Effect of probiotic bacteria isolated from the gut of *Dawkinsia filamentosa* (Valenciennes, 1844) on survival of *Channa striatus* (Bloch, 1793) fingerlings challenged with *Aeromonas hydrophila*

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ABSTRACT

Probiotics are living microorganisms that confer positive effects on the health of the digestive system when incorporated with feed in a defined dosage and help to stimulate the immune response in fish against virulent fish bacterial pathogens. Therefore, focusing on the gastrointestinal health of fish is more essential. This study aimed to determine the antagonistic activity of bacteria isolated from the gut of freshwater fish *Dawkinsia filamentosa* and the immune response in *Channa striatus* challenged with *Aeromonas hydrophila*. In total, 15 bacterial strains with different colony morphology were isolated; further screened for antibacterial activity using the agar overlaying and well diffusion method against *Aeromonas hydrophila, Pseudomonas aeruginosa, Vibrio cholerae*, and *Staphylococcus aureus*. In the preliminary screening, four isolates showed considerable activity. Among them, DF12 was found to have prominent antagonistic activity against fish pathogens. In the agar overlaying method, the strain DF12 showed the highest inhibitory zone of 8 mm against *Pseudomonas aeruginosa, Staphylococcus aureus, Aeromonas hydrophila* and 6 mm against *Vibrio cholerae*. In the well diffusion method, the isolate DF12 showed the highest activity against *S. aureus* (10 mm), *V. cholerae*, and *P. aeruginosa* with 8 mm each, the active bacterial strains were identified up to genus level as *Enterococcus* sp. (DF6), *Lactobacillus* sp. (DF8), and *Bacillus* sp. (DF12) based on morphological and biochemical characterization. The isolate *Bacillus* sp. possesses a high ability to tolerate low to high pH range of 4 to 8 and the bile salt concentration of 0.0, 0.15, and 0.30%, This present investigation substantiates that the fish gut can be explored as a promising source of antagonistic bacteria and develop an immune response that produces a wide spectrum of bioactivity. These probiotic bacteria have warranted much research interest because of their broad specificity on antibacterial potential and even immunostimulant effects.

Keywords: *Dawkinsia filamentosa*, Gut probiotics, Antagonistic activity, Immune response, *Channa striatus*

1. INTRODUCTION

Aquaculture has evolved as the fastest growing sector as it offers a high source of protein for humans all over the world [1]. Fisheries play a vital role in food security and livelihood and are a source of income and social development in developing countries. Developing technological advances and increased demands for fish as a source of animal protein are the main reasons for the intensification of fish culture practices. Because of the expansion of the industry, the cultural methods have become more intensive for producing higher yields [2, 3]. However, the development of aquaculture practices requires cultivation at high densities, which has caused significant damage to the environment due to discharges of concentrated organic wastes, that deplete dissolved oxygen in ponds, giving rise to toxic metabolites (Such as hydrogen sulfide, methane, ammonia, and nitrites), that often are responsible for mortality [4, 5]. Moreover, under these conditions of intensive production, aquatic species are subjected to high-stress conditions, increasing the incidence of diseases and causing a decrease in productivity [6]. Outbreaks of microbial infections have caused devastating economic losses worldwide, significant stock mortality has been reported due to poor environmental surroundings on farms, unbalanced nutrition, generation of toxins, and genetic factors [7]. In the modern cultural method, prevention and control of fish diseases have focused on the use of chemical drugs and veterinary medicines, especially antibiotics, which generate significant risks to public health by promoting the selection, propagation, and persistence of bacterial strains [8, 9 & 10].

Biological disease control in aquaculture is one of the best approaches to infectious disease management. Probiotics are bacterial strains that are non-pathogenic to fish. The other definition of probiotics is that live microorganisms are administered to fish to develop a beneficial effect on health, growth factor, immune response, and nutrition for a healthy gastrointestinal system [11]. After being treated fish with probiotics, they start to multiply themselves to occupy the gastrointestinal tract of the fish, enhance normal microflora, and maintain microbial balance in the fish. Many probiotic products have been researched as evidenced by their efficacy in aquaculture, beneficial bacterial inoculums that were species-specific probiotics have become widely available to the aquaculture industry, and these preparations have been refined to have a more effective function as applied probiotics [12]. The application of new analysis methods, including molecular methods, for the evaluation of probiotic products and for *in vivo* validation, was expected to significantly improve both the quality and functional properties of probiotics [13]. As a result, the probiotic usage in the aquaculture sector is an excellent strategy for the management of infectious bacterial diseases and to replace the chemicals, drugs, antibiotics, and chemotherapeutics.

