NaCl: 0.85 %). The washed gut samples were homogenized (1: 10 w/v) using normal saline solution. The resultant supernatant of gut samples was serially diluted (10^{-2} ; 10^{-3} ; 10^{-4} ; 10^{-5} ; 10^{-6} and 10^{-7}) and 100 μ L of each dilution were spread on nutrient agar and De Man, Rogosa, and Sharpe (MRS) agar plates for 48 h at 37°C. The microbial colonies were isolated using the quadrant striking method. On MRS agar medium, the total number of bacterial counts (Maximum count: 1.6×10^6 to minimum count: 2.4×10^4 CFU/ml) obtained from the gut samples of fish was determined using serial dilution (up to 10^{-7}). The glycerol stocks of individual isolates were stored at -80°C until further experimental analysis. This study was approved and all the experiments were carried out at department of Marine Biology as per the recommendations of the Institutional Animal Ethics Committee, Vikrama Simhapuri University, Nellore-524324.

Characterization of bacteria

The characterization of bacteria was performed based on gram staining, biochemical and molecular analysis. Briefly, the bacterial isolates obtained at 10^{-7} dilution were subjected to hemolysis assay according to the method described previously using blood agar base No.2 plates containing defibrinated sheep blood [17]. The gamma-hemolytic strains (27 isolates) were subjected to hydrophobicity analysis. Out of 27 isolates, two gamma hemolytic strains were selected based on high hydrophobicity values and further biochemical and molecular characterization was performed. The vegetative cells were observed using a phase-contrast microscope (Make: Olympus, Model: CH25i). Gram staining technique was performed in accordance to the standard protocol.

The molecular identification of selected strains was based on 16S rDNA sequencing method. In brief, bacterial DNA was isolated using phenol-chloroform method [18]. The quantity and purification of DNA isolated from the bacterial samples were analyzed by using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientifics) and the purity was also analyzed on agarose gel electrophoresis (1.0 %). After quantitative and qualitative analysis of DNA, partial 16S rDNA was amplified using polymerase chain reaction (PCR; Thermal Cycler: Eppendorf Nexus Gradient, Eppendorf). For amplification, of partial 16S rDNA fragment universal primers (Forward primer: 5'-CCAGAGTTTGATCMTGGCTCAG-3' and reverse primer: 5'-AGAGTTTGATCMTGGCTCAG-3') using the PCR conditions (*Initial denaturation* at 95° C for 4 minutes, followed by 35 cycles of *denaturation* at 94°C for 30 sec, *annealing* at 58° C for 20 sec, *extension* at 72° C for 60 sec, followed by *final extension* at 72° C for 10 min.). The primers were

selected based on the studies of Molina et al., (Molina F et al., 2015). The amplified products were analyzed on 1 % agarose gel electrophoresis. The amplicons (1500 bp) obtained were purified from the gel using Gel extraction kit (Fermentas, USA). The purified PCR products were directly sequenced with PCR primers using ABI Big DYE terminator method (Xcelris lab, Ahmedabad, India). The sequence was manually assembled using Gene Runner V 3.0 software and to avoid sequencing errors, assembled sequences were terminated to a homologous region. The consistency of the sequence was tested by evaluating the Phred score (Q value), and a value of > 35 for bases is taken as a threshold. The trimmed sequence obtained from the bacterial isolate was subjected to NCBI's Mole-BLAST (<https://blast.ncbi.nlm.nih.gov/moleblast/moleblast.cgi>) to query the submitted sequence against Nucleotide collection (nr/nt)" database to identify nearest neighbors and while computing, uncultured/environmental samples were excluded. The phylogenetic tree was constructed based on the multiple sequence alignment using nearest-neighborhood method.

Hydrophobicity test

Bacterial cell surface hydrophobicity analysis of gamma hemolytic strains was determined in accordance to the protocol described by Borah et al.[19]. In brief, the pellet obtained through centrifugation (10,000 Xg) from the overnight grown bacterial culture was used for bacterial cell surface hydrophobicity analysis. The pellet was washed with phosphate buffer saline and suspended in 0.1 M KNO₃ solution (3.0 ml) and the absorbance was recorded at 600 nm (A0). This was followed by the addition of toluene (1 ml), which resulted in a two-phase system. After vortex for 2 min. The two-phase system was incubated for 30 min. at room temperature. The aqueous phase was carefully separated and the absorbance was measured at 600 nm (A1). The hydrophobicity (H) was expressed in terms of percentage and was determined using the formula $H = [1 - (A1/A0)] \times 100$.

