Probiotic Properties of *Enterococcus faecium* Isolated from *Gallus gallus domesticus* and Its Anti–Microbial, Anti–Biofilm and Growth Enhancing Potential in *Danio rerio*

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ABSTRACT

This study focused on isolation and characterisation of potential probiotic bacteria from a non–aquatic source (*Gallus gallus domesticus*). *Enterococcus* sp. was isolated from the midgut of *G. gallus domesticus* and was characterised for its ability to survive in artificial gastric juice, trypsin, varying pH and temperature, different concentrations of organic solvents, and was evaluated for its ammonia reduction potential. Notable inhibition of biofilm against *Vibrio harveyi* (41.0±3.2 %), *Escherichia coli* (33.0±4.0 %), *Pseudomonas aeruginosa* (37.8±4.0 %) and *Staphylococcus aureus* (41.7±2.0 %) was observed. The study showed that the isolate improved the survival rate of *Danio rerio* against *V. harveyi* and *E. coli* in challenge studies using survival analysis. The weight and length gains observed were 4.9±0.1 % and 0.3±0.2 % ($p>0.05$), respectively. The use of probiotics from non–aquatic sources can increase the diversity of available probiotics for aquaculture practices.

Keywords: Anti-biofilm activity, Anti-microbial activity, Aquaculture, *Enterococcus faecium*, Probiotic

INTRODUCTION

World aquaculture production stands at over 170 million tonnes, and the fish consumption rate has surpassed meat consumption from all terrestrial animals combined (FAO, 2018). Despite the increasing demand for human consumption and the rising plea for economic profits from non–food products from the aquaculture sector, a major challenge to the fisheries industry comes from the threats caused by viral, bacterial, and fungal infections (Assefa and Abunna, 2018). The extensive use of antibiotics to combat pathogens has resulted in the microbial population acquiring antibiotic resistance (Watts *et al*., 2017). Longstanding farming practice requires suitable alternatives to antibiotics (Balcázar *et al*., 2007). Probiotics which possess similar therapeutic properties are now receiving more attention as potential candidates in managing aquatic health. A probiotic is a cultured product or live microbial feed supplement that beneficially affects the host by improving the intestinal microflora (Fuller, 1989). Some common probiotics used in aquaculture are *Bacillus* sp. (Thankappan *et al*., 2015), *Carnobacterium divergens* (Leisner *et al*., 2007), *Lactobacillus helveticus* (Balcázar *et al*., 2006), *Enterococcus faecium* (Wang *et al*., 2008), *L. acidophilus* (Gioacchini *et al*., 2010), *Vibrio alginolyticus* (Thompson *et al*., 2010), and *Pediococcus acidilactici* (Castex *et al*., 2009). Probiotics conferring resistance to pathogens, extending survival rate and enhancing feed conversion ratio are getting more industrial applications in aquaculture (Balcázar *et al*., 2006; 2007). Other benefits of probiotic supplementation in the aquaculture sector include enhancement of the immune system and reproductive ability in fishes (Ghosh *et al*., 2007; Gioacchini *et al*., 2010;

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Hoseinifar *et al*., 2018). Probiotics possessing the property of higher replication rate naturally colonise the intestinal mucosa and exert beneficial action by the secretion of antagonistic compounds like proteases, amylases, lipases, vitamins, fatty acids, free amino acids, organic acids, bacteriocins, exopolysaccharides and bioactive peptides (Balcázar *et al*., 2006; Kanmani *et al*., 2011a; Kumar *et al*., 2011). Adhesive, co–aggregating and inhibitory properties of probiotics and their secretory products negatively influence the microbial ecology of biofilms formed by pathogens (Sayem *et al*., 2011; Vuotto *et al*., 2014). *Enterococcus* sp., one of the common inhabitants of the intestinal tract has been used as starter cultures, probiotics and as a growth enhancer in aquaculture (Bogut *et al*., 2000; Chang and Liu, 2002). Species of *Enterococcus* such as *E. faecium*, *E. lactis*, *E. faecalis* and *E. hirae* have been used as probiotics and are reported to have reduced mortality rates in both juvenile and adult organisms due to their resistance to wide ranges of pH and temperature, and their ability to synthesize enterocin–like bacteriocins (Wang *et al*., 2008; Hoseinifar *et al*., 2018). This study aimed at isolation, identification and characterisation of a potential probiotic bacterium from a non–aquatic source (*G. gallus domesticus*) and attempted to deduce the functional benefits of the isolated strain in tackling fish pathogens and for growth enhancement in aquatic systems.

