Indian Journal of Geo Marine Sciences
Vol. 49 (07), July 2020, pp. 1175-1183

Efficacy studies on commercially available probiotics to induce immune response and resistance in tilapia fish (*Oreochromis mossambicus*), against *Vibrio* infection

R Fatima[a], K Karthikeyan[a], R Sudhakaran*[,a], K Anuja[b], R Prafull[b] & K Ganesh[b]

[a]Aquaculture Biotechnology Laboratory, School of Bio Sciences and Technology, Vellore Institute of Technology, Vellore, Tamil Nadu – 632 014, India

[b]Organica Biotech Pvt. Ltd., Mumbai, Maharashtra – 400 088, India

[E-mail: sudhakaran.r@vit.ac.in]

Received 23 July 2019; revised 24 September 2019

The current research was undertaken to determine the potency of two distinct probiotics Biogut® Aqua (feed probiotic) and Bioclean® Aqua (water and soil probiotic) to induce immunity and protection faced due to *Vibrio* pathogen during cultivation of Mozambique tilapia under controlled laboratory trials. Assessment was carried out using immune gene expression and immunological assays. During the initial phase of treatment, nitro blue tetrazolium (NBT) assay, antiprotease assay and peroxidase assay displayed a convincing raise in serum activity of the probiotic managed feed when compared to the control groups. In addition, qPCR real time analysis displayed in which nutritional implementation with feed probiotic vitally increased the interpretation of immune related genes, particularly TNF and MCP gene expression which are involved in immune response. The results revealed that the probiotics Bioclean® Aqua and Biogut® Aqua have beneficial effects through efficiently enhancing immune system and protection in tilapia model facing Vibriosis. Hence it can be stated that the application of probiotics like Biogut® Aqua and Bioclean® Aqua in aquaculture environment may significantly reduce the antibiotic usage, thereby preventing the entry of these chemicals into the food chain. Additionally it was demonstrated that the ammonia level in water can be reduced significantly with the application of Bioclean® Aqua (water and soil probiotic). The toxicity studies proved that both the probiotic are non-toxic and safe for the use in aquaculture environment.

[**Keywords**: Disease resistance, Immune genes, *Oreochromis mossambicus*, Probiotics]

Introduction

Aquaculture refers to the practice of culturing animals and plants in water environments. It is also referred as aqua farming. Fishes are reared for various commercial, domestic and ethical purposes like consumption, aesthetics, as model organisms, species protection and much more[1]. Aquaculture has developed into a commercial activity drawing immense attention across the globe as it provides a solution to overfishing wild populations[2]. Aqua farming benefaction to global feed manufacture, natural resources for technical and medicinal usage, and marine species used in industry has heightened perilously in past few years. It has been estimated that the worldwide manufacture of marine feed obtained from aqua farming for human utilization entered 52.5 million in 2008[3].

Vibrio parahaemolyticus is a rod shaped gram negative bacteria which is asporogenous and halophilic in nature. It is a pathogen known to infect marine organisms like codfish, sardine, crab, lobster, crawfish, scallop, oyster and much more. It is found to be highly prevalent when the temperature exceeds 15 □ and peaks at 25 □[(ref. 4)]. The virulence of this pathogen is due to the presence of TDH gene which encodes thermostable direct haemolysin (TDH) protein, a toxin[5]. *V. parahaemolyticus* was initially recognized after a major outbreak in poisoning of food occurred in Osaka, Japan which affected 272 people killing 20[(ref. 6)]. Since then around 42 outbreaks have been documented which spans across the globe. In US, around 5000 cases of illnesses are recorded because of this *V. parahaemolyticus* pathogen[7]. The effects observed include gastroenteritis with diarrhea, nausea, vomiting, headache, severe abdominal pain and low fever. In such cases, the most common practice is to use anti-microbial agents to prevent the spread of the disease. Although, in current date there have been extensive records regarding the emergence of antimicrobial resistant pathogens which has forced people to explore other methods to control a disease outbreak[8]. The former reason coupled with the

environmental hazards resulting from the application of antibacterial agents has driven the discovery of a new class in treatment strategies called probiotics. The traditional definition of probiotics says that "probiotics are organisms and substances which contribute to intestinal microbial balance". Probiotic microorganisms hold the competence to discharge chemical matter with antibacterial effect on pathogenic bacteria present in the host intestine, hence creating an impediment across the multiplication of opportunistic pathogens[9,10]. As in mainly, the prophylactic response emanates from the ensuing aspects: production of antibiotics, bacteriocins, siderophores, and enzymes occasionally with the abdominal pH owing to the formation of organic acids.