Antimicrobial activity

The isolated bacteria were analyzed for its antibacterial efficacy against *Vibrio harveyi* using well diffusion method. *V. harveyi* was responsible for white tail disease in shrimps and hence, as a preliminary analysis, *V. harveyi* was selected as test organism. The pathogenic strain of *V. harveyi* was obtained from Microbial Type Culture Collection, Chandigarh, India (Ref No: MTCC 7475). The bacteria were inoculated in Tryptone Soy Broth (TSB; HiMedia, India) and incubated for 24 h at 36°C. After completion of incubation period, *V. harveyi* (1.5×10^8 CFU/ml in accordance to the 0.5McFarland Standard) was spread

on to the Tryptone Soy Agar (HiMedia, India) using sterile swabs. On the other hand, the overnight grown culture of selected bacterial strain isolated from *L. calcarifer* in TSB at 37°C was centrifuged and the resultant supernatant was used for antibacterial activity using well diffused method [20].

In vivo probiotic assay

To test the probiotic ability of selected bacterial isolate from fish gut, an *in vivo* assay was carried out in experimental cultures of *L. vannamei* following *V. harveyi* infection

Shrimp collection

Juvenile shrimps (n = 300) were obtained from shrimp hatchery near Gangapatnam, Nellore District, India weighing about 1.8 ± 0.3 g. The shrimps were carefully analyzed for the presence of any viral or bacterial diseases using PCR and other microbiological methods before their arrival to the animal house [21]. After their arrival, all the shrimps were acclimatized to the laboratory conditions (10 days). They were maintained in sterile sea water in concrete tanks. All the parameters such analysis of salinity (30 psu), dissolved oxygen (5.7 ± 1.0 mL L⁻¹ DO) and temperature ($26 \pm 2.0^\circ\text{C}$) were regularly monitored. During their sojourn, the shrimps were given standard commercial shrimp feed (BLANCA, CP Aquaculture India Pvt, Ltd., Chennai, India).

Probiotic feed preparation

Probiotic feed preparation was performed based on the methods described previously with minor modifications. The selected strain from fish gut was cultured in nutrient agar and was inoculated into the tryptic soy broth at 150 rpm at 28°C and kept in shaking incubator. After 24 h, the bacterial cells were harvested by centrifugation at 5000 rpm for 5 min, discarding the supernatant and the bacterial pellet was washed with sterile saline 0.85 % NaCl. After thorough washing (three times), the selected strain was mixed thoroughly with the commercial diet [4 g wet bacterial weight per one kilogram of commercial CP Shrimp feed (BLANCA, CP Aquaculture India Pvt, Ltd., Chennai, India)] and dried at room temperature. In this study, two types of probiotic feed preparations (PFP) were performed: PFP1 (1×10^4 CFU g⁻¹ fodder) and PFP2 (1×10^6 CFU g⁻¹ fodder). The probiotic bacteria were mixed with CP shrimp feed “BLANCA” (CP Aquaculture India Pvt Ltd., Chennai, INDIA). In order to minimize the leaching of bacteria, a final coating of feed with cod liver oil and soybean oil was used. The probiotic feed was given by calculating the shrimp average body mass.

Pathogen preparation

Vibrio harveyi (MTCC No 7954) purchased from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India were revived, cultured

and maintained on Tryptic Soy Agar (TSA) (Growth medium No: 54; Pancreatic digestive casein: 17.0 g; Papaic digest of soyabean meal: 3.0 g; Sodium Chloride: 5.0 g; Dipotassium hydrogen phosphate: 2.50 g; Dextrose (Glucose): 2.50 g; Agar: 15.0 g; Growth condition: Aerobic; Temperature: 37°C; Incubation time: 12 hrs; Medium components were dissolved @ 45 g/ 1 lit. of distilled water) as suggested by MTCC. They were harvested, washed and finally re-suspended in half strength aged sea water diluent as described by Abraham et al. [22].

