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Research Article

Probiotic Properties of *Bacillus subtilis* Isolated from Dried Anchovies (*Stolephorus indicus*) and Evaluating its Antimicrobial, Antibiofilm and Growth-Enhancing Potential in *Danio rerio*

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Abstract | The study was aimed at isolating and characterising a potential probiotic bacterium from dried anchovies (*Stolephorus indicus*) and evaluating its antibacterial, antibiofilm and growth enhancing potential in *Danio rerio*. The isolate was identified as *Bacillus subtilis* using 16S rRNA sequencing and phylogenetic analysis. Probiotic properties were characterised based on the ability of the isolated strain to survive in simulated gastric juice and trypsin. Isolated strain was further subjected to varying pH, temperature, different concentrations of organic solvents to evaluate its potential to tolerate stress. Biofilm inhibition against *Vibrio harveyi* (31.5±4.6%), *Escherichia coli* (28.8±4.2 %), *Pseudomonas aeruginosa* (34.8±3.1%) and *Staphylococcus aureus* (34.4±3.75%) was noted. The study showed that the isolate improved the survival rate of *Danio rerio* against *Vibrio harveyi* and *Escherichia coli*. The weight (12.77±0.06) and length (11.413±0.18) gain percentage was numerically (p> 0.05) improved in probiotic supplemented groups as compared to control. The use of probiotics from non-conventional sources can improve the diversity of the available probiotics for aquaculture practices

Keywords | *Bacillus subtilis*, Probiotic, Aquaculture, Anti-bacterial, Anti-biofilm, Growth enhancement

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INTRODUCTION

Among the various food production sectors, aquaculture is the most rapidly growing. The average annual increase in global consumption of fish (3.2%) outpaced the average growth in population (1.6%) between 1961 and 2016 (FAO, 2018). Coupled with this growth are the various concerns related to the safety and quality of the fish due to overproduction to meet the needs of billions of people around the globe. One of the major constraints in the field of aquaculture is the surge in disease outbreaks. Antibiotics have been the go-to option to combat disease outbreaks (Satish et al., 2011). Over-prescription of antibiotics in clinical setups and in the food industry to the emergence of antibiotic resistant microorganisms such as *Staphylococcus aureus, Enterococci,* and *Streptococcus pneumoniae* (Hols et al., 2019). Due to the growing concerns about the use and abuse of antibiotics, alternatives such as probiotics are emerging as a safer and beneficial alternative (Satish et al., 2011).

A probiotic is a cultured product or live microbial feed supplement, which beneficially affects the host by improving the intestinal microflora (Fuller, 1989). Probiotics are mostly associated with the gastrointestinal tract, where they are antagonistic to pathogenic bacteria (Hoseinifar et al., 2018). Some of the mechanisms by which the probiotics confer beneficial effects to the host are by causing

the exclusion of pathogenic microorganisms (Gayathri and Rashmi, 2016), improving the host immune response by producing antibacterial substances such as antibiotics, bacteriocins (Gaspar et al., 2018; Hoseinifar et al., 2018), siderophores (Panda et al., 2017), alteration of pH by organic acid production and by the production of hydrogen peroxide under aerobic growth conditions (Pavlova, 2020). Probiotics are also known to reduce mutagenesis by directly binding to mutagenic compounds thereby reducing their absorption in the intestine (Orrhage et al., 1994), and reduce cholesterol production through the production of short-chain fatty acids (Pereira and Gibson, 2002).

Researchers have relied on different sources for the isolation of potential probiotic bacteria. The conventional sources are dairy products and the gastrointestinal tract of healthy humans. However, research conducted over the years has highlighted the use of alternate sources such as the gastrointestinal tract of other animals, traditional fermented vegetables, fruits, and dried fish (Zielińska and Kołożyn, 2018; Singh et al., 2012). One example of such an unconventional source, dry fish, has been gaining popularity. There has been an increase in the production of dry fish with about 17% of the total catch in Indian fisheries being used for the production of dry fishes (Bharda et al., 2017). Mackerel, tuna, oil sardines, lesser sardines, silver bellies, mullets, ribbon fishes, and anchovies are some of the species commonly subjected to dry fish production in India (Siriskar et al., 2013, Logesh et al., 2012). Dried anchovies (*Stolephorus indicus*) form an important component of purse-seine fishery of the Indian coast that are found mostly along the coastal areas of India and are widely consumed as salted or in dried form (Siriskar et al., 2013). Traditionally dried anchovies are a potentially unique source of probiotic bacteria (Alkalbani, 2019). Thus, the current study was conducted with the following objectives: (1) to isolate potential probiotic bacteria from dried anchovy fish and characterize their probiotic properties and (2) to decipher its use in combating pathogens, enhancing the growth and survival rates in fishes.