MATERIALS AND METHODS

Isolation, identification and biochemical characterisation

A midgut sample (5 g) of *Gallus gallus domesticus* was procured from a poultry farm and brought to the laboratory under sterile conditions. The sample was taken from a Sindhu Assel breed (5–month–old male, weighing around 2 kg).

MRS (De Man–Rogosa–Sharpe) media was used for isolation. The isolate was identified using 16s rRNA sequencing employing forward primer (27F) and reverse primer (1492R) via BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The evolutionary history was inferred in MEGA X using the Neighbor–Joining method and Maximum Composite Likelihood method (Saitou and Nei, 1987; Tamura *et al*., 2004). The isolate was characterised biochemically (Talib *et al*., 2019) and analysed for its antimicrobial (Balouiri *et al*., 2016) and haemolytic activity (Halder *et al*., 2017) following standard protocols.

pH, temperature and salt tolerance assays

Tolerance to pH, temperature and varying salt concentration was characterised following the protocol of Yadav *et al*. (2016) with slight modifications. Briefly, the isolate was exposed to varying pH (2–10), temperature (5–55 ºC), NaCl concentrations (2–10 %), solvent concentrations (2–6 %), and phenol concentrations (0.2–0.6 %). After incubation for 24 h, the culture was plated onto MRS agar plates and the Log ($CFU \cdot mL^{-1}$) was calculated (Zheng *et al*., 2013).

Artificial gastric juice tolerance and trypsin tolerance tests

Artificial gastric juice [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] was prepared according to the protocol of Corcoran *et al*. (2005). All components of gastric juice were added as stock in the media prior to inoculation of the culture. The effect of artificial gastric juice was examined by incubating MRS broth inoculated with 100 µL of overnight grown culture at different time intervals (2, 4 and 6 h). The cell viability was expressed as Log ($CFU \cdot mL^{-1}$) after plating onto MRS agar for 24 h at 37 ºC. Survival percentage of the isolated probiotic under varying concentrations (0.2–0.6 %) of the digestive enzyme trypsin was tested. The isolate was inoculated into MRS broth containing trypsin for a period of 6 h, after which the culture was spread plated on MRS agar at 37 ºC and Log ($CFU \cdot mL^{-1}$) was calculated.

Ammonia reduction test

The ability of the isolate to reduce ammonia was measured using an API ammonia test kit (LR 8600, API). Standard ammonia solution (8 ppm) was prepared using ammonium sulphate. Treatment

groups contained known concentrations of ammonia with 100 µL of isolate. Sterile media containing known concentrations of ammonia without inoculum served as a blank. The effect of probiotic on reduction of ammonia supplemented in nutrient broth was estimated after 24 h and the percentage reduction was estimated using standards prepared using 2, 4, 6 and 8 ppm concentrations of ammonia solution.

Safety assessment and challenge studies

Challenge studies were performed according to the protocol of Wang *et al*. (2008) with slight modifications. A total of 180 healthy *Danio rerio* weighing 0.2–0.25 g and approximately 3 cm in length were segregated into groups: Group 1 was treated only with *Enterococcus* sp. isolate; Group 2 was treated with pathogens (*Vibrio harveyi* and *Escherichia coli*); Group 3 was treated with the two pathogens along with the *Enterococcus* sp. isolate; Group 4 was treated only with saline. In the challenge studies (Group 2), 5 Log CFU of the isolate was supplemented to determine the survival rate of *D. rerio* upon challenge with 8 Log CFU of *E. coli* and *V. harveyi*. Survival parameters analysed after the study period included cumulative mortality and relative percentage survival (RPS) (Amend, 1981). Growth parameters of the fish, including feed conversion ratio, weight gain percentage and length gain percentage were monitored for a period of 15 days in both the control and the treatment groups.