Tilapia cultivation is highly advantageous in India and is growing day by day. Tilapia has developed into the second best prominent seafood after crab, owing to which its farming is expanding. However, bacterial disease emergence remains a nagging problem in the commercial culture of these fishes[11,12]. This study deals with the testing of formulated probiotics against *V. parahaemolyticus* infected tilapia fishes in a laboratory setting. There were two probiotics tested, one was incorporated into the feed while the other was added to the water in which the fishes were reared. The probiotic also aimed at reducing the ammonia level of the water in which the fishes were reared. It has been estimated that ammonia accounts for around 40-90 % of nitrogenous excretions. Ammonia exists in ionized form and unionized form but, only the un-ionized form is toxic to the animals being cultured[13]. At times when the ammonia level builds up to a very high degree, it affects the growth of the cultured organisms and also results in its mortality if not properly controlled[14]. The current analysis was initiated to assess the efficacy of two distinct commercially available probiotics, Biogut®Aqua (feed probiotic) and Bioclean® Aqua (water and soil probiotic) to induce immunity and defiance against infection caused due to *Vibrio* during cultivation of Mozambique tilapia under controlled laboratory trials. Bioclean® Aqua is a soil and water probiotic, highly suitable for use in aquaculture. It contains various beneficial species of *Bacillus* and yeasts that are capable of degrading ammonia and other toxic waste material accumulated in hatcheries and aquaculture ponds over time. This also alleviates the onset and increase in pathogen load in the water and soil found within aquaculture ponds. Biogut®Aqua consists of various beneficial species of *Bacillus*, *Lactobacillus* and yeast that benefits fish by improving their immune response and restricting the colonization of pathogenic microbes in the fish gut.

Materials and Methods

Probiotics

The products evaluated in this study were Bioclean® Aqua and Biogut® Aqua (Organica Biotech, Mumbai, India). Bioclean® aqua is a soil and water probiotic suitable for use in aquaculture. Bioclean® Aqua mainly consists of various beneficial species of *Bacillus* and yeast. BioGut® Aqua consists of various beneficial species of *Bacillus* including *Lactobacillus* and yeast. Biogut® Aqua is applied as fish feed probiotic that benefits fish by improving their immune response and restricting the expansion of microbes in the fish gut. The microorganisms present in both the formulations were initially evaluated for their antimicrobial activity (against a variety of fish pathogens including *Vibrio* sp.), enzymatic activity and safety. The cultures were also assessed for their compatibility with each other before being processed into their respective solid formulations.

Estimation of ammonia (NH_3)

Total three groups containing five fishes in a tank having 10 L of water were maintained for a week with zero water exchange. Group I (Control) having water with no treatment, Group II (Bioclean® Aqua I) having water mixed with Bioclean® Aqua (5 mg/L) a Nessler'S reagent was used to measure free ammonia (NH_3) level present in the water samples one day post immersion. Briefly, 50 mL of water from both the groups were taken in a separate beaker and mixed with 1 mL of Nessler's reagent. Components were mixed properly and OD was measured after 10 minutes at 420 nm. The concentration of ammonia present in the unknown samples was determined by plotting a standard curve by using five different concentrations of standard ammonia (0.05 to 0.25 mg/50 mL)[15].

Brine shrimp (*Artemia salina*) toxicity analysis

Artemia nauplii model was used for toxicity testing of probiotics. Six different concentrations of product Bioclean® Aqua ranging from 0 to 80 ppm were introduced in a test tube with 5 mL of sea water and 10

nauplii were introduced in each tube and maintained for 24 h to check the mortality. Each concentration was tested in triplicates. After 24 hours, survivors were counted using 3 X magnifying glasses[16].

Tilapia fingerlings bioassay for toxicity analysis

In this experiment, five different concentrations (10, 50, 100, 500 and 1000 ppm) of Bioclean® Aqua were tested and five tilapia fingerlings were introduced in a beaker containing 1 L of water and were maintained for one week to check the survival of fishes. Commercial fish feed were fed twice a day. Unutilized feed, fecal matter and dead fishes were removed before next feeding. The water level was maintained throughout the experiment with corresponding concentration of Bioclean® Aqua product.

Bioassay for ammonia stress induced mortality and qPCR gene expression analysis of Corticotropin-releasing hormone (CRH)

Bioassay covered two different groups with five fishes in a tank having 10 L of water and was maintained for one week with zero water exchange. Group I (Control) having water with no treatment and Group II having water mixed with Bioclean® Aqua (5 mg/L). Commercial fish feed was fed to the fish twice in a day. Mortalities were observed twice a day throughout the experimental period. Brain tissues were dissected aseptically for all three groups and preserved in RNA later solution for isolation of RNA. RNA was isolated utilizing trizol method following the manufacturer's instructions (TaKaRa, Japan). cDNA was synthesized from 1 µg of RNA following the obtained protocol using the complementary DNA Reverse Transcription Kit (TaKaRa, Japan). Relative gene expression analysis was done using SYBR® Master Mix in Applied Bio systems Step One instrument. *Ef1-alpha* was used as an endogenous control and the analysis was performed using comparative cycle-threshold (CT) method[17]. The sequence of primers *Ef1-alpha* and corticotropin-releasing hormone (*CRH*) was mentioned in Table 1. Primers were synthesized and obtained from Sigma Aldrich, Bangalore, India.

Therapeutic experiment

Blood Collection

Clove oil was employed for anesthetizing fish (0.1 mL in 1 liter of water) for blood collection without sacrificing the fish. Blood was obtained from the gills, clotting was performed at 4 ºC followed by centrifugation at 10,000 g for 10 min. The serum was separated and stored at -80 ºC for further detection and quantification of immune assays (antiprotease assay, peroxidase assay, nitric oxide activity and NBT) parameters.