2. MATERIALS AND METHODS

2.1 Fish collection: 10 live fish samples were collected from 3 to 4 m depth of Thamirabarani River (Latitude: 8.7597^{o} N and Longitude: 77.8183^{o} E), Tirunelveli District using a cast net and was transported to the Centre for Aquaculture Research and Extension (CARE), Department of Zoology, St. Xavier's College (Autonomous), Palayamkottai, through plastic containers. Overcrowding and rough handling of fish were avoided to reduce the stress.

2.2 Collection of Gastrointestinal tract of fish: To isolate a stable aerobic, heterotrophic bacterial population from the gastrointestinal tracts, the collected fishes were starved for 24 h to make their intestinal tract clear and also to eliminate the bacteria that were transit in nature. After the starvation period (24 h), the fishes were sacrificed and the gastrointestinal tract was removed aseptically. 1 g of the gut was well macerated using mortar and pestle with sterile distilled water (10 ml) and then centrifuged at 3000 rpm for 15 minutes. The homogenate solution was used as inoculums.

Figure1. Dissection of gastrointestinal tract of *Dawkinsia filamentosa* (Valenciennes, 1844)

2.3 Isolation of bacteria - Serial dilution: Homogenized gut sample of experimental fishes was serially diluted up to 10^{10} dilution by adding 1 ml of the sample into a 9 ml of dilution blank, mixed well, and from the first dilution, 1ml was transferred into another 9 ml to get dilution 10^2, Likewise up to 10^{10} dilutions were made with sterile and fresh 1ml pipette. Diluted samples (1ml) were inoculated on sterilized nutrient agar (Himedia, India) using the pour plate method. Then the plates were incubated at 37°C for 48 h. After incubation, the number of bacterial colonies in each plate was counted. The bacterial colonies were divided into different types according to the colony characteristics such as shape, size, elevation, structure, surface, edge, colour and opacity, and the number of colonies of each recognizable type was also counted and recorded. Representatives of each colony type were then streaked on additional Nutrient plates repeatedly until pure strains were obtained. Pure strains on Nutrient slants were kept at 4°C for further study.

2.4 Test microorganism: The test bacterial fish pathogens viz., *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Aeromonas hydrophila* were collected from the Centre for Aquaculture Research and Extension (CARE), St. Xavier's College (Autonomous), Palayamkottai, Tirunelveli.

2.5 Antagonistic Activity using Agar Overlaying Method: Antibacterial activities of 15 isolated strains were tested by the agar overlaying method [14]. Macrocolonies of isolated bacteria were cultured on Mueller-Hinton agar (1.2% agar) plates by inoculating on 18 h old nutrient broth culture with a micropipette and incubated at 37ºC for 24–48 h. A pathogenic strain of *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Aeromonas hydrophila* was grown separately in nutrient broth and incubated at room temperature for 18 h. 100µl of the culture was suspended in 100 ml of Mueller-Hinton agar (0.7% agar) maintained at 40-45ºC and poured immediately over the macrocolonies of the test culture on the Mueller-Hinton agar. The plates were incubated for 24 h at room temperature. The clear zone around the macrocolony of the bacteria was measured (Edge of the macrocolony to end of the zone).

2.6 Evaluation of antibacterial activity using the Well diffusion method: Antibacterial activities of isolated bacterial strains (cell free supernatant) were evaluated using the well diffusion method on Mueller-Hinton agar (MHA) by Jahangirian *et al.*, [15]. *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Aeromonas hydrophila* were used as references for the antibacterial assay. Mueller-Hinton Agar plates were seeded with bacterial strain under aseptic conditions; active strains were inoculated in 20 ml of nutrient broth, then the cultures were centrifuged at 10,000 rpm for 10 minutes to get cell free supernatant. The collected supernatant was concentrated, from which 50 µl were loaded onto the well (diameter = 8mm) and incubated at 37°C for 24 h. After the incubation period, the inhibition zones were measured in millimeter (mm) from the edge of the well to the end of the clear zone. The DMSO was used as negative standards while Streptomycin 10mcg and Tetracycline 10mcg were used as positive standards.