Pathogen challenging test:

Shrimps were challenged by immersion method at a concentration of $\sim 10^7$ CFU/ml of *V. harveyi* in rearing water [23]. The test concentration was determined based on preliminary trials by infecting the shrimps at concentrations ranging from 10^3 to 10^8 CFU/ml. During the preliminary experimental infection study, the shrimp pathogen *V. harveyi* MTCC 7954 caused > 95 % mortality at 10^7 CFU/ ml concentration in 14 days. Based on the preliminary study, pathogen challenging test was performed using shrimp infected with pathogen, *V. harveyi* supplemented with or without *Staphylococcus* spp. LCF15 feed formulations. Shrimps were randomly allocated into three groups (every treatment group was performed in triplicates and each treatment was performed with n=30 shrimps per treatment per diet). The shrimps were maintained in 80 L capacity concrete tank with sterilized sea water, continuous aeration and water was changed for every four days. The experimental design was as follows: shrimp in group 1 were fed with feed without any probiotic bacteria, shrimps in groups 2 and 3 were maintained on PFP1 and PFP2, respectively were considered as experimental groups and were fed with probiotic feed every alternative day over a period of 30 days. Uneaten food was siphoned carefully and water was changed every four days. Briefly, after 30 days of culture a short-term laboratory experimental infection study was carried out. All water quality variables were similar to those in culture ponds. Feeding schedules were also similar. The duration of the experiment was 14 days. During immersion test survival rates and mortality percentage were recorded to assess disease resistance of *L. vannamei* supplemented with or without probiotic bacteria.

Hemato-Immunological Parameters in Shrimp

Hemolymph collection

Hemolymph sampling was performed at different time points (1, 10, 20 and 30 days). Briefly, hemolymph (300 µl) collected from ventral sinus of shrimps using 21 G needle coupled with a 1 mL syringe was diluted with 2700 µl of anticoagulant solution (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7.0) was used to

determine THC, phenol oxidase and lysozyme activity, while hemolymph collected from ventral sinus of shrimps using 19 G needle coupled with 1 ml syringe was diluted with ice cold 5 ml of anticoagulant solution (MAC solution: 0.45 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA; pH 4.6) was used to determine the phagocytic activity.

Total hemocyte count (THC)

Briefly, a drop of hemolymph sample [50 µl of diluted hemolymph-anticoagulant mixture combined with 150 µl of 6% formaldehyde solution (1:3 ratio)] was placed on hemocytometer to determine total hemocyte count using microscope (Make: Olympus; Model: CH 25i). THC was expressed as number of cells per ml.

Phenoloxidase activity (PO)

PO activity was determined in accordance to the method described previously [24]. Briefly, the hemolymph-anticoagulant mixture was placed on ice and the resultant clot was subjected to frozen state. After thawing thrice (facilitates to break the hemocytes), the sample was centrifuged at 10000 x g for 10 min. to obtain the serum which was used as a source for PO activity. The reaction mixture comprising of 50 µl of serum [10 µl of serum diluted in 90 µl TBS buffer (1 mM Tris, 336 mM NaCl, 5 mM CaCl₂, 10 mM MgCl₂ pH 7.4)], and 50 µl of elicitor, trypsin was incubated for 5 min followed by the addition of 50 µl of L-dihydroxyphenylalanine (L-DOPA). Finally, 50 µl of TBS buffer was added to obtain reaction mixture volume to 0.2 ml. The absorbance obtained due to the oxidation of substrate L-DOPA to form a pigment, dopachrome was monitored spectrophotometrically at 490 nm. against the blank sample which comprise of all the reaction mixture contents as mentioned above except 50 µl of elicitor was replaced by 50 µl of TBS buffer. Each test was performed in triplicate. The units for PO activity were expressed as dopachrome formed per mg protein per min. The serum protein concentration was calculated using bovine serum albumin (BSA) as standard [25].

Lysozyme activity

Lysozyme activity was determined in accordance to the method described earlier [26]. Briefly, 1 ml of dried probiotic bacteria isolated from *L. calcarifer* (4 mg/ml), 1 % agarose was thoroughly mixed in 14 ml of 50 mM Tris-HCl buffer (pH 5.2) and poured in to petri dish (90 mm in diameter; 15 mm in height). After solidification, wells with a size of 6 mm diameter were sunk on to the agarose followed by the addition of control and experimental samples. Human saliva [diluted with NaCl (1:9 ratio)] and Tris-HCl buffer were used as positive and negative controls, respectively. Lysozyme activity was determined by

subtracting the diameter of the well from the diameter of each clearance zone formed around the respective samples. The results were expressed as units per mg protein wherein 1 unit is equal to 0.1 mm diameter. All the experiments were performed in triplicates.