Antibiofilm activity

Crystal violet assay was performed according to the protocol of Costa *et al*. (2018) with minor modifications. *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio harveyi* and S*taphylococcus aureus* were grown on nutrient broth in a microtiter plate and were allowed to form biofilm. Cell–free supernatant (100 µL) of the isolate was added and the plate was incubated for 4 h at 37 ºC. Antibiofilm activity was measured using a microplate reader (iMark™, Biorad) set at 450 nm and the percentage inhibition was calculated.

Percentage inhibition
= 100 – [(A450 in experimental well with isolate/ A450 in control well without isolate) × 100].

Statistical analysis

The results are presented as mean±SD of triplicates. Antibiofilm activity of the isolate was evaluated using one–way ANOVA (SAS 9.4) and independent t–tests were conducted on the growth parameters including feed conversion ratio, weight gain and length gain percentage, with $p<0.05$ being considered significant. A Kaplan–Meier plot and survivorship curve were applied to the survival data in the challenge study.

RESULTS AND DISCUSSION

The genus *Enterococcus* contains a wide variety of Gram–positive bacteria, but very few have been documented as probiotics (Baccouri *et al*., 2019). However, new varieties like *Enterococcus durans* and *Enterococcus hirae* are being incorporated into studies for their role as feed additives (Adnan *et al*., 2017; Li *et al*., 2018). The isolated probiotic in this study was identified as *Enterococcus* sp. using 16s rRNA sequencing and the partial sequence was deposited in NCBI (Accession number MN044445.1). Based on percentage identity and total score, the closest match was found to be with *Enterococcus faecium*. The percentage identity was 100 % for all hits, with a maximum score of 985/985 on a 100 % query cover (Figure 1). Biochemical profiling showed the isolate to be Gram–positive, cocci shaped, non–motile, catalase–negative, non–haemolytic, with high coagulation efficacy and strong antimicrobial activity. The isolate was able to grow in 0.6% phenol and tolerated up to 0.6% trypsin treatment. *E. faecium* is a proven probiotic and has been evaluated for its effects in stimulating the immune response in both human and animal models (Marteau *et al*., 2001). Antimicrobial activity of *E. faecium* has been evaluated against major fish pathogens such as *Edwardsiella tarda*, *Pseudomonas* spp., *Vibrio parahaemolyticus* and *Aeromonas* spp. (Kumar *et al*., 2011). *E. faecium* has also been used to improve immune response in *Paralichthys olivaceus* by increasing myeloperoxidase and lysozyme activities (Lee *et al*., 2013). The present study utilised the antimicrobial potential of *Enterococcus* sp. isolate against fish pathogens such as *Escherichia coli*, *Vibrio harveyi*, *Pseudomonas aeruginosa* and *Staphylococcus*

aureus. The cell mass as well as the cell–free supernatant of *Enterococcus* sp. exhibited a clear zone of inhibition against the pathogens.

The isolate in this study was able to tolerate temperature up to 40 ºC, and the survival rate gradually declined when stored beyond 40 ºC (Figure 2). Minimal or nil growth was reported at lower temperature ranges of 5–15 ºC (data not shown). Very few probiotics have been reported to tolerate temperature changes above 50 ºC (Ding and Shah, 2007). Thermal resistance is an important facet for probiotic characterisation since fluctuations in temperature in external or internal environments can cause cell injury or cell death (Champagne *et al*., 1993). Temperature tolerance of any probiotic strain is an essential trait for its application as a forage additive (Mao *et al*., 2020).