2.7 pH tolerance test: Acid tolerance of selected bacteria was investigated with nutrient broth with different pH including 3, 4, 5, 6, 7, 8, and 9 which were prepared using 1% HCl and 1 N NaOH and divided into universal bottles following the method of Samelis *et al.*,[16]. The broth media along with control bottles were autoclaved at 121°C for 15 min and then inoculated with an overnight culture of the selected strains in nutrient broth and incubated at room temperature. Optical density (OD) was measured by a UV-spectrophotometer at 600 nm after 4 h incubation to calculate the growth rate of bacteria. The viability of the isolates was also assessed by duplicate inoculation on nutrient agar [17, 18 & 19].

2.8 Bile salt tolerance: Bile salt tolerance was further tested in nutrient broth which included 0.0, 0.15, and 0.3% (w/v) Oxgall bile salt. Duplicate bottles of nutrient broth containing filtered different concentrations of bile salt were inoculated with 30 µl of cultured strains and incubated at room temperature. The growth rate was assessed by measuring the optical density with a UV spectrophotometer at 600 nm after 4 h incubation [17, 18 & 19].

2.9. Identification of active strains: Based on the antagonism against fish pathogens; the tolerance towards the pH and bile salt the active strains were selected for the feed mixture. These active strains were identified up to genus level by biochemical characterization following Bergey's manual of systematic biology [20]

2.10 Fish Feed preparation: Experimental semi-solid feed were prepared separately using known quantities of ingredients; Fish meal, Jawala, and Anchovy fish were purchased from the local fish market and washed in tap water and boiled at 60ºC, then dried under the Sun for 30 days and after drying, powdered using the mixer and sieved to the required size. The protein content of this fish meal was 40%. Flours: wheat flour, soybean flour, rice flour, and tapioca flour were used. Rice flour

constitutes 8g, wheat flour 9g, soy flour 24g, and 10g of tapioca flour which was used as a binder to improve the stability of the feed. Sunflower oil of 1ml was also used in fish feed formulation. Selected ingredients were powdered and sieved to get fine particles of uniform size. Ingredients were then weighed according to the formulation and hand-kneaded by adding a sufficient quantity of distilled water and finally made into dough. Then the probiotic pellets of selected strains were mixed with the feed whereas the control feed was without the probiotics. When compared with live feeds, semi-moist feeds were widely used in experiments on *C. striatus* because of their high feed conversion efficiency, easy preparation, less consumption, and easy digestion [21].

2.11 Experimental design: *C. striatus* with an average weight of 4 ± 0.5 g were selected and reared in a cement tank of 600 litre capacity before the experiment. Fifteen fish were introduced into each tank of 130 litre capacity. The experimental setup was divided into 4 groups namely control (I), DF6 (II), DF8 (III), and DF12 (IV). The test fish were fed with the control feed without probiotics for 20 days. After 20 days, the probiotic mixed feed was provided for another 45 days except for control. Tanks were cleaned and refilled with fresh water every 10 days.

2.12 Disease Challenge Study: The disease challenge study was done on the 45^{th} day by injecting pure culture of *A. hydrophila* at the load of 7.5 x 10^6 CFU/ml intra-peritoneally (IP) [22]. To observe the clinical signs of the disease and mortality rate, all groups were kept under observation for 7 days. To finalize the fact of the death of the experimental fishes the mortality was recorded. The results were computed and graphed using Microsoft Office Excel 2013.

3. RESULTS

3.1 Bacterial density: In this study, the total bacterial density in the gut of *Dawkinsia filamentosa* was enumerated to be 118 x 10^4 CFU/ml. From the obtained bacterial cultures, 15 bacterial strains were selected based on different colours and morphology and assessed for antibacterial screening against four fish bacterial pathogens. All the selected bacterial strains were given designated codes.

3.2 Antibacterial activity using agar overlaying method: The isolates DF12, DF11, DF9, and DF2 showed considerable antagonistic activity, DF12 showed the highest level of activity against *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Aeromonas hydrophila*, the zone of inhibition was 6, 8, 8, and 8 mm respectively. A moderate level of activity was found in DF11 against *V. cholerae*, *P. aeruginosa*, *S. aureus*, and *A. hydrophila*, the zone of inhibition was 2, 4, 2, and 2 mm respectively. The strain DF9 and DF2 showed a minimal level of activity against *V. cholerae* and *P. aeruginosa* with the inhibition zone of 3, and 2 mm; 4, and 4 mm respectively. Antibacterial activities of gut bacteria from *Dawkinsia filamentosa* using the agar overlaying method are given in Table 1.