MATERIALS AND METHODS

Isolation, Identification and Characterisation

The isolation source for probiotics was dried Anchovy fish (*Stolephorus indicus*). MRS (De Man-Rogosa-Sharpe) media was used for isolating the culture. The isolate was identified using 16SrRNA sequencing using forward (16SrRNA-F) and reverse (16SrRNA-R) primers by BDT v3.1 cycle sequencing kit on ABI 3730xl Genetic Analyzer. Gram's staining, IMViC test, motility and catalase tests were performed as preliminary evaluation (Hoque et al., 2010). The isolate was screened for its antimicrobial (Kavitha et al., 2018) and haemolytic activity (Halder et al., 2017) before further characterisation.

pH, temperature and tolerance test

Characterisation was done according to the protocol of Yadav et al. (2016) with slight modifications. The isolate was exposed to varying pH (2-10), temperature (15°C-55 °C), sodium chloride concentrations (2 %-10%) and solvent concentrations (0.2% - 0.6%). After incubation for 24 h, the culture was plated onto MRS agar plates and the Log (CFU•mL^{-1}) was calculated (Zheng et al., 2013).

Artificial gastric juice tolerance and trypsin tolerance test: The test was performed according to the protocol described by Corcoran et al. (2005) with slight modifications. The composition of the artificial gastric juice was Glucose (0.35 g), NaCl (0.20 g), KH_2PO_4 (0.06 g), $CaCl_2$ (0.01 g), KCl (0.03 g) and Pepsin (0.03 g) per 100 mL. All components of gastric juice were added as stock in the media prior to inoculation of culture. An overnight culture of the isolate was inoculated into artificial gastric juice and incubated at 37 °C for time duration of 2, 4 and 6h. Similarly, the isolate was subjected to varying concentrations (0.2% - 0.6 %) of trypsin. The isolate was treated with trypsin for a period of 6h. The cell viability was expressed in Log (CFU•mL^{-1}) after plating onto MRS agar for 24 h at 37°C.

In vivo challenge and growth studies

Challenge studies were performed according to the protocol of Wang et al. (2008) with slight modifications. A total of 180 healthy Zebrafish (*Danio rerio*) weighing (0.1-0.15 g) were segregated into triplicates in the following groups; Group treated with saline, group treated with probiotic only, group treated with pathogens (*Vibrio harveyi* and *Escherichia coli*) and a final group constituting pathogens along with the isolate. The survival rate in *D. rerio* was determined by supplementing 6 Log (CFU•mL^{-1}) of the isolate against *Escherichia coli* and *Vibrio harveyi* (6 Log (CFU•mL^{-1}). Parameters such as feed conversion ratio, weight gain percentage and length gain percentage were monitored for a period of 15 days.

Antibiofilm Activity

Escherichia coli, Pseudomonas aeruginosa, Vibrio harveyi and *Staphylococcus aureus* were allowed to form biofilm in a microtiter plate. The antibiofilm activity was measured using a microplate reader (iMark™ Microplate Absorbance Reader, Biorad) set at 450 nm and the percentage inhibition was calculated (Costa et al., 2018).

Statistical Analysis

The results are presented as Mean±SD of triplicates. The various parameters analyzed were subjected to statistical analysis using one way ANOVA and independent t-test with $p < 0.05$ being considered significant.

Table 1: General characteristics of the isolated *Bacillus subtilis*

Colony morphology	**White,round,opaque, medium and smooth**	**(Lu et al., 2018)**
Gram's staining and Shape	**Gram positive, bacilli**	**(Seenivasan et al., 2012)**
Catalase test	+	(Lu et al., 2018)
Motility test	Motile	(Liu et al., 2009)
IMViC test*	+--+**	(Seenivasan et al., 2012)
Haemolysis assay	Gamma Hemolytic	(Zulkhairi et al., 2019)
Milk coagulation efficacy	-	(Wu et al. 2013)
Growth at 0.6% trypsin	++	(Nithya and Halami, 2012)
Antimicrobial well diffusion test	++	(Kavitha et al. 2018)
Organic acid production	-	(Hoque et al. 2010)

*Indole, Methyl red, Voges-Proskauer and Simmon citrate test.
**Consecutive values according to IMViC test

