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Note

Use of commercial probiotics for the improvement of water quality and rotifer density in outdoor mass culture tanks

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ABSTRACT

An experimental study was carried out to evaluate the efficacy of three commercially available probiotics P1, P2 and P3 consisting of mainly *Bacillus* spp. and nitrifying bacteria against *Vibrio* loads in mass culture tanks of the rotifer *Brachionus plicatilis*. Triplicate tanks were maintained for each of the probiotic treatment as well as for control group. All the tanks were inoculated with 50 rotifers ml^{-1} and were fed with *Nannochloropsis oculata* at a density of 1 x 10^7 cells ml^{-1}. Every alternate day, all the experimental tanks were treated with probiotics at a concentration of 1 x 10^4 cfu ml^{-1} and the experiment was carried out for one week. The study showed a significant increase in rotifer density ($p<0.05$) in all the tanks treated with the probiotics and a maximum density of 400 nos. ml^{-1} was observed in the tanks treated with P3. After 5th and 6th day of culture, total elimination of *Vibrios* was also recorded in the tanks treated with P3 and P2 respectively. The study revealed that P3, with a combination of *Bacillus, Thiobacillus, Acetobacter* and *Paracoccus* supplemented with enzymes, was found to be most effective in the enhancement of rotifer density and also in the elimination of *Vibrios* in rotifer mass culture tanks.

Keywords: *Brachionus plicatilis*, Commercial probiotics, Rotifer density, *Vibrio*

Brachionus plicatilis is the most commonly used live feed as a first feed organism for larval rearing in marine finfish hatcheries. Many studies were carried out on the mass culture of rotifers with different microalgal diets and at different temperatures and salinity conditions (Fielder *et al*., 2000; Hotos, 2002, 2003; Savas and Guclu, 2006; Kostopoulou and Vadstein, 2007; Jabeur *et al*., 2013; Abou-Shanab *et al.,* 2016) in the rotifer tanks**.** Several researchers reported variations in rotifer density due to *Vibrio* infections which is a major constraint for high density production of rotifers (Skjermo and Vadstein, 1993; Verdonck *et al*., 1994, 1997; Gomez-Gil *et al*., 2000; Rombaut *et al.,* 2001; Prol-Garcia *et al*., 2010, Jayasree *et al*., 2016). Use of antibiotics in culture systems will lead to the development of antibiotic resistant strains of pathogenic bacteria and will also hinder the growth of cultured organisms. Use of probiotics has been proved to be the best alternative to antibiotic application in aquaculture systems (Gomez-Gill *et al.,* 2000; Planas *et al*., 2004; Vine *et al*., 2006; Merrifield *et al*., 2010). Although there are many reports on the antagonistic activity of *Lactobacillus* spp. and *Bacillus* spp. against pathogenic *Vibrio* spp. (Gatesoupe, 1994, 1999; Vadstein, 1997; Ringo and Birkbeck, 1999; Murillo and Villamil, 2011), very few studies investigated the effect of application of commercially available probiotics on production of live feed (Douillet, 2000; Benetti *et al.,* 2008; Rotman *et al*., 2011).

The present study was carried out to investigate the efficacy of three commercially available probiotics as antagonistic to *Vibrio* infections and also on their efficacy in enhancement of rotifer density in outdoor mass culture of *B. plicatilis*. The study was carried out at the marine hatchery complex, Karwar Research Centre of ICAR-Central Marine Fisheries Research Institute, Karwar.

Three commercially available probiotics designated as P1, P2 and P3 (Source: NEOSPARK Drugs and Chemicals Private Limited, India) were selected for the study. All the three probiotics used for the experiment were having a combination of several bacterial strains with major contribution of *Bacillus* spp. and nitrifying bacteria Table 1. The control group (C) was maintained without addition of any probiotic. All the treatment and control groups were triplicated.