Phagocytic activity

The hemolymph-anticoagulant mixture was transferred to 20 ml ice-cold MAC solution and centrifuged at 2000 g for 10 min at 4°C. The resultant pellet was re-suspended in 5 ml ice-cold MAC and washed, counted and re-suspended in a volume of sterile marine saline. Cell suspension (100 µl equivalent to 5×10⁵ cells) was dispensed on pre-marked slides followed by incubation step over a period of 20 min. at 25 °C. The excess saline was carefully drained and replaced with 100 µl of the bacteria solution and incubated in the dark for 1 hour at 25 °C followed by rinsing step using marine saline. The slides were then fixed in the fixative of 5 percent of Baker solution (5 percent formalin, and 1 percent calcium acetate in marine saline, by volume) for 2 hours before being dried overnight and kept dry in the dark. The slides were mounted using Permafix HRF (Citi fluor Ltd., Leicester, UK) and stored at 4°C for examination using a Zeiss LSM 510 META laser scanning confocal microscope. The phagocytic activity was expressed in percentage and calculated using the formula: total number of phagocytes/total number of hemocytes X 100).

Results

Characterization studies

The findings indicated that out of 27 isolates from gut of *L. calcarifer* (LCF), two strains which exhibited greater hydrophobicity (Fig. 2). Both the strains, LCF9 and LCF15 were gram positive and morphological analysis revealed that they appeared as cocci in shape. Phylogenetic analysis using Neighbor joining tree showed that the isolated strains of bacteria (LCF9 and LCF15) formed a separate clade and both the strains belong to firmicutes (Fig. 3). In this study, a non-*Lactobacillus* species was identified from the gut of *L. calcarifer* as indicated by molecular analysis (Sequences of LCF-9 and LCF-15 were provided in supplementary material). The sequences were submitted to gene bank with an ID Numbers 2911370 for LCF15 and 2911372 for LCF9. BLAST analysis showed >90 % sequence similarity with *Staphylococcus sps* as well as uncultured bacterium. The divergence values between isolated sequences and reported sequences showed < 2 % divergence values. However, based on less genetic divergence values with reported species, LCF 9 and LCF15 could be species of *Staphylococcus*. This notion required further authentication and the relationship between LCF 9 and LCF15 with reported species could be resolved by using a specific marker.

Antibacterial activity

After characterization, their antibacterial efficacy of isolated bacteria was evaluated using test bacterial pathogen, *V. harveyi*, a causative organism of white tail disease in shrimps. Antimicrobial efficacy of both the selected strains isolated from fish gut against *V. harveyi* were shown in Fig 4. Both the isolated strains exhibited antibacterial efficacy as indicated by zone of inhibition against *V. harveyi*. Since, LCF15 showed 9 mm of zone of inhibition over LCF9 (8 mm diameter zone) and also exhibited high hydrophobicity, further probiotic efficacy experiments were evaluated using LCF15 strain.