Table 1 — List of oligonucleotides used for gene expression analysis

Target gene	Sequence (5'-3')	Size (bp)
MCP -Tilapia-F	CGG GTT AGC TGT TGG GCA TTG T	198
MCP-Tilapia-R	AAG CAA GCA GAG AAA ACC ACT TCA	
EF-1alpha-Tilapia-F	CCC AGA AAC ACC GAA ACT AAA	157
EF-1alpha-Tilapia-R	TGT CGA TTC CTC CGC ACT	
MyD88-Tilapia-F	GGT ATG TTG TGC TGT AGA CTT CCG A	454
MyD88-Tilapia-R	GTA GTT CTT TAT TTC CAG GTA GTT G	
TNF-F	GCTGGAGGCCAATAAAATCA	339
TNF-R	CCTTCCGTCAGTCTCCAGCTC	
LBP-F	GGCGCAGCTGGGGAAAGAA	269
LBP-R	TGGGGACATCAGTGAGAGGAAGG	

Peroxidase assay

The serum peroxidase assay was analyzed following Quade & Roth[18]. In brief, 5 µl of blood serum was reduced employing 45 µl of phenol red-free Hank's buffer in 96-well plates. Followed by addition of 100 µl of tetramethylbenzidine hydrochloride and 5 mM H_2O_2 were added. The change in color of reaction was interrupted after two minutes by addition of 50 µl 2 M Sulphuric acid and OD was observed at 450 nm. Samples with absence of serum were considered as blank. Peroxidase assay was calculated as one unit of peroxidase which generates an absorbance change of 1 OD.

Antiprotease activity

Inhibition of trypsin by blood serum was analyzed by antiprotease assay[19]. In brief, 10 µl of trypsin procured from sigma was added with 10 µl of serum sample solution and incubated for ten minutes at 22 ºC. Followed by the addition of 100 µl 0.1 M phosphate buffer (pH 7.0) and 120 µl of 2 % azocasein, further incubation was carried out for 2 h at 22 ºC, followed by inclusion of 250 µl of 10 % trichloro acetic acid, and incubated for 30 min at 22 ºC. Centrifugation was carried out at 6,000 g for 5 min. Briefly 100 µl of supernatant was moved to a 96-well plate holding 100 µl of 1 N NaOH, the OD was recorded at 450 nm employing a plate reader. For the positive control, the serum was retrieved by buffer while for the negative control; the serum along with trypsin was retrieved by buffer.

Nitric oxide assay

To determine the amount of nitric oxide generated in serum sample we employed the previous protocol[20]. Phosphoric acid (2.5 %), Sulphanilamide (1 %) and N-Naphthylethylenediamine (0.1 %) were utilized to prepare Griess solution to determine the nitric oxide in fish serum samples. About 100 µl of serum sample along with the negative control was left for incubation with the same volume of Griess solution in a 96 micro titer plate and incubation was carried out for 10 minutes at 27 °C. Following which, OD was read at 570 nm employing an ELISA reader.

NBT assay

Nitrobluetetrazolium (NBT) assay was used for analyzing the production of superoxide anion employing the protocol explained as previous article[21]. Employing 1 ml of syringe, 300 µl of blood was drawn and added to a 0.5 ml vial containing heparin, to prevent clotting of blood. The heparinized blood sample (100 µl) was added in a micro titer plate and followed by addition of 100 µl of 0.2 % NBT and mixed well. After 30 minutes of incubation at 37 ºC, 50 µl of Nitrobluetetrazolium-blood suspension was mixed to a 1.5 ml vial containing 1 ml of N, N dimethyl formamide solution. The vial was subjected to centrifugation at 3,000 rpm for 5 minutes and the supernatant was obtained. Approximately 200 µl of supernatant from each sample was introduced into microtiter plate and absorbance was read at 540 nm using an ELISA reader.

RNA extraction and immune gene expression analysis

RNA was obtained from the intestine of the fish on completing the feed duration of thirty days. RNA was quantified using spectrophotometer. Approximately 1 µg of RNA was converted to cDNA by reverse transcription. PCR was demonstrated employing the primers for *EF1-α*, that was used as an endogenous control for the qPCR. The expressions of tumor necrosis factor alpha (*TNF-α*), Lipopolysaccharide binding protein (LBP), Mast cell protease (MCP) and Myeloid differentiation factor (MYD88) were tested employing the primer sequences given in Table 1.

Experimental pathogenicity

V. parahaemolyticus culture was procured from Microbial Type Culture Collection (MTCC-451) and purity was accepted following observation with biochemical traits as described in Bergy's standards of Bacteriology. The bacterial culture was grown on tryptic soya agar and further cultured in Brain heart infusion broth to enhance the pathogenicity which was used for experimental infection in fish[22]. Culture broth (50 µl) was infected into *O. mossambicus* intra-muscularly to determine the infection of *Vibrio*. The lethal dose of the pathogen was found to be 9×10^{-5} cells/fish. Healthy *O. mossambicus* were divided into four tanks with ten fishes in every group. The experiment was carried out in triplicates. Group one received phosphate buffer and served as negative group. Group 2 fishes were injected with the pathogen and served as positive group. Group 3 and 4 were treated with Bio clean Aqua and Bio gut Aqua (5 mg/L). The experimental fishes were nourished with commercial pellets throughout the experimental course.