3.3 Antibacterial assay using well diffusion method: Cell-free supernatant of 15 selected strains was further examined using the well diffusion method. The highest level of activity was observed in isolate DF12 against *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Aeromonas hydrophila*, the zone of inhibition was observed as 8, 8, 10, and 4 mm respectively. A moderate level of activity was observed in the DF6 and DF8 against *V. cholerae*, *P. aeruginosa*, *S. aureus*, and *A. hydrophila*, the zone of inhibition was observed as 4, 2, 8, and 4 mm; 4, 10, 2, and 3 mm respectively. Antibacterial activities of gut bacteria from *Dawkinsia filamentosa* using the well diffusion method are given in Table 2. The isolates which showed prominent activity in both agar overlaying and well diffusion methods were selected and checked for their pH and bile salt tolerance which are considered major criteria for the selection of effective probiotics.

3.4 pH tolerance: The isolated strains were tested for their tolerance to different pH levels; 4 h culture was examined through a UV-spectrophotometer. The strain DF12 showed the highest growth rate at pH 6 and pH 7, when the pH level decreases below 5 and increases above pH 7 the growth rate gets fluctuates. Whereas the strain DF6 and DF8 showed better growth in pH 6 and 7 but pH levels lower than 5 and greater than 8 showed a very low growth rate. Isolate DF12 only showed viability at pH 3 and pH 9 whereas the DF6 and DF8 did not show any growth at pH 3 and pH 9 (Graph 1). The obtained results showed that the pH could remarkably affect the growth of selected isolates.

3.5 Bile salt tolerance: Bile salt tolerance of isolated culture in different percentages (0.0, 0.15, and 0.3%) at 4 h incubation period was recorded by UV- spectrophotometer for 4 h cultures. At 0% bile concentration, all the strains showed a good growth rate followed by 0.0%, at 0.15 concentration moderate growth was observed, but at 0.30 % the growth rate of DF6 and DF8 was very low. Remarkably the isolate DF12 only showed moderate growth in 0.30% bile concentration. As bile salt concentration increased, the bacterial growth decreased significantly. Furthermore,

cultures showed different abilities to survive and grow in bile salt (Graph 2).

3.6 Survivability of *Channa striatus*: Based on the above biological activity, DF6, DF8, and DF12 were selected for the disease challenge study, after the experimental period (feed trial) of 45 days; the treated fishes were injected with *Aeromonas hydrophila*. While comparing the survival rate of the experimental group with the control group, the highest survival rate was observed in DF12 (IV) the survival rate was 86.66%, followed by DF12, DF8-III showed a moderate level of survival rate of 73.33%, and the lowest survival rate was observed in DF6-II the percentage was 66.66 %. The control group showed only a 13.33% of survival rate.

3.7 Biochemical characterization of active isolates: The isolated active bacterial cultures were identified to be gram-positive (rod and cocci). The gram-positive cocci (DF6) resemble *Enterococcus* sp. and the rod shape gram-positive bacteria resemble *Lactobacillus* sp. (DF8) and *Bacillus* sp. (DF12) (Table 3). The results revealed that *Enterococcus* sp. (DF6), *Lactobacillus* sp, and *Bacillus* sp. were the most abundant gut-associated probiotic bacteria in fishes *Dawkinsia filamentosa*. The presence of *Bacillus* sp. was attributed to maintaining a healthy digestive system and protecting the host from the diseases.

4. DISCUSSION

Probiotics should be normal inhabitants of the host and able to survive and grow at the site of application while exerting their beneficial effect. Increasing the demand for eco-friendly aquaculture, the use of probiotics as immuno-stimulants has received more attention. Probiotic usage can improve the immunity of cultured animals against pathogenic organisms and induce growth performance. In the present study, 15 different bacterial colonies were isolated from the gut of *Dawkinsia filamentosa* belonging to the Cyprinidae family. In which the isolates such as DF1, DF6, DF8, DF12, and DF15 showed considerable activity. Among them, DF12 showed prominent activity with the highest inhibition zone of 8 mm against *Staphylococcus aureus*, *Aeromonas hydrophila*, and *Pseudomonas aeruginosa*. By using the morphological and biochemical characterization the isolate DS12 was identified as *Bacillus* sp. Similarly, Vijayabaskar and Somasundaram [23] reported that the probiotic bacteria *Bacillus subtilis* isolated from the gut of *Oreochromis niloticus* belonging to the Cichlidae family showed the highest antibacterial activity with the inhibition zone of 8mm against *Staphylococcus aureus*. Bacterium belonging to the genus *Bacillus* is widely used as a probiotic because they are capable of spore formation and are extremely resistant to physical and chemical effects and this determines its longevity in the environment [24, 25].