Effect of probiotic supplementation on hemato-Immunological Parameters in shrimp

Based on hydrophobicity tests and antibacterial activity, LCF15 strain was used as probiotic organism. In this study, two feed formulations, probiotic feed preparation 1 (PFP1) and PFP2 were prepared. Supplementation of PFP1 and PFP2 caused a significant improvement in the immune responses of juvenile *L. vannamei* especially with reference to THC, lysozyme, PO, and phagocyte activity against *V. harveyi* (Table 1). The average THC (10^6 cells per ml) in control, PFP1 and PFP2 groups were 10.85 ± 0.75 , 14.02 ± 0.67 and 17.21 ± 0.93 , respectively. Statistical analysis revealed that a significant THC was observed in PFP1 (29.21 %; $p < 0.05$) and PFP2 (58.61 %; $p < 0.05$) supplemented shrimp infected with *V. harveyi* over controls. Further, THC in PFP2 supplemented shrimp challenged with pathogen showed 22.75 % ($p < 0.05$) increase in THC as compared to PFP1 group. The average phagocytic cells (per 100 cells) in control, PFP1 and PFP2 groups were 41.21, 52.23 and 65.21, respectively and a statistically significant increase ($p < 0.05$) in the phagocytic activity was observed in pathogen infected shrimp supplemented with PFP2 (58.23 %; $p < 0.05$) and PFP1 (26.74 %; $p < 0.05$) over controls. On the other hand, phagocytic activity in PFP2 group was also statistically significant (24.85 %; $p < 0.05$) over PFP1 group. The average phenol oxidase activity (units per mg protein) in control, PFP1 and PFP2 groups were 10.31 ± 0.98 , 20.20 ± 1.57 and 20.95 ± 1.97 , respectively and a statistically significant increase in the PO activity was observed in pathogen infected shrimp supplemented with PFP2 (103.20 %; $p < 0.05$) and PFP1 (101.96 %; $p < 0.05$) over controls. However, no significant change (3.71 %; $p > 0.05$) was observed in PO in PFP2 vs PFP1 groups. The average lysosomal activity (Units) in control, PFP1 and PFP2 groups were 0.205 ± 0.041 , 0.351 ± 0.032 and 0.491 ± 0.023 , respectively and a statistically significant increase in the lysosomal activity was observed in pathogen infected shrimp supplemented with PFP2 (139.51 %; $p < 0.05$) and PFP1 (71.21 %; $p < 0.05$) over controls.

Whereas, a significant change (39.88 %; $p > 0.05$) was observed in PO in PFP2 vs PFP1 groups.

Mortality analysis

Feeding of diet supplemented with LCF15 caused a significant effect on the cumulative mortality percentage of the shrimp, regardless of the dose of LCF15 in the feed that was used (Table 2). The mortality rate in control group was 100 % while, the mortality rates in PFP1 and PFP2 groups were 61 % and 75 %, respectively suggesting that the supplementation of PFP1 and PFP2 increased survival rate of infected shrimp of about 39 % and 25 %, respectively.

Discussion

Molecular and phylogenetic analysis revealed that LCF15 (15th isolate from the intestine of sea bass) could be a non-*Lactobacillus* organism and exhibited close similarity to *Staphylococcus* spp. Before determining its probiotic efficacy, the hydrophobicity and antibacterial activity were analyzed. It is well known that the cell surface hydrophobicity is a key factor associated with adhesion and persistence of a probiotic activity once they have entered the intestinal cavity [27,28]. The findings of current study affirm that the ability of LCF15 to bind the intestinal epithelium of shrimp increases via hydrophobic interactions. Adhesion of LCF15 to the intestinal epithelium favors colonization which in turn exhibit antagonistic activity against enteropathogens [29,30]. Hydrophobic interactions might be linked to various cell surface proteins like S-layer proteins, divalent cations and polycarbohydrate of probiotic bacteria [31]. However, which of the factors would influence the hydrophobic interactions between the epithelium of shrimp and LCF15 needs to be clarified. Next, we analyzed the antibacterial potential of LCF15. LCF15 isolated from the intestine of sea bass showed antibacterial activity against *V. harveyi*. This could be ascribed to the occurrence of bacteriocin in LCF15. The results are in consonance with previous studies [14,15,32]. It has been shown that the antimicrobial metabolites produced by probiotic organisms such as organic acids, hydrogen peroxide and bacteriocins might be associated with antimicrobial activity [33]. This notion requires additional research to characterize active compounds in order to substantiate the antibacterial activity against *Vibrio* spp.

Since, LCF15 exhibited strong hydrophobicity and *in vitro* antibacterial activity against *V. harveyi*, we sought to investigate its probiotic efficacy. It is believed that haemato-immunological responses are one of the host primary physiological mechanisms to fight against diseases in animals [34]. In our study, supplementation of probiotic feed containing LCF15 resulted in the amelioration of host immunity as indicated by a

significant increase in total hemocyte count, elevated number of phagocytes, and enhanced activity levels of lysozyme and phenol oxidase (PO). Hemocytes are one of the mediators of cellular response in crustaceans and they are primarily involved in phagocytosis, non-self-recognition mechanisms and promotion of melanization process to trap foreign materials [35]. During bacterial infections, the reduction in total hemocyte count might reflect active participation of hemocytes to promote rapid immunity. Studies have shown that during infections, the hemocyte count number increases in shrimp [36,37] and an increase in THC could be associated with enhanced immune capability and amelioration of disease resistance in the infected shrimps [38]. Our findings support this notion. Studies also indicated that a reduction in THC might be associated with body defense mechanisms to infiltrate the infected hemocytes and consequently promotion of hemocyte cell death via apoptosis [39].