Results

Ammonia estimation

Each group containing five fishes in a tank having 10 L of water and were maintained for a week with zero water exchange to check the ammonia level. Compared to control after one day post immersion of Bioclean® Aqua product with concentration of 5 mg/L, the ammonia level in water was significantly reduced (Fig. 1)

Brine shrimp toxicity analysis

Brine shrimp toxicity assay is the most favorable method for detecting biological properties of different compounds. It is used to assess the toxic potential of different compounds rapidly with limited requirement of the test compounds. The Brine shrimp toxicity analysis shows that at 5-10 ppm concentrations of Biogut® Aqua and Bioclean® Aqua products were nontoxic to the fish (Fig. 2).

Toxicity experiment in Tilapia fingerlings

The toxicity results indicate that 10 ppm concentration of Bioclean® Aqua product were

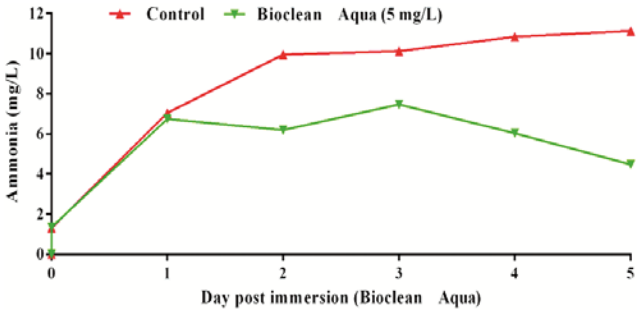

Fig. 1 — Estimation of ammonia level in water shown a reduction after treatment with Bioclean® Aqua

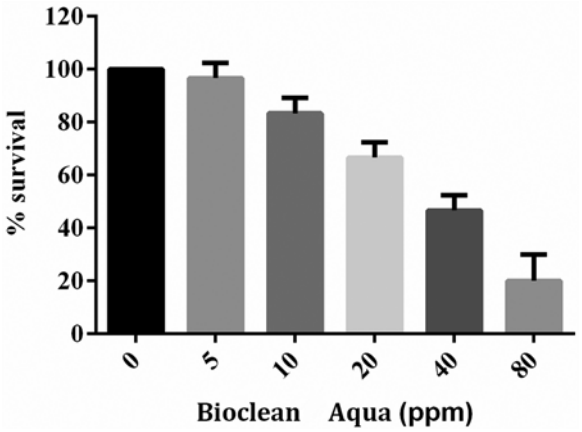

Fig. 2 — Brine shrimp toxicity analysis shows nontoxic level from 5 to 80 ppm of Bioclean® Aqua

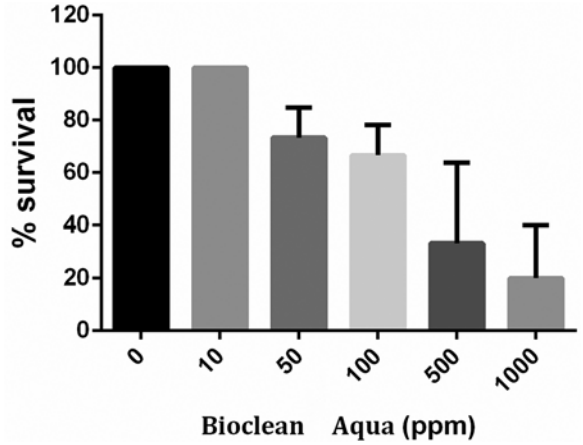

Fig. 3 — Toxicity analysis in Tilapia fingerlings resulting with 10 ppm is optimal and nontoxic for bioassay in tilapia fish

nontoxic and optimal for other biological assay in tilapia fish (Fig. 3).

Stress related gene (CRH) expression analysis in Tilapia fish

Corticotropin-releasing hormone is a hormonal peptide and a neurotransmitter responsible for stress reaction. The product Bioclean® Aqua have mild expression of and up regulated with 0.4 fold compared to control and is statistically not significant. Hence Bioclean® Aqua is considered better in activity in both gene expression and stress response analysis (Fig. 4).

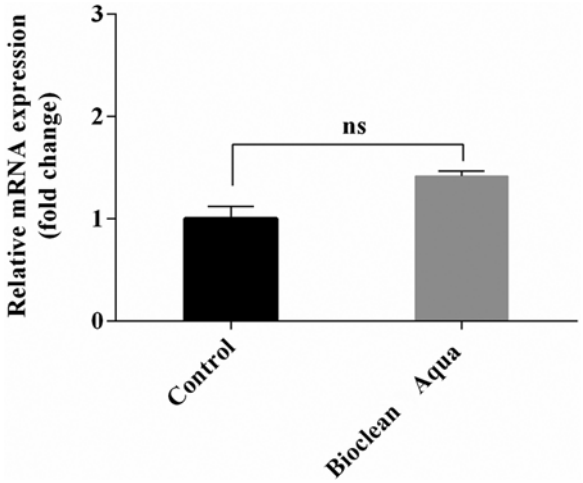

Fig. 4 — Stress related gene (*CRH*) expression analysis in Tilapia fish. Bioclean® Aqua shows mild expression and no significant changes compare to control group