Enterococcus faecium can tolerate a pH range of 4–10 (Chauhan and Singh, 2019). The isolate showed evident growth at pH of 4–7, and maximum growth at pH 6–7 with 8.2±0.2 Log CFU·mL^{-1} and minimum growth (7.1±0.1 Log CFU·mL^{-1}) at pH 4. Nil growth was observed at pH 2 and growth declined at pH ranges of 8–10 (Figure 3). Adaptability and ability of *Enterococcus* strains to grow at varied pH and temperature conditions have been reported previously (Morandi *et al*., 2005). To be classified as a probiotic it is essential for the isolate to survive in the gastric environment (Ding and Shah, 2007). The isolate was tested for its ability to survive in simulated artificial gastric juice for a time duration of 2, 4 and 6 h, mimicking the amount of time to be spent by the culture in the GI tract (Figure 4). The final growth observed after 6 h was 4.9±0.6 Log (CFU·mL^{-1}).

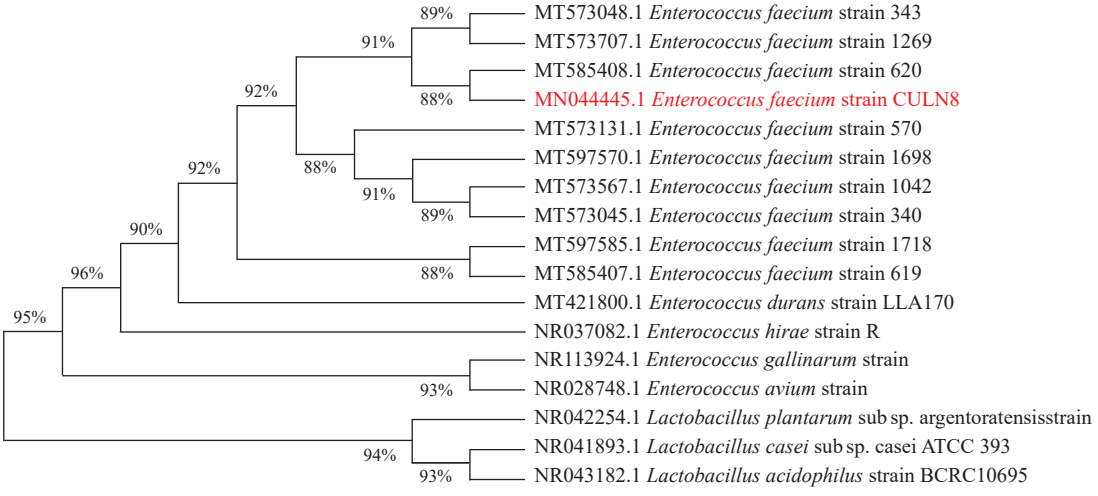

Figure 1. Molecular phylogenetic relationship of the *Enterococcus faecium* strain CULN8 and other *Enterococcus* spp. The evolutionary history was inferred using the Neighbor–Joining method. The optimal tree with the sum of branch length = 0.528 is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. This analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1,608 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

Presence of resistance genes such as cytolysin and enterococcal surface protein could aid *Enterococcus* to combat oxidative stress (Domann *et al*., 2007). This could be the reason for the tolerance factor of Enterococcus to combat extreme environment during gut colonisation in the gastric micro-niche.

Further characterisation was done based on the ability of the strain to tolerate different concentrations of NaCl and also solvents like phenol, acetone and toluene. Growth at higher concentrations of NaCl (10%) was 7.22±0.07 Log (CFU·mL^{-1}) and nil growth was reported at 6% concentrations of toluene and chloroform (Figures 5 and 6).

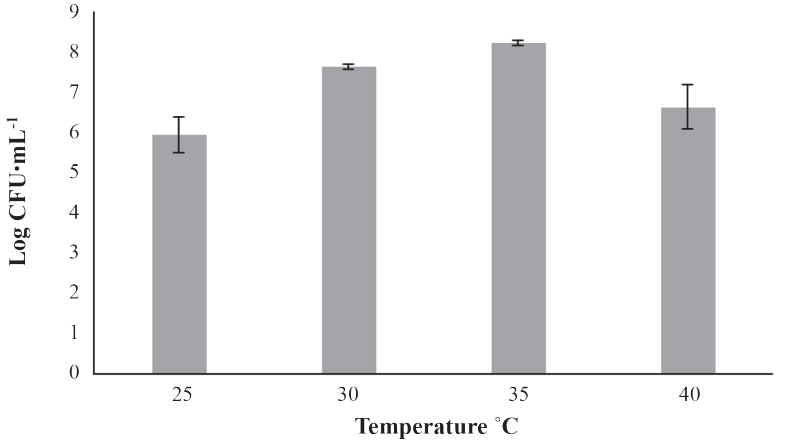

Figure 2. Thermotolerance of the *Enterococcus* sp. to temperature upshifts and downshifts (±5 ºC); error bars indicate standard deviations.