PO is an enzyme that plays a key role in pigmentation/melanization process and also defense system. The increased activity levels of PO in infected shrimp supplemented with LCF15 might be associated with phagocytosis and accordingly elevation of phagocytic activity. It has been shown that PO can able to induce oxidation of phenols to quinones which eventually elevate melanin content and these cascade of events trigger phagocytosis and cytotoxic reactant production [38,40]. Lysozyme is one of the innate immune system tools which fundamentally acts as antibacterial proteins [41]. The finding, increased activity of lysozyme associated with enhanced THC and PO activity in the hemolymph of infected shrimp supplemented with LCF15 might reflect boosting of non-specific immune defense in shrimp. Piecing the data, we suggest that the reduction in mortality rate could be associated with an improvement in selected immune effector molecules in LCF 15 supplemented infected shrimp groups.

Conclusion

A novel bacterial strain of LCF15 was isolated from the gut of sea bass, *L. calcarifer* and its probiotic effects in shrimp challenged with *V. harveyi* was investigated. 16S RNA sequencing analysis indicated that the LCF15 could be a member of *Staphylococcus* species. We have shown that an overall survival rate in shrimp challenged with *V. harveyi* could be associated with an improvement in immune response. The findings of current study indicated that LCF15(*Staphylococcus* sp.) isolated from the gut of Asian sea bass, *L. calcarifer* exhibited a significant increase in total hemocytes, phagocytic cells, phenol oxidase and lysosomal activity levels in hemolymph associated with a cumulative reduction in the mortality rates of shrimp infected with the

pathogen. Hence, the isolated strain of bacteria from *L. calcarifer* could be used as a probiotic tool that can promote productivity in shrimp aquaculture. Moreover, we report a *non-lactobacillus* bacterial species from the intestine of sea bass. Previously, occurrence of lactic acid bacteria from the intestine of sea bass [14,15,32] was reported. This could be attributed to the ecological habitat of *L. calcarifer*, which influences its associated microbiota.

Given the importance of probiotics in shrimp aquaculture, identification of probiotic bacteria that provide health benefits to the shrimp against the pathogens is one of the emerging areas of aquaculture research. The findings of this study contribute to the literature and acts as a platform to promote sustainable shrimp aquaculture. Therefore, future research is warranted to better contextualize our results.

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Conflict of interest

Nothing to disclose

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Table 1: Effect of probiotic bacteria on haemato-immunological properties in shrimp. *L. vannamei* challenged with *Vibrio harveyi* over a period of four weeks

Parameters	VH Treated	VH + PFP1	VH + PFP2
Total hemocyte count ($\times 10^6$ cells per ml)	10.85 ^a $\pm$ 0.75	14.02 ^b $\pm$ 0.67	17.21 ^c $\pm$ 0.93
Phagocytic activity			
Number of phagocytic cells	169.0 ^a $\pm$ 2.0	185 ^b $\pm$ 4.0	194 ^c $\pm$ 2.0
Number of non-phagocytic cells	31.0 ^a $\pm$ 2.0	15 ^b $\pm$ 4.0	6 ^c $\pm$ 2.0
Phenoloxidase activity (units/mg protein)	10.31 ^a $\pm$ 0.98	20.2 ^b $\pm$ 1.57	20.95 ^b $\pm$ 1.97
Lysozyme activity (units/mg protein)	0.205 ^a $\pm$ 0.041	0.351 ^b $\pm$ 0.032	0.491 ^c $\pm$ 0.023

Values are mean $\pm$ S.D. of 90 individual shrimps (experiments repeated thrice with a sample size of n=30 per treatment; 3 x 30 = 90 shrimps per group). For evaluation of statistical analysis, VH (*Vibrio harveyi* treated shrimps; 10^7 CFU/ ml) served as controls for VH+PFP1 (Probiotic feed preparation1 supplemented: 1×10^4 CFU g⁻¹ fodder) VH treated group) and VH + PFP2 (Probiotic feed preparation 2 supplemented: 1×10^6 CFU g⁻¹ fodder) VH treated group). Mean values with different letters in a row differ significantly from each other.