RESULTS AND DISCUSSION

The isolate was identified as *Bacillus subtilis* using 16S rRNA sequencing and the partial sequence was deposited in NCBI (Accession number -MN960600). The evolutionary history was inferred using the Maximum Likelihood method and Tamura-Nei model and a phylogenetic tree was constructed (Tamura and Nei, 1993) (Figure 1) and the closest relative was identified as *Bacillus subtilis*. The genus *Bacillus* comprises a diverse group of organisms that offer a wide range of nutritional, physiological and metabolic diversity (Sen et al., 2015). *B. subtilis* has been widely used due to its antimicrobial activity to tackle fish pathogens in commercial aquaculture (Wu et al., 2018; Kong et al., 2017). The isolate in the study was biochemically characterised (Table 1) and screened for its anti-microbial activity against pathogens like *E. coli* and *V. harveyi*.

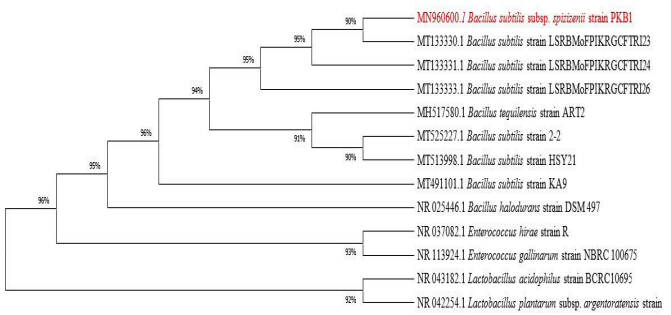

Figure 1: depicts MEGA-X phylogenetic analysis of *Bacillus* sp. isolate. The tree with the highest log likelihood (-4108.98) is shown. Initial tree(s) for the heuristic search were obtained by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) method and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site.

The isolate in this study was able to tolerate temperature up to 45°C and the growth subsided beyond 45°C (Figure 2). Minimal or no growth was reported at lower temperature ranges below 25°C (data not shown). Probiotics can rarely tolerate temperature changes beyond 50°C (Ding and Shah, 2007). Thermal resistance in probiotics is key in determining survival ability since fluctuations in temperature in external or internal environments can lead to cell death (Champagne et al., 1993).

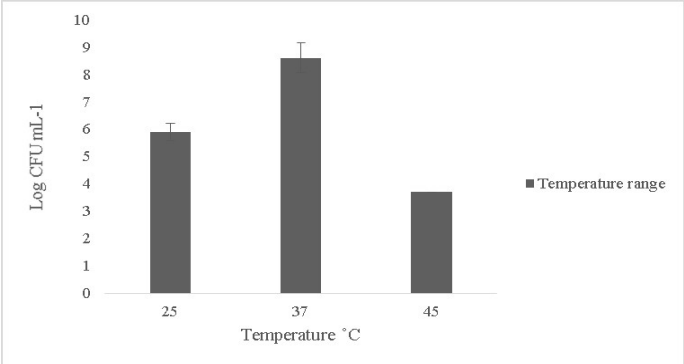

Figure 2: Thermal tolerance of *Bacillus subtilis*-MN960600 on upshifts and downshifts from 37°C *Error bars indicate the standard deviation, the values are expressed as Mean±SD of triplicates

The isolate showed maximum growth at pH 6-7 with 8.1±0.4 Log CFU·mL^{-1} and growth was 7.6±0.1 Log CFU·mL^{-1} at pH 4. No growth was observed at pH 2 and the growth dropped by over 1.1±0.2 Log CFU·mL^{-1} at pH ranges of 8-10 (Figure 3). *B. subtilis* is able to tolerate varied ranges of pH and the optimum pH showing maximum activity ranges between 5 to 7 (Cotter and Hill, 2003). *Bacillus* sp. are known to tolerate acidic conditions (pH 2.5) and such candidates are suitable to be employed as starter cultures as they are able to survive the internal gastric environment and possess lesser or equal enzymatic activities (Krulwich et al., 1985; Jeon et al., 2017). The ability of the isolate to tolerate wide ranges of pH fluctuations exerts its

antimicrobial activity in the extreme pathogenic environment.