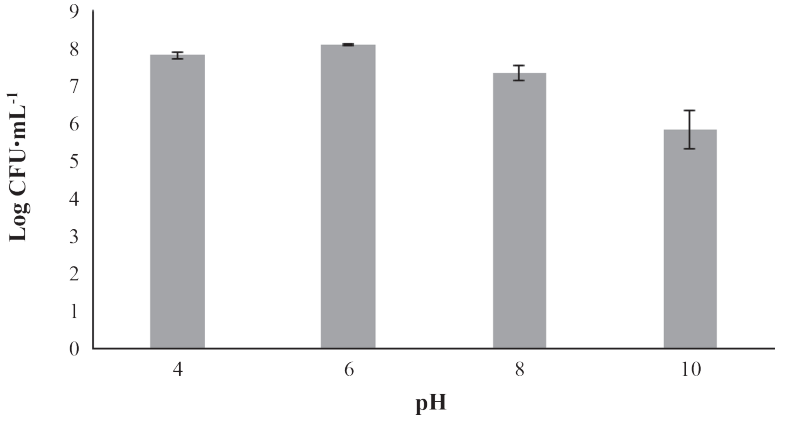

Figure 3. Effect of varying pH on survival of the *Enterococcus* sp. in MRS broth; error bars indicate standard deviations.

Ammonium, nitrite and other toxic metabolites originating in the feces, underused feed, and waste in aquatic systems can result in huge losses to aquaculture (Gross *et al*., 2004). Probiotics like Bacillus licheniformis, *Nitrobacter* sp. and *Nitrosomonas* sp. have been used to decrease levels of ammonia, starch and protein from underutilized feed in wastewater (Feng *et al*., 2011; Padmavathi *et al*., 2012). Application of the isolate for water quality improvement was assessed by testing the ammonia reduction potential under *in vitro* conditions. Probiotics have been proven effective as an eco-friendly additive to improve water quality by degrading ammonia (Guo *et al*., 2016). The ammonia in the test samples was found to be around 4.3±0.6 ppm, whereas the concentration of ammonia remained the same in the control samples.

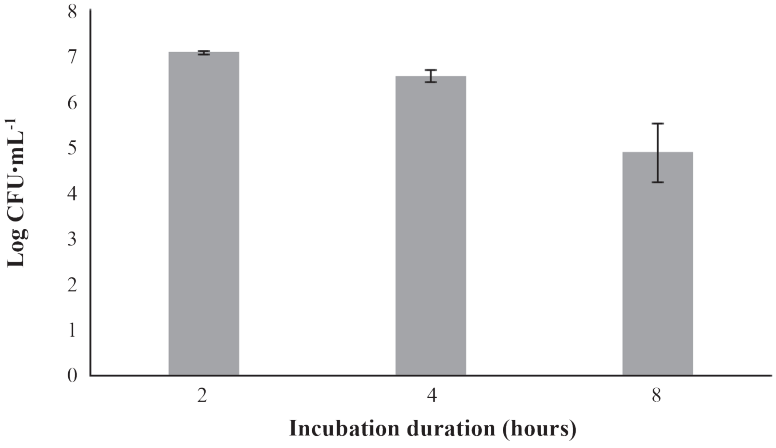

Figure 4. Survival of *Enterococcus* sp. in simulated gastric juice at different time points (2 h, 4 h and 6 h); error bars indicate standard deviations.