Therapeutic experiments

The fishes treated with probiotics showed a decline in mortality as compared to untreated but injected with *V. parahaemolyticus* fishes. A total of 21 out of 30 healthy fishes that were injected with *V. parahaemolyticus*, died during the experiment tenure giving 70 % mortality. In contrast to this the fishes in treated tanks did not show high mortality. In treated group Biogut® Aqua, 9 out of 30 fishes died with a mortality rate of 30 %, whereas in group Bioclean® Aqua I, 6 fishes out of 30 died of infection giving a 20 % mortality rate. This could be indicative of the effect of probiotic treatment that helped fishes in reducing the pathogenic bacterial load and thus escaping the infection. The probiotics given as water treatment might have a stimulatory effect on beneficiary natural microbial population by balancing them and giving an enhanced immune response.

Peroxidase activity

Serum of the fish blood was employed to study the activity of the peroxidase enzyme described by Quade & Roth[18]. Serum peroxidase activities of Tilapia species exposed to *V. parahaemolyticus* obtained a different observation with more decline to a clear range in each treatment group (Fig. 5). The corresponding serum peroxidase activities three days after bacterial infection were consequently higher in comparison to the negative group, and then relatively reduced after the 3rd day in Biogut® Aqua group. Bioclean® Aqua showed a sharp drop in activity and then achieved a stable level from 9th day.

Antiprotease activity

The physiological reaction, stressors and cortisol are important immune suppressors. Ability to modulate anti-protease activity and subsequently their ability to resist bacterial pathogen was clearly visible in time course elevation patterns of treated groups

(Fig. 6). Trypsin inhibition was significantly elevated after 72 h of infection in the group treated with probiotics, in comparison to the time-matched control fish. Similarly anti-protease activity went on increasing day after day after the probiotic treatment. The highest anti-trypsin activity was seen in the Bioclean® Aquaon 12th day, when compared to Biogut® Aqua. The lowest activity was observed in the Biogut® Aqua at the 3rd day.

Nitric oxide activity

At the 3rd day post infection, nitric oxide concentration in serum samples were elevated, then relatively reduced on 6th day. An increase in nitric oxide activity on 9th day, Biogut® Aqua was observed. Bioclean® Aqua was consistent with constant decline in level from 3rd to 12th day. The reduction in NO concentration was highly significant at 12th day in Biogut® Aqua treatment group. Negative control group value was less than those of Positive control as expected (Fig. 7).

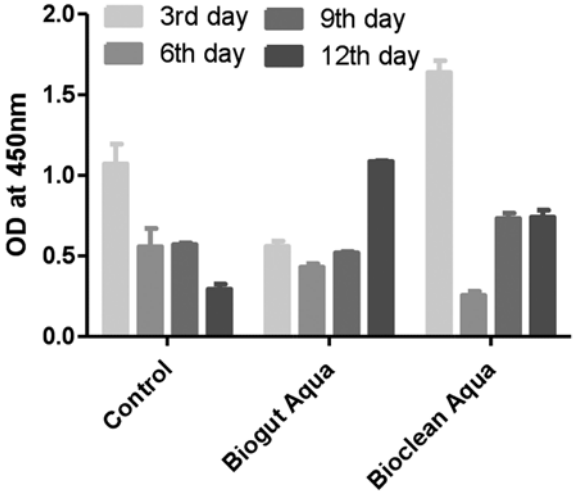

Fig. 5 — Serum peroxidase activity in serum of Mozambique Tilapia treated with Biogut® Aqua and Bioclean® Aqua

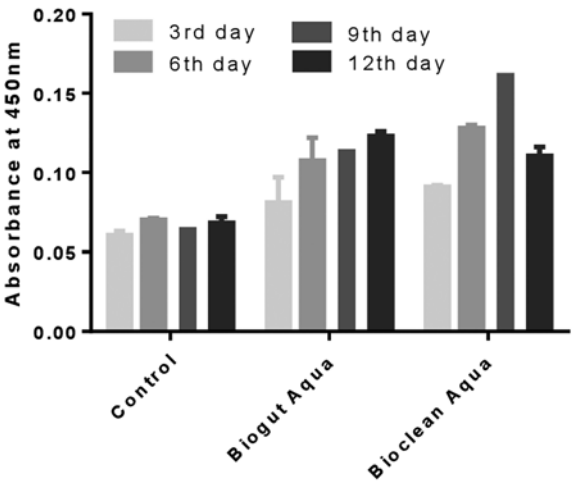

Fig. 6 — Antiprotease activity in serum of Mozambique Tilapia treated with Biogut® Aqua and Bioclean® Aqua

NBT assay

NBT is a reagent that can absorb superoxide and change its color to purple (absorbed at 540 nm). NBT assay in blood serum of fish defined to Biogut® Aqua and Bioclean® Aqua probiotic treatment was compared to that of control group (Fig. 8). Serum activity of fish in treatment group employing probiotics was comparatively greater when compared to the respective positive group value at each point of time. Time course elevation pattern among the treated groups indicates that the probiotic fed group induced elevation of NBT activity was at peak value on 9th day of post infection. Prior to 9th day, the NBT activity