The experimental tanks (1 t circular tanks) were disinfected with chlorine for 12 h and were then filled with 50 l of the microalga *Nannochloropsis oculata* at a concentration of 1 x 10^7 cells ml^{-1}. Probiotic inocula were prepared by dissolving 10 mg of each probiotic in 100 l of seawater and mixed thoroughly for 30 min. Further dilutions were made to get a final concentration of 1 x 10^4 cfu ml^{-1}. Three days before the initiation of the experiment, all the experimental tanks other than the

Table 1. Details of the commercial probiotics used for rotifer culture experiment

Code	Product	Bacterial strains	Concentration (cfu g^{-1})
P1	BioClear	*Bacillus* sp., *Nitrosomonas* sp., *Nitrobacter* sp., *Cellulomonas* sp., *Acetobacter* sp. (impregnated on granular Zeolite)	3 billion
P2	BioRemid-Aqua	*Bacillus* sp., *Nitrosomonas* sp., *Nitrobacter* sp., *Aerobacter* sp., *Cellulomonas* sp. and biochemical accelerators with high enzyme activity of lipase, hemicellulase, lactase, proteases and amylase	184 billion
P 3	QBac	*Bacillus subtilis, Bacillus megaterium, Bacillus licheniformis, Bacillus polymyxa, Bacillus pumilus, Thiobacillus denitrificans, Paracoccus denitrificans, Acetobacter* and Enzymes protease, amylase, cellulase, hemicellulase and lipase	180 billion

control were treated with probiotics at a concentration of 1 x 10^4 cfu ml^{-1}. On the 3rd day, rotifers (*B. plicatilis*) were inoculated in the experimental tanks at an initial density of 50 nos. ml^{-1}. Rotifers in all the tanks were fed daily with 5 l of *N. oculata* at 1.0 x 10^7 cells ml^{-1}. On every alternate day, all the experimental tanks were treated with uniform concentration of probiotics (1 x 10^4 cfu ml^{-1}) before feeding with *N. oculata*. The experiment was carried out for 7 days, with continuous aeration and with no water exchange during the experimental period.

Rotifer density was determined every day during the experimental period by taking 10 ml of sample from each tank, subsequently five 1 ml subsamples were prepared and counted under microscope. Water quality parameters *viz.*, temperature, salinity, dissolved oxygen (DO), pH and ammonia levels in the experimental tanks were analysed during the experimental period following standard protocols (APHA, 2004). Total bacterial (Zobell marine agar) and *Vibrio* loads (Thiosulphate citrate bile salt sucrose agar) were estimated as per standard methods (APHA, 2004). Triplicates were made for each sample and mean values of the replicates were analysed statistically by using two-way analysis of variance (ANOVA).

Results of the study revealed that the rotifer density was significantly higher ($p<0.05$) in P3 treated tanks on termination of the experiment. P3 was found to be the most effective probiotic, with a maximum rotifer density of 400 numbers ml^{-1}. On 7th day of culture, the rotifer density remained almost similar in other tanks treated with P1 and P2, with rotifer densities of 295 and 288 nos. ml^{-1} respectively and the mean density in the control tanks was significantly low (95 nos. ml^{-1}) (Fig. 1). In control tanks, the density increased gradually reaching a maximum of 150 nos. ml^{-1} on 3rd day and then started decreasing from 4th day onwards (Fig. 1). The rotifer density in all the treated tanks increased gradually throughout the culture period. The tanks treated with three different probiotics exhibited significant variations in the rotifer densities ($p<0.05$). Rotifer density on 1st day of the culture was very low in P1 and P2 treated tanks, whereas, a significant increase was observed in P3 treated tanks.

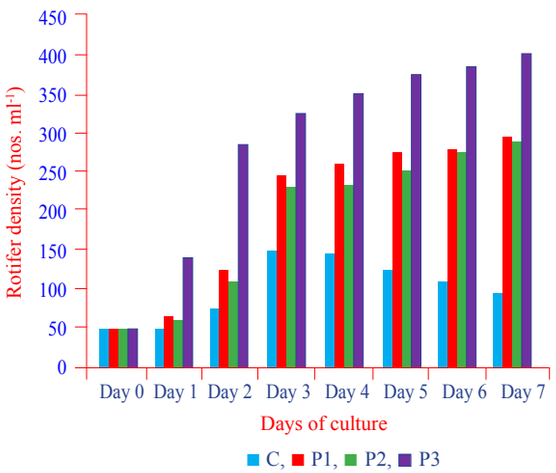

Fig. 1. Density of rotifers fed with *N. oculata* supplemented with three different commercial probiotics