Vendrell *et al.*, [26] reported that the great interest in the use of probiotics in aquaculture, probiotics *B. subtilis*, and *Lactobacillus plantarum* have been shown to improve growth performance and modulate intestinal microbiota in different fish species Similar to their report, in our study the highest activity was observed in *Bacillus* sp. followed by, *Lactobacillus* sp., further showed higher survival rate and immune response on freshwater air-breathing fish *Channa striatus* challenged with *Aeromonas hydrophila*. In this study, the *Bacillus* sp., (DF12) has a wide range of activity against the selected fish pathogens, especially against *A. hydrophila*, which was found to be more virulent fish pathogens that cause the epizootic ulcerative syndrome, fin rot, tail rot, and hemorrhagic septicemia, in Indian major carps [27].

Khanmohammadi Otaghsara *et al.*, [28] reported that the *Lactobacillus acidophilus* isolated from the gut of *Rutilus kutum* showed the highest zone of inhibition against *E.coli* (12 mm) and *P. aeruginosa* (14 mm). Comparatively in our study also the *Lactobacillus* sp. showed the highest activity against the *P. aeruginosa* with the zone of inhibition of 10 mm. Tah-Wei Chu *et al.*, [29] examined the antimicrobial activity status of *Oreochromis niloticus* fed with *Enterococcus* sp. isolated from goldfish. The antimicrobial activity of the active probiotic *Enterococcus* sp. towards several pathogens exhibited the activity against all tested pathogens. The minimal activity was observed in *S. aureus*, *P. aeruginosa*, *L. anguillarium, and E. tarda.* In our study also the isolated *Enterococcus* sp. showed minimal activity against selected fish pathogens; however, showed a better response in the disease challenge study and the survival rate was 66 % on *Channa striatus* challenged with *Aeromonas hydrophila*.

The *Bacillus* sp. (DF12) has a high ability to tolerate even low and high pH and also tends to tolerate the 0.3% bile salt which is a major criterion for the selection of probiotics. Similarly, Muthukrishnan *et al.*, [30] reported the antagonistic activity of gut-associated bacteria from *Etroplus maculates* in which the isolate EM9 (*Bacillus* sp.) showed the highest antagonist activity and showed the high ability to tolerate even low and high pH and high bile salt concentration. In the present investigation, among the selected isolates, the *Bacillus* sp. had a high ability to tolerate high to low pH and high bile salt

tolerance and possess biopotential against freshwater fish bacterial pathogens as a desirable beneficial probiotic bacterium that could be used to maintain and increase gastrointestinal health of host animal.

Table 1: Antibacterial activity of gut bacteria by Agar overlaying method

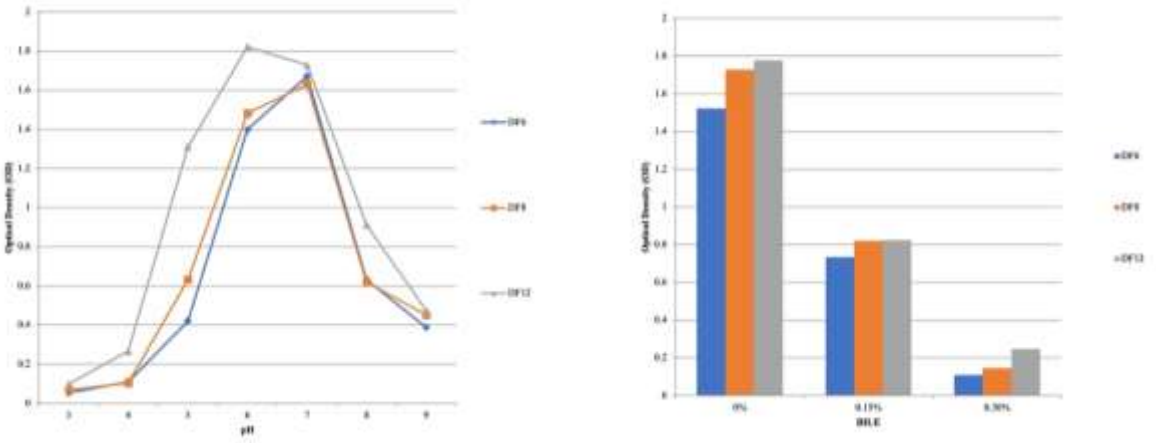
Gut Bacterial isolates From *Dawkinsia filamentosa*	Name of Pathogen and Zone of Inhibition (mm)			
	Vibrio cholerae	***Pseudomonas aeruginosa***	***Staphylococcus aureus***	***Aeromonas hydrophila***
DF1	-	Trace	-	-
DF2	4	4	-	-
DF3	-	-	-	-
DF4	-	-	-	-
DF5	-	-	-	-
DF6	-	-	-	-
DF7	-	-	-	-
DF8	-	-	-	-
DF9	3	2	-	T
DF10	-	-	-	-
DF11	2	4	2	2
DF12	6	8	8	8
DF13	-	-	-	-
DF14	-	-	-	-
DF15	-	-	-	-