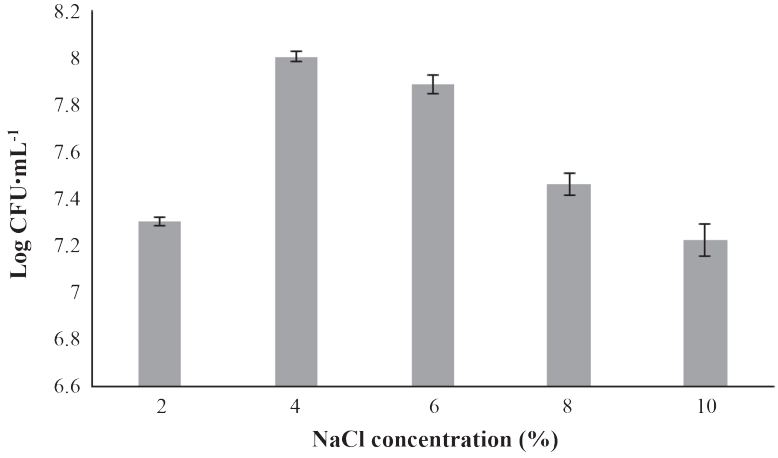

Figure 5. NaCl tolerance of the *Enterococcus* sp. isolate. The culture was inoculated in different concentrations of NaCl (2–10%). Error bars indicate standard deviations.

The antibiofilm activity of the *Enterococcus* sp. was measured based on its ability to inhibit the growth of pathogens. The percentage inhibition against *Escherichia coli* was 33.0±4.0 %. Similarly, the percentage of inhibition against *Vibrio harveyi*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* was 41.0±3.2 %, 37.8±4.0 % and 41.7±2.0 %, respectively ($p<0.05$) (Figure 7). The results suggest that the isolated culture is a potential biofilm inhibitor. Certain compounds like enterocins secreted by *E. faecium* have been reported to suppress the growth of *Vibrio* and *Aeromonas* spp. (Kanmani *et al*., 2011b). Secretory proteins such as enterocin A,enterocin B, enterocin P–like bacteriocin extracted from *E. faecium* JCM 5804T inhibit the growth of pathogens like *Clostridium perfringens*, *Escherichia coli*, *Salmonella enteritidis*, *Listeria monocytogenes* and *Clostridium difficile* (Park *et al*., 2003). Higher antibiofilm activity can also be attributed to the organic acids produced by the probiotic (Kanmani *et al*., 2011b; Sharma *et al*., 2017).

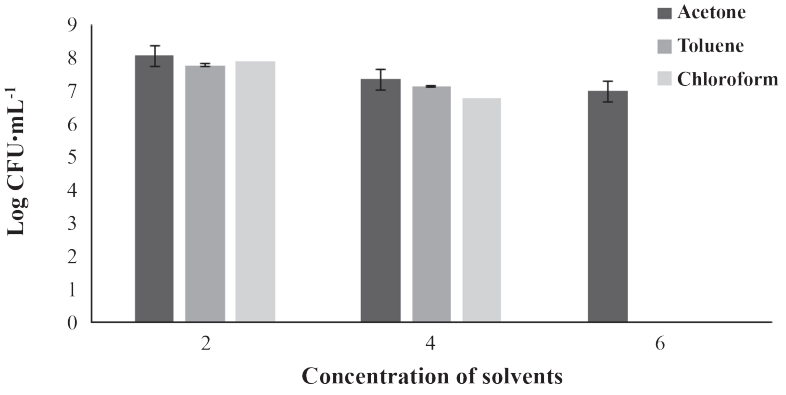

Figure 6. Tolerance to various concentrations (2-6 %) of acetone, toluene and chloroform by *Enterococcus* sp. isolated from *Gallus gallus domesticus*. Error bars indicate standard deviations.