Total bacterial counts in all the treatments increased continuously from 2nd day onwards, till the completion of the experiment. Total bacterial loads of control tanks ranged between 0.19 x 10^4 to 0.39 x 10^4 cfu ml^{-1}, whereas, the bacterial loads in probiotic treated tanks were found significantly high with a maximum of 0.21 x 10^5 cfu ml^{-1} in P3 treated tank. Total bacterial loads of water in P1, P2 and P3 treated tanks varied between 0.33 x 10^4 to 0.15 x 10^5 cfu ml^{-1}, 0.39 x 10^4 to 0.16 x 10^5 cfu ml^{-1} and 0.34 x 10^4 to 0.21 x 10^5 cfu ml^{-1} respectively (Fig. 2). From 2nd day onwards, significantly higher total bacterial loads were recorded in all the probiotic treated tanks compared to control tanks.

It was observed that probiotics played a significant role in controlling *Vibrios* in rotifer culture tanks. *Vibrio* loads of water in control tank ranged from 0.25 x 10^2 to 0.26 x 10^3 cfu ml^{-1} (Fig. 3). In probiotic treated tanks, the *Vibrio* loads reduced significantly with the days of culture. A significant variation between the three probiotic treatments and also between the days of culture ($p<0.05$) was observed. Out of the three probiotic treatments, P3 was found more effective in reducing the *Vibrio* loads and it completely eliminated the *Vibrios* from 5th day onwards. In P1 treated tanks, *Vibrio* loads started decreasing from

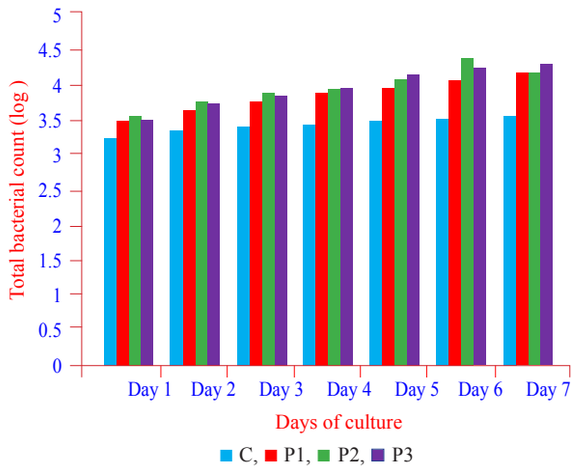

Fig. 2. Total bacterial count (cfu ml^{-1}) of water in rotifer tanks treated with three different commercial probiotics

2nd day onwards. At the end of the experiment, total *Vibrio* loads in the control and P1 treated tanks were 0.26 x 10^3 and 0.2 x 10^1 cfu ml^{-1} respectively (Fig. 3). However, on termination of the experiment, P2 and P3 treated tanks recorded zero *Vibrio* loads. *Vibrio* loads of P1 and P2 in the initial two days were almost similar but later, loads gradually reduced and found to be zero in P2 treated tanks. Complete elimination of *Vibrios* in P2 and P3 treated tanks was recorded on 6th and 5th day of culture respectively (Fig. 3). Complete elimination of the *Vibrios* was not observed in P1 treated tanks.

The water quality parameters recorded in the experimental tanks are given in Table 2. No significant difference was recorded in water temperature and salinity, between the treatments ($p>0.05$). But ammonia levels showed significant variation between treatments ($p<0.05$). A clear positive correlation was observed between the

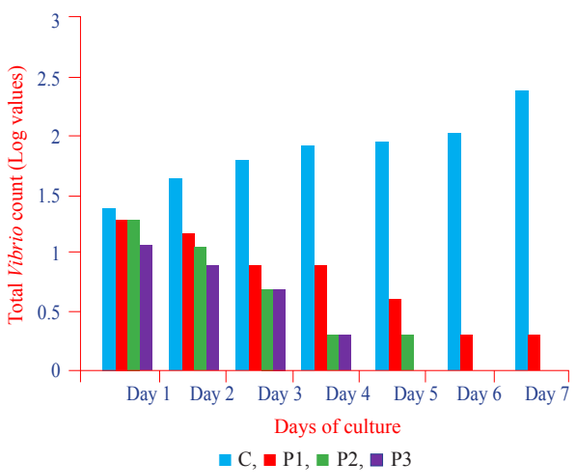

Fig. 3. Total *Vibrio* count (cfu ml^{-1}) of water in rotifer tanks treated with three different commercial probiotics

ammonia levels and occurrence of *Vibrios* in water of rotifer culture tanks in the different treatment groups (with r values of 0.89, 0.4, 0.88 and 0.79 for C, P1, P2 and P3 respectively). Higher *Vibrio* loads were recorded with increase of ammonia levels.