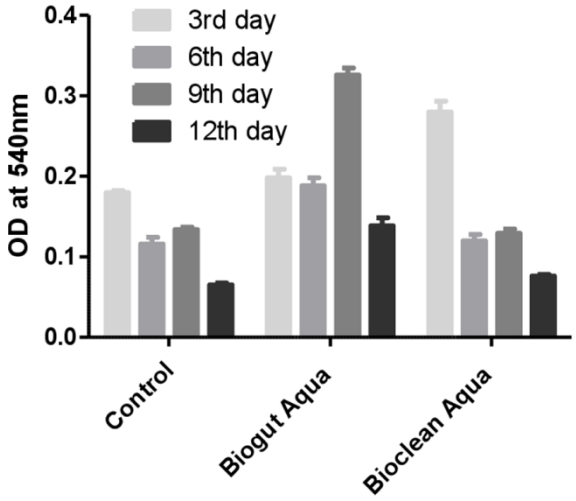

Fig. 7 — Nitric oxide activity in serum of Mozambique Tilapia treated with Biogut® Aqua and Bioclean® Aqua

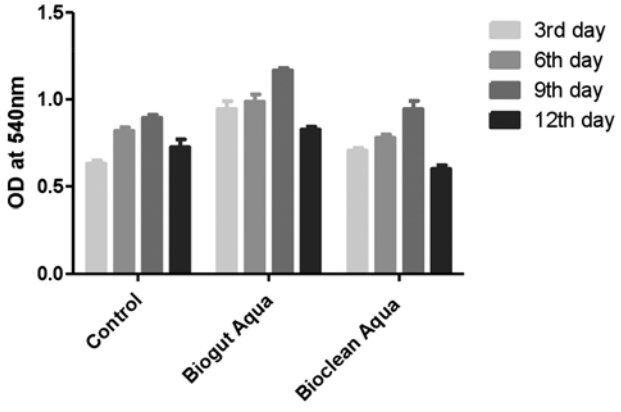

Fig. 8 — Nitroblue tetrazolium assay in serum of Mozambique Tilapia treated with Biogut® Aqua and Bioclean® Aqua

showed an increase and after that the activity declined.

Gene expression analysis

mRNA expression of LBP seems to be increasing with increase in duration of time. It displays its function immunity particularly during response in acute phase (Fig S1). From the obtained results we infer that Bioclean® Aqua treatment seems to be efficient in decreasing the inflammation. The expression of this gene is been regulated well in this method of treatment than the control. This treatment seems to be better among other group. TNF is a cytokine responsible for systemic inflammation and is involved in acute phase response. From the mRNA expression analysis it is disclosed that during initial phase of treatment with Bioclean® Aqua the TNF-alpha level is increased in its regulation and at the end of the treatment course it resolves to normal suggesting that TNF-alpha efficiently inhibits the pathogenesis (Fig. S2). MCP has varied roles such as inflammatory and anti-inflammatory tissue homeostasis and innate immunity, its deregulated release contribute to the pathogenesis to a number of inflammatory conditions. Again Bioclean® Aqua and its treatment method seem to be under regulation because it is increased during the initial phase of infection and regulated at the end of treatment course (Fig. 9). Myeloid differentiation factor is a universal adapter protein which activates transcription factor NF kappa B. It shows resistance to common viral and bacterial infections. The expression of this gene has been increased during the initial phase and has been regulated well by Bioclean® Aqua at the end of the course (Fig. S3).

Cumulative mortality assay

The signs and symptoms of infection generally appear having inactivity and anorexia. Discoloration, redness and necrosis occur on the outer layer of the fish as the infection is progressed in the positive control. Blood filled blemishes appear over the mouth and fin. Boil-like sores appeared on the body, the skin surface resulted in large, open sores. The fishes commenced to display mortality and signs of infection following injection with *V. parahaemolyticus.* Anomalous behavior and deficit activity were visualized in the positive group. Fall in appetite and abrasions were found on the surface of the skin. Absence of infectious signs was recorded in the negative group injected with PBS. Bioclean Aqua and Biogut Aqua were employed for protection and defense of bacterial infection in *O. mossambicus.* A 100 % survival was recorded in both Bioclean and Biogut Aqua (5 mg/L) with mild clinical symptoms similar to positive group. Group 3 and Group 4 that remained as treated groups were checked for 30 days post infection and it declined to bring mortality. Cumulative mortality results are depicted in Figure 10. Mortality was absent in negative control receiving PBS buffer.

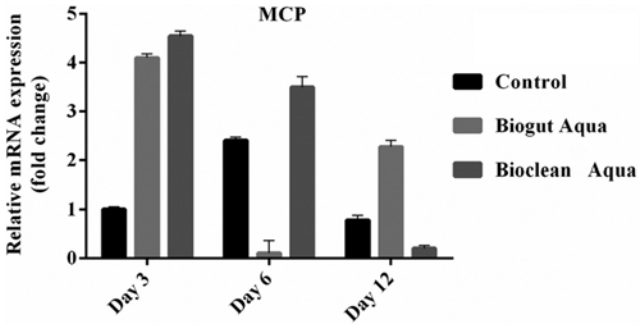

Fig. 9 — Gene expression analysis of Mast cell protease encoding gene in three different time durations