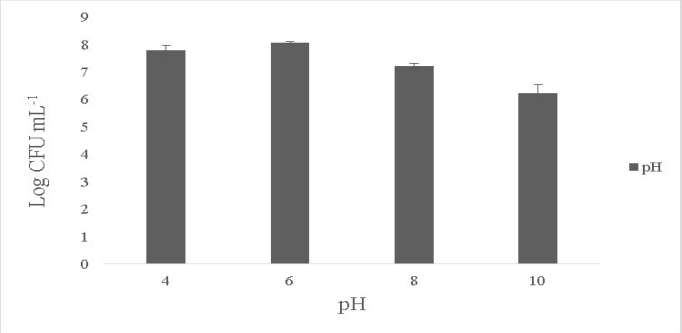

Figure 3: Effect of pH on the survival of the *Bacillus subtilis*-MN960600. *The data are means of triplicate experiments and error bars indicate standard deviations.

Another probiotic characteristic is the ability to survive in the internal gastric environment (Ding and Shah, 2007). The isolate was checked for its ability to survive in simulated artificial gastric juice for time duration of 2, 4 and 6h mimicking the estimated time to be spent in the gastrointestinal tract (Figure 4). The final growth observed after 6h was 7.0±0.31 Log (CFU·mL^{-1}).

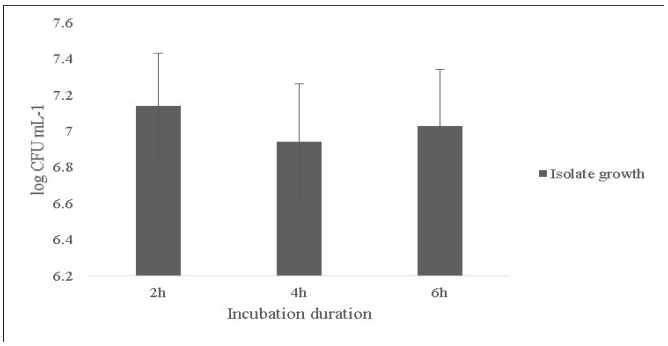

Figure 4: Survival of the *Bacillus subtilis*-MN960600 in artificial gastric juice at durations 2h, 4h and 6h. *Error bars indicate the standard deviation; the values are expressed as Mean±SD of triplicates

Salt tolerance can influence the water and enzymatic activity of bacterial strains and confers high osmotolerance to withstand excessive external osmotic pressure (Adnan and Tan, 2007). *Bacillus* sp. is highly tolerant to salt concentrations ranging from 2% -10% (Ragul et al., 2017). At 2% concentration, growth was 7.98±0.56 Log (CFU•mL^{-1}) and at 10% concentration, growth was found to be 6.98±0.29 Log (CFU•mL^{-1}) (Figure 5). An important facet for a probiotic is its ability to survive the action of toxic metabolites like phenol and organic solvents like toluene. Primary phenols are the by-products of the digestive process. Some probiotics retain added bacteriostatic activity by metabolising aromatic amino acids into phenols (Kumar et al., 2019; Suskovic et al., 1997). In this study, isolate was able to tolerate phenol and toluene at 0.6% concentrations [7.8±0.08 Log (CFU•mL^{-1}) and 8.5±0.09 Log (CFU•mL^{-1})] respectively (Figure 6).

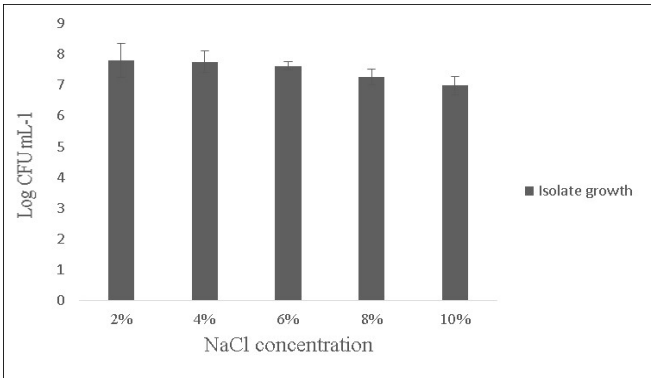

Figure 5: showing the effect of NaCl (2-10%) tolerance on the growth of *Bacillus* subtilis -MN960600. *Error bars indicate the standard deviation; the values are expressed as Mean±SD of triplicates