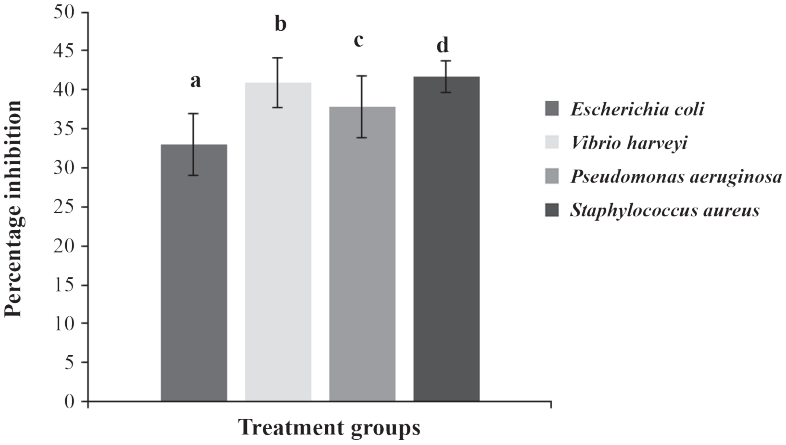

Figure 7. Antibiofilm activity of *Enterococcus* sp. against indicator strains presented as percent inhibition against *Escherichia coli*, *Vibrio harveyi*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Different letters above error bars show significant difference.

The Kaplan–Meier survival plots shown in Figure 8 indicate initial mortality in challenge groups; thereafter, the survival rate in challenge groups with probiotic supplementation was higher compared to challenge alone. The survivorship curve for probiotic+challenge showed the same trend with a lesser slope compared to the challenge group ($p<0.05$) (Figure 9). The relative percentage survival (RPS) in the probiotic+challenge treatment was found to be 45.2 %. Growth parameters such as FCR, length gain and weight gain were not significantly different ($p>0.05$) between the control and treatment group (Table 1).

Enterococcus faecium isolated from aquatic (Chauhan and Singh, 2019) and non–aquatic sources (Wang *et al*., 2008) has been used in enhancing the survival rate of *Cyprinus carpio* and *Oreochromis*

Table 1. Growth performance (mean±SD) of *Danio rerio* treated with *Enterococcus* sp.

Parameters	Control	*Enterococcus* supplemented group
Feed conversion ratio	2.19±0.03	2.02±0.07
Weight gain percentage	3.47±0.10	4.89±0.06
Length gain percentage	0.60±.06	0.87±0.15

Note: Non–significant difference was observed between control and treated group.