Discussion

Probiotics have shown an excellent ability to resist disease and prevent pathogens. Probiotic supplementation results in improvements in microvilli density and length, which can increase the absorptive surface of the fish intestines and ultimately enhance the host's physical barrier against potential pathogens. Probiotics have an essential part which stimulates the immune response in fish. In the current analysis, tilapia treated with feed probiotics showed significant increase in non-specific immune response, which includes respiratory bursts, antiprotease, serum peroxidase and nitric oxide assay at initiative stage of

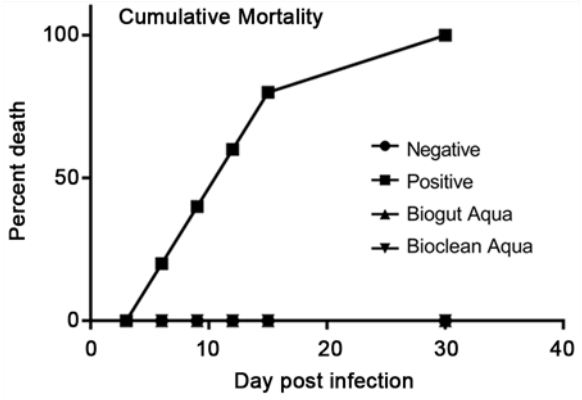

Fig. 10 — Cumulative mortality of groups treated with Bioclean and Biogut Aqua (5mg/L)

treatment and reduced at the deadline of treatment course. Nitric oxide has varied significance in immune response acting as lethal agent against pathogenic organisms. The activity employing NBT decline to identify oxidative radical generation in the process of phagocytosis is a potentimmune response and is broadly employed to detect protection against infectious microbes[23-25]. Dietary supplementation of Biogut® Aqua has notably enhanced the survival of Tilapia fed diets. Gene expression analysis reveals that regulation of immune genes are increased during the initial stage and well regulated by probiotic treated group at the end of the treatment course suggesting the inhibition of pathogenesis which indicates Bio clean aqua has better efficiency than Biogut aqua. Various analyses have identified the valuable properties of probiotics on the development in growth of tilapia. Antibacterial assays were persuaded by Probiotics against *Vibrio parahaemolyticus* in this study. Probiotic treated group showed 85 % of survival whereas bacterial infected group showed only 20 % of survival following *in vivo* treatment. A related inhibitory assay for *Bacillus subtilis* and *Bacillus* S11 culture were determined formerly. Similar antibacterial assay could be associated to the certainty that the *bacillus* can be aroused to challenge with different rapid expanding microbes for nourishment[26]. It's widely understood that probiotics are living microbial supplement giving beneficial effects in developing the progress, survival and overall well-being of the commercially cultivated organisms use good framing[27]. Probiotics are employed to treat diseases essentially due to their activity in immune system and anti-inflammatory response. Probiotics used in our study showed reduction in expression of pro inflammatory cytokine. In completion, the current study shows the early approach to assess the outcome of immersion and nutritional consumption of probiotic bacteria on disease resistance and innate status of tilapia fish. The data added support about the possible usage of probiotics, as an interesting immunostimulant for farmed fish. Our study validates that applicable forms of Biogut Aqua and Bioclean® Aqua, which was Biogut is feed probiotic and Bioclean is for water and soil application, adequately improved Mozambique tilapia immunity and apparently increased 65 % survival and disease resistance against bacterial infection in lab experiments, which could be further implemented in field levels.

Supplementary Data

Supplementary data associated with this article is available in the electronic form at http://nopr.niscair.res.in/jinfo/ijms/IJMS_49(07)1175-1183_SupplData.pdf

Acknowledgements

The authors are grateful to the management of Vellore Institute of Technology for providing necessary facilities to carry out this study.

Conflict of Interest

There is no conflict of interest among the authors.

Author Contributions

RF executed the experiment. KK was critically involved in performing and analyzing quantitative PCR assays. KA, RP and KG were involved in suggesting the parameters and dosages of the probiotics utilized in the experiment. RS designed the whole experiment; monitored the execution of the experiment; and was also involved in the preparation of manuscript.

References

1 Primavera J H, Overcoming the impacts of aquaculture on the coastal zone, *Ocean Coast Manage*, 49 (9-10) (2006) 531-545.

2 Diana J S, Aquaculture production and biodiversity conservation, *Bioscience*, 59 (1) (2009) 27-38.

3 Chatterjee S & Haldar S, *Vibrio* related diseases in aquaculture and development of rapid and accurate identification methods, *J Marine Sci Res Dev*, S1 (002) (2012), pp. 7.

4 Croci L, Suffredini E, Cozzi L, Toti L, Ottaviani D, *et al*., Comparison of different biochemical and molecular methods for the identification of *Vibrio parahaemolyticus*, *J Appl Microbiol*, 102 (1) (2007) 229-237.

5 Yanagihara I, Nakahira K, Yamane T, Kaieda S, Mayanagi K, *et al*., Structure and functional characterization of *Vibrio parahaemolyticus* thermostable direct hemolysin, *J Biol Chem*, 285 (21) (2010) 16267-16274.