(- no activity, 1-3 low, 4-6 moderate, 7-9% high, T-trace activity) DF- *Dawkinsia filamentosa*

Table 2: Antibacterial activity of gut bacteria by well diffusion method

Gut Bacterial Strains From *Dawkinsia filamentosa*	Name of Pathogen and Zone of Inhibition (mm)			
	Vibrio cholerae	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Aeromonas hydrophila*
DF1	2	2	4	4
DF2	-	2	2	TC
DF3	TC	TC	8	TC
DF4	TC	-	6	TC
DF5	-	-	4	2
DF6	4	2	8	4
DF7	2	TC	4	-
DF8	4	10	2	3
DF9	TC	TC	6	-
DF10	4	-	-	2
DF11	-	3	6	TC
DF12	8	8	10	4
DF13	TC	4	8	TC
DF14	2	2	-	4
DF15	4	-	4	2

'- No activity, TC- Trace activity, DF- ***Dawkinsia filamentosa***



Graph 3 & 4: Tolerance of active bacterial isolates at different pH level and different bile concentrations (OD)

Table 3: Biochemical characterization of active isolates

Biochemical Test	Isolate DF6	Isolate DF8	Isolate DF12
Gram staining	Positive	Positive	Positive
Shape/ motility	Cocci/ Non-motile	Rod/ Non-motile	Rod/ motile
Indole	-	-	-
Methyl red	-	-	-
Voges Proskaur	+	-	+
Citrate	-	-	+
Urease	-	-	-
Oxidase	-	-	+
Carbohydrate fermentation of glucose	±	±	+
Gas production	-	-	-
Catalase	-	-	+
H_2S	-	±	±
Glucose	+	+	+
Arabinose	+	+	+
Lactose	+	+	±
Maltose	+	+	+
Sucrose	+	+	+
	Enterococcus sp	***Lactobacillus*** sp	***Bacillus*** sp

Table 4: Survival rate of *Channa striatus* fed with different probiotic feed challenged with *A.hydrophila*

Feed Trial	Total Number of Fish	Mortality	Survival Rate	
			Total Number of Fish Survived	%
CONTROL	15	13	2	13.33
DF6	15	5	10	66.66
DF8	15	4	11	73.33
DF12	15	2	13	86.66

5. CONCLUSION

Probiotics are alive or dead bacteria that confer health benefits to the fish when incorporated with the fish through feed and water, improve disease resistance, growth performance, feed conversion ratio (FCR), and stress resistance. Due to the beneficial effect and eco-friendly manner probiotic treatment is rapidly increasing in the aquaculture sector. The present study aims to isolate and biochemically characterize active probiotics prevailing in the gut of freshwater fish *Dawkinsia filamentosa*. The isolated strains were preliminarily subjected to antagonistic activity by Agar overlying method and then antibacterial assay by disc diffusion methods. In these methods, the strain DF12 showed the highest zone of inhibition (8mm) against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Aeromonas hydrophila*, and it was identified as *Bacillus* sp followed by DF12, isolates DF6 and DF8 showed a moderate level of activity against the selected fish pathogens they were identified as *Enterococcus* sp. and *Lactobacillus* sp. using morphological and biochemical characterization. All those strains satisfy the basic criteria for the selection of probiotic strains. Observed results indicate isolates produce antibacterial metabolites to inhibit fish bacterial pathogens. Further studies are in progress to improve the beneficial effects of probiotic feed in fish to maintain a healthy intestine and develop an immune response in cultured freshwater fish. Mining the hidden treasures from the universe of microorganisms like bacteria is crucial for the better survival of the human race. It unlocks diverse areas of research for the betterment of aquaculture thereby achieving the global need for nutritional food.

ACKNOWLEDGEMENT

We sincerely thank Rev. Fr. Principal and Secretary for having provided necessary facilities for carrying out the work at St. Xavier's College (Autonomous), Palayamkottai.

CONFLICT OF INTEREST

The author declares he has no conflict of interest.

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