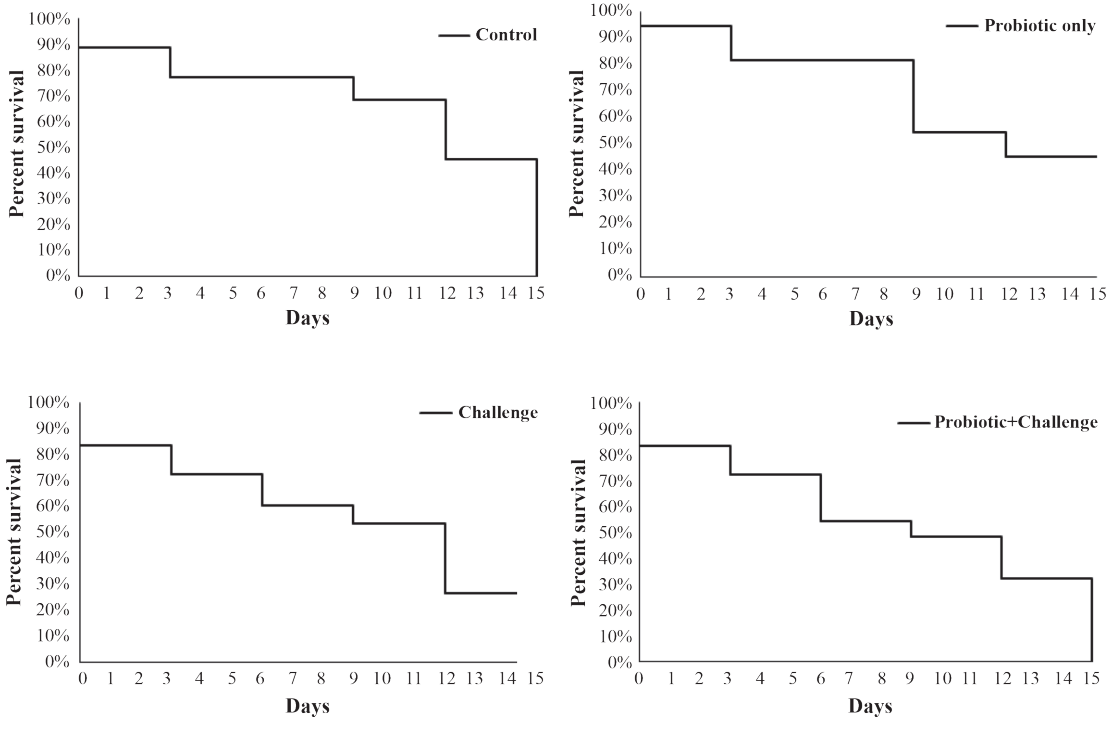

Figure 8. Kaplan–Meier survivorship curves (survival probability over time) for *Danio rerio* after challenge with *Escherichia coli* and *Vibrio harveyi* (survivorship with both pathogens combined). *Enterococcus* sp. isolate was supplemented in Challenge+probiotic group prior to bacterial challenge.

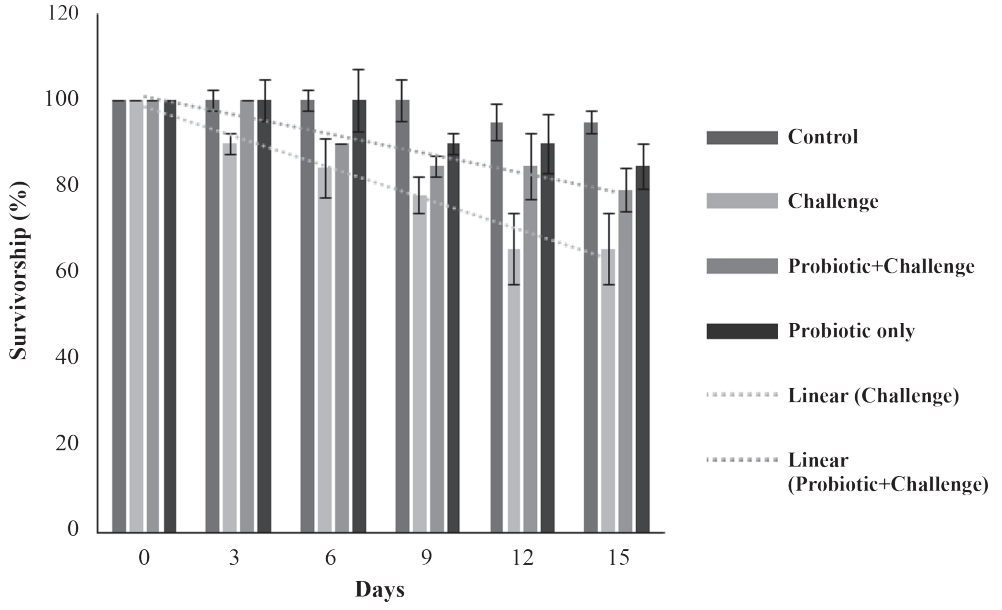

Figure 9. Survivorship curve of *Danio rerio* upon probiotic supplementation in challenge trail for 15 days (Control, challenge, probiotic + challenge and probiotic only).

niloticus. Immunoprotection by probiotics could occur by a mechanism such as bacteriostasis, which plays an important role in the determination of the dominant bacterial communities within the intestine (Tulumoglu *et al*., 2013). The use of *Gallus gallus domesticus* as the probiotic source was an attempt to analyse the potential of strains from non–aquatic sources in aquaculture as an aid for improving the efficiency of already available probiotics. The study promotes the use of probiotics isolated from non–aquatic sources to be used as a potential probiotic supplements in aquaculture sectors. This study highlights the antimicrobial, antibiofilm and growth–enhancing properties of *Enterococcus* sp. and promotes its usage for sustainable aquaculture as a potential alternative for better aquaculture practices.

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