6 Su Y C & Liu C, *Vibrio parahaemolyticus*: a concern of seafood safety, *Food Microbiol*, 24 (6) (2007) 549-558.

7 Cook D W, Leary P O, Hunsucker J C, Sloan E M, Bowers J C, *et al*., *Vibrio vulnificus* and *Vibrio parahaemolyticus* in US retail shell oysters: a national survey from June 1998 to July 1999, *J Food Prot*, 65 (1) (2002) 79-87.

8 Spellberg B, Guidos R, Gilbert D, Bradley J, Boucher H W, *et al*., I.D.S.O. America, The epidemic of antibiotic-resistant infections: a call to action for the medical community from the Infectious Diseases Society of America, *Clin Infect Dis*, 46 (2) (2008) 155-164.

9 Zokaeifar H, Balcázar J L, Saad C R, Kamarudin M S, Sijam K, *et al*., Effects of *Bacillus subtilis* on the growth performance, digestive enzymes, immune gene expression

and disease resistance of white shrimp, *Litopenaeus vannamei*, *Fish Shellfish Immun*, 33 (4) (2012) 683-689.

10 Otles S, Cagindi O E & Kefir: A probiotic dairy-composition, nutritional and therapeutic aspects, *Pak J Nutr*, 2 (2) (2003) 54-59.

11 Suresh A V & Lin C K, Tilapia culture in saline waters: a review, *Aquaculture*, 106 (3-4) (1992) 201-226.

12 Bondad-Reantaso M G, Subasinghe R P, Arthur J R, Ogawa K, Chinabut S, *et al*., Disease and health management in Asian aquaculture, *Vet Parasitol*, 132 (3-4) (2005) 249-272.

13 Sutton M, Dragosits U, Tang Y & Fowler D, Ammonia emissions from non-agricultural sources in the UK, *Atmos Environ*, 34 (6) (2000) 855-869.

14 Chin T S & Chen J C, Acute toxicity of ammonia to larvae of the tiger prawn, *Penaeus monodon*, *Aquaculture*, 66 (3-4) (1987) 247-253.

15 Wu H Z & Cao A, Preparation and adding methods of Nessler's Reagent having effects on determination of water quality ammonia nitrogen, *Adv Mater Res*, *Trans Tech Publ*, 2013, 1362-1366.

16 Hossain M J, Khaleda L, Chowdhury A, Arifuzzaman M & Forkan M, Phytochemical screening and evaluation of cytotoxicity and thrombolytic properties of *Achyranthes aspera* leaf extract, *J Phar Biol Sci*, 6 (2013) 30-38.

17 Van Enckevort F, Pepels P, Leunissen J, Martens G, Bonga S W, *et al*., *Oreochromis mossambicus* (tilapia) corticotropin-releasing hormone: cDNA sequence and bioactivity, *J Neuroendocrinol*, 12 (2) (2000) 177-186.

18 Quade M J & Roth J A, A rapid, direct assay to measure degranulation of bovine neutrophil primary granules, *Vet Immunol Immunopathol*, 58 (3-4) (1997) 239-248.

19 Hanif A, Bakopoulos V & Dimitriadis G, Maternal transfer of humoral specific and non-specific immune parameters to sea bream (*Sparus aurata*) larvae, *Fish Shellfish Immun*, 17 (5) (2004) 411-435.

20 Rajaraman V, Nonnecke B, Franklin S, Hammell D & Horst R, Effect of vitamins A and E on nitric oxide production by blood mononuclear leukocytes from neonatal calves fed milk replacer, *J Dairy Sci*, 81 (12) (1998) 3278-3285.

21 Anderson D & Siwicki A, Basic hematology and serology for fish health programs, (1995).

22 Maftuch M, Toban Y R & Risjani Y, Administration of marine algae (*Gracilaria verrucosa*) immunostimulant enhances some innate immune parameters in black tiger shrimp (*Penaeus monodon Fabricus*) against *Vibrio harveyi* infection, *J Appl Sci Res*, 8 (2) (2012) 1052-1058.

23 Shiu Y L, Lin H L, Chi C C, Yeh S P & Liu C H, Effects of hirami lemon, *Citrus depressa* Hayata, leaf meal in diets on the immune response and disease resistance of juvenile barramundi, *Lates calcarifer* (bloch), against *Aeromonas hydrophila*, *Fish Shellfish Immun*, 55 (2016) 332-338.

24 Chiu S T, Shiu Y L, Wu T M, Lin Y S & Liu C H, Improvement in non-specific immunity and disease resistance of barramundi, *Lates calcarifer* (Bloch), by diets containing Daphnia similis meal, *Fish Shellfish Immun*, 44 (1) (2015) 172-179.

25 Giri S S, Sukumaran V & Oviya M, Potential probiotic *Lactobacillus plantarum* VSG3 improves the growth, immunity, and disease resistance of tropical freshwater fish, *Labeo rohita*, *Fish Shellfish Immun*, 34 (2) (2013) 660-666.

26 Moriarty D, Control of luminous *Vibrio* species in penaeid aquaculture ponds, *Aquaculture*, 164 (1-4) (1998) 351-358.

27 Tan L T H, Chan K G, Lee L H & Goh B H, *Streptomyces* bacteria as potential probiotics in aquaculture, *Front Microbiol*, 7 (2016) pp. 79.