Table 2: Effect of probiotic bacteria on cumulative Mortality and survival rates in shrimp. *L. vannamei* challenged with *Vibrio harveyi* over a period of 10 days

Groups	Mortality rate (%)*	Survival rate (%)**
VH-treated	100 ^a ± 0.00	0
VH+PFP1	61.0 ^b ± 4.0	39.0 ^a ± 4.0
VH+PFP2	75.0 ^c ± 3.0	25.0 ^b ± 3.0

Values are mean ± S.D. of 90 individual shrimps (experiments repeated thrice with a sample size of n=30 per treatment; 3 x 30 =90 shrimps per group). *For evaluation of statistical analysis, VH (*Vibrio harveyi* treated shrimps; 10⁷CFU/ ml) served as controls for VH+PFP1 (Probiotic feed preparation1 supplemented: 1 x 10⁴ CFU g⁻¹ fodder) VH treated group) and VH + PFP2 (Probiotic feed preparation 2 supplemented: 1 x 10⁶ CFU g⁻¹ fodder) VH treated group). **For evaluation of statistical analysis, VH+PFP1 (Probiotic feed preparation1 supplemented: 1 x 10⁴ CFU g⁻¹ fodder) VH treated group) served as control for VH + PFP2 (Probiotic feed preparation 2 supplemented: 1 x 10⁶ CFU g⁻¹ fodder) VH treated group). Mortality and survival rate: mean values with different letters in a column differ significantly from each other.

Figures



Figure 1: Asian sea bass, *Lates calcarifer*

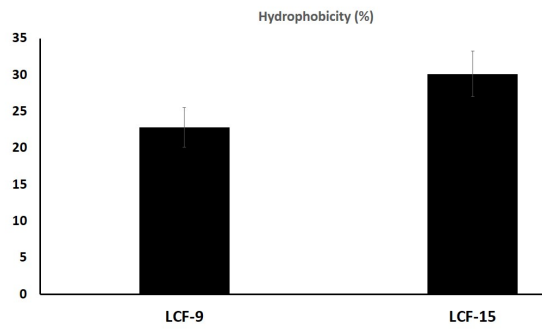


Figure 2: Cell surface hydrophobicity of the tested probiotic strains
Bars represent mean $\pm$ SD of four replicate experiments

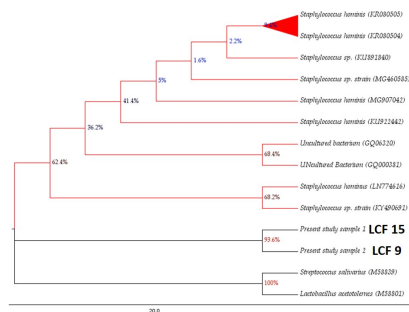


Figure 3: NJ tree of 16SrRNA of present and reported sequences

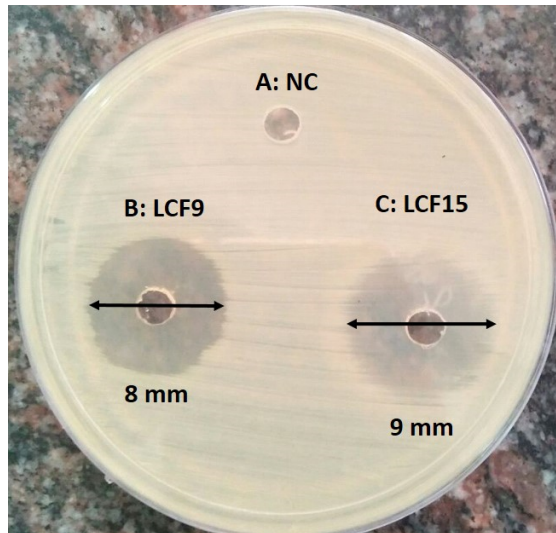


Figure 4: Antibacterial activity of selected isolates on against *Vibrio harveyi*.

A: Negative control; B: Bacteria isolated from *L. calcarifer* (LCF9); C: Bacteria isolated from *L. calcarifer* (LCF15); Zone of inhibition was recorded for five replicate experiments.