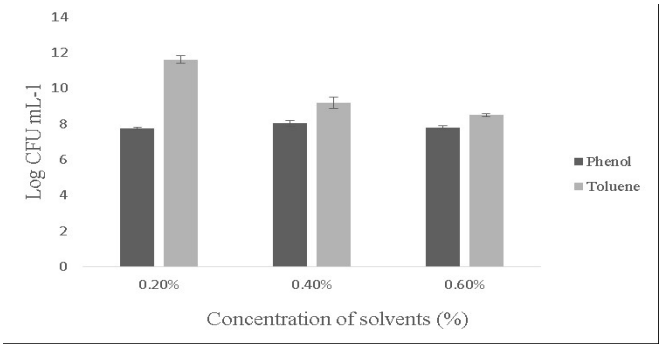

Figure 6: showing the effect of solvents (Phenol and toluene) on the growth of *Bacillus subtilis*-MN960600. *Error bars indicate the standard deviation; the values are expressed as Mean±SD of triplicates

The anti-biofilm forming activity of the isolate was evaluated against *Escherichia coli, Vibrio harveyi, Pseudomonas aeruginosa* and *Staphylococcus aureus* and was found to be 28.8±4.2%, 31.5±4.6%, 34.8±3.1% and 34.4±3.75% respectively (Figure 7). *Bacillus* sp. possess remarkable antimicrobial properties which can be attributed to the production of bacteriocins like fengycin, surfactin, bacilysin, butirosin, macrolactin, bacillaene, difficidin, bacillibactin, lanthipeptides and LCI. Antimicrobial peptides are also produced by genes like LCI, YFGAP and hGAPDH and gene clusters for secondary metabolites (Wu et al., 2018). Bacterial motility is crucial for colonization processes of pathogens. *Bacillus* sp. isolated from a marine niche has been used to decrease *Vibrio alginolyticus* motility and thereby reduce its pathogenicity against *Danio rerio*. The potential lead compounds from *Bacillus* sp. targeting bacterial motility and biofilm inhibition are encouraged to be used in aquaculture. *Bacillus* sp. have a low potential for eliciting antibiotic resistance since they are not involved in horizontal gene

transfer and are unlikely to acquire genes pertaining to antibiotic resistance (Xiu et al., 2017).

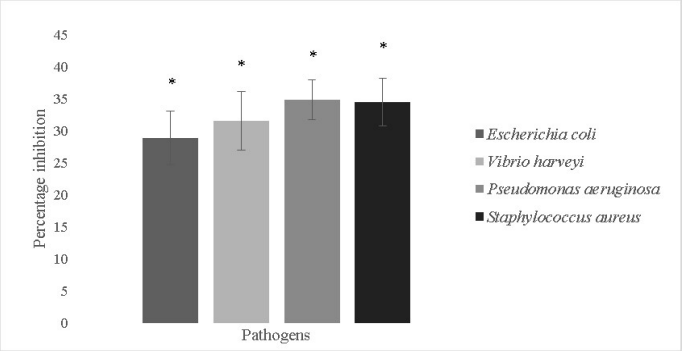

Figure 7: Antibiofilm activity of *Bacillus subtilis*- MN960600 against *Escherichia coli, Vibrio harveyi, Pseudomonas aeruginosa* and *Staphylococcus aureus*. **The error bars indicate standard deviation and statistical significance indicated by * (P<0.05)

Feed conversion ratio (FCR) was lesser compared to the control sample indicating a potential of improved nutrition absorbability. Similarly, other growth parameters like length gain and weight gain percentages were found to be better in probiotic supplemented samples but no significant difference in the growth parameters between the control and treatment groups was noted (Table 2).

Table 2: Growth parameters in *Danio rerio* upon treatment with *Bacillus subtilis* isolate

Parameters	Control	Probiotic	P- value
Feed conversion ratio	0.32±0.07	0.27±0.09*	0.24
Weight gain percentage	12.3±0.08	12.77±0.06*	0.18
Length gain percentage	8.9±0.32	11.413±0.18*	0.43

Note: The values are Mean±SD of triplicates, the statistical significance was verified using independent student t-test and the non-significant (p> 0.05) difference between rows are highlighted using *

Bacillus sp. have been widely used as growth promoters and contribute for improved survival rate (Gatesoupe, 1999). In this study, *Bacillus* sp. was used to assess the survival of *Danio rerio* against fish pathogens as described in the survivorship cure (Figure 8). *Bacillus* spp. improve food absorption by enhancing protease levels, providing resistance against pathogens like *V. harveyi* and luminescent vibrio spp. in both fishes and penieds (Irianto and Austin, 2002; Moriarty, 1999; Lakshmi et al., 2013). *Bacillus subtilis* stimulates mRNA expression of both pro-inflammatory and anti-inflammatory cytokines in dendritic cells of grass carp via cytokine-related pathways (Zhou et al., 2019). *Bacillus amyloliquefaciens* G1 has been used against a wide spectrum of pathogenic *Aeromonas hydrophila* strains by having a protective antimicrobial action in *Anguilla anguilla* (Lu et al., 2011). Furthermore, combination cultures of *Bacillus* sp. have been used to enhance innate immune response in *Perca flavescens,* thereby contributing to lesser risk of disease susceptibility. Similarly, a combination mixture of *Bacillus subtilis* and *Bacillus licheniformis* (BioPlus2B) has been used against *Yersinia ruckeri* infected trout to improve their survival rate (Raida et al., 2003; Salim and Reda, 2015). *Bacillus subtilis* has also been used against *A. hydrophila*, *Edwardsiella ictaluri*, *Streptococcus* sp. in *Cirrhinus cirrhosus*, *Oncorhynchus mykiss*, *Ictalurus punctatus*, *Pangasianodon hypophthalmus*, *Epinephelus summana* and *Oreochromis mossambicus* to improve their survival rate

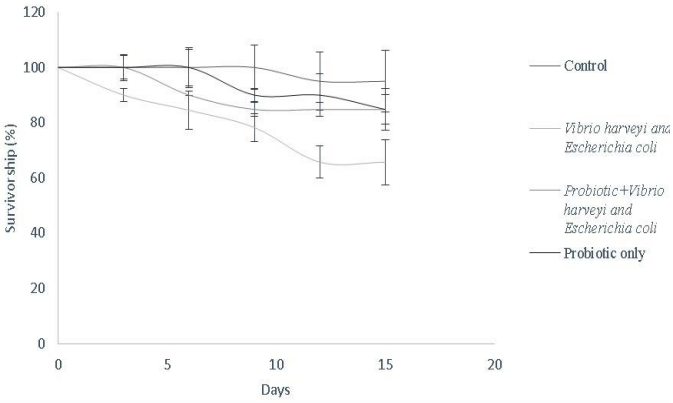

Figure 8: Survivorship curve of *D. rerio* depicting the effect of *B.subtilis*- MN960600 supplementation against pathogen challenge in a total duration of 15 days (Control, *Vibrio harveyi* and *Escherichia coli*, probiotic+*Vibrio harveyi* and *Escherichia coli* and probiotic only). Error bars indicate the standard deviation; the values are expressed as Mean±SD of triplicates

(Kumar et al., 2008; Newaj et al., 2007; Ran et al., 2012). *Bacillus licheniformis, Bacillus pumilus* and *Bacillus circulans* have been used against *Streptococcus agalactiae* and *A. hydrophila in Catla catla*, *Paralichthys olivaceus* and *Oreochromis niloticus* to improve immune health status and disease resistance (Aly et al., 2008; Babdyopadhyaya and Mohapatra, 2009; Cha et al., 2013; Han et al., 2015). *Bacillus* sp. S11, NL 110 and *B. coagulans* have been supplemented to *Penaeus monodon, Ictalurus punctatus, Macrobrachium rosenbergii*, *Cyprinus carpio Litopenaeus vannamei* and *Fenneropenaeus indicus* to improve nutrient digestibility (Queiroz and Boyd, 1998; Rahiman et al., 2010; Rengpipat et al., 1998; Lin et al., 2012; Heizhao et al., 2004). *B. subtilis*, *L. acidophilus*, *S. cerevisiae* have been used in *Paralichthys olivaceus* to improve stress tolerance (Taoka et al., 2006). *Bacillus subtilis* has also been used in *Poecilia reticulata*, *Xiphophorus maculatus* for reproductive enhancement (Ghosh et al., 2007).

CONCLUSION

Our study revealed the promising effect of *Bacillus subtilis* on growth and disease resistance of *Danio rerio*. Dietary supplementation of *Bacillus subtilis* as an eco-friendly alternative to antibiotics gained enormous attention in the fisheries sector. Results of our study confirmed the biocontrol potential of *Bacillus subtilis* against aquaculture pathogens. Widespread research should be conceded out to analyze the species specific interaction of the host and *Bacillus subtilis*.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

AUTHOR CONTRIBUTION

Thejaswi Bhandary was involved in the experimental design, data collection, data analysis and revised this manuscript. Riyaz Ali.L was involved in experimental design, data analysis and drafted this manuscript. Kuppusamy Alagesan Paari conceptualized the idea, involved in funding and revised this manuscript. All authors read and approved this manuscript.

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