Results of the present study revealed significant influence of probiotic treatment on the rotifer density and also on the elimination of *Vibrio* loads of water in the culture tanks. *Bacillus* strains are the most suitable probiotics for aquaculture owing to their availability in all the environments and also in the gut of aquatic organisms (Hong *et al.,* 2005). Rotifer density was found enhanced with application of the probiotics in all the treatments, with a maximum of 400 nos. ml^{-1} in P3 treated tanks. Significantly ($p<0.05$) lower rotifer density was noticed in control tanks (95 nos. ml^{-1}) as compared to probiotic treated tanks on 7th day of culture. Zink *et al*. (2013) reported significantly high population density in probiotic treated rotifer batch cultures. Yu and Hirayama (1986) stated that *Bacillus* spp. could reduce production of pathogenic bacteria and improve rotifer population growth.

Murillo and Villamil (2011) recorded decrease in heterotrophic bacterial levels in rotifer cultures when supplemented with *Bacillus* strains. They found significant decrease in *Vibrio* levels after 3 and 6 days of treatment with *B. subtilis*. Our results also recorded a decreasing trend in *Vibrio* loads in the tanks treated with probiotics and complete elimination of *Vibrios* was observed from 5th day onwards in P3 treated tanks. The inhibition of harmful pathogenic bacteria with the application of probiotics could be due to the enzymes released by probiotic bacteria which might help to improve digestion in rotifers (Murillo and Villamil, 2011). Probiotic inclusion was found effective in the manipulation of bacterial communities in rotifer cultures (Zink *et al*., 2013). The total bacterial loads of water were found high in all the treatments during the present investigation, but the *Vibrio* loads were found to decrease in probiotic treated rotifer tanks with complete elimination of *Vibrio*s observed from 5th day of culture in P3 treated tanks.

The water quality parameters recorded in the present study were all within the limits but showed variations with days of culture and between the treatments ($p<0.05$). Ammonia (mg l^{-1}) levels were found to show impact on the occurrence of *Vibrio* loads in rotifer culture tanks. A strong positive correlation between ammonia levels and total *Vibrio* loads was recorded during the present experiment. Earlier reports also stated that *Bacillus* spp. reduces ammonia to levels that inhibit rotifer population

Table 2. Water quality parameters (Mean ± SD) recorded in experimental rotifer tanks

Days of culture	Treatment	Temperature (°C)	Salinity (ppt)	pH	Ammonia (mg l^{-1})
0	C	28.5±0.02	25±0	7.8 ± 0.01	0.01±0.002
	P1	28.6±0.01	25±1	7.8 ± 0.02	0.01±0.001
	P2	28.5±0.05	25±0	7.8 ± 0.02	0.01±0.0005
	P3	28.5±0.02	25±0	7.8 ± 0.02	0.01±0.001
1	C	28.5±0.05	25±1	7.8 ± 0.01	0.02±0.006
	P1	28.5±0.1	25±1	7.8 ± 0.02	0.02±0.005
	P2	28.6±0.05	25±1	7.8 ± 0.02	0.02±0.001
	P3	28.6±0.05	25±1	7.8 ± 0.02	0.01±0.001
2	C	29.1±0.2	26±0	7.9± 0.01	0.02±0.0025
	P1	29.3±0.05	26±1	7.9± 0.05	0.02±0.0028
	P2	29.2±0.4	26±0	7.9± 0.05	0.01±0.0005
	P3	29.1±0.05	26±1	7.9± 0.02	0.01±0.008
3	C	28.8±0.05	26±0	7.8 ± 0.02	0.04±0.005
	P1	28.7±0.1	26±1	7.9 ± 0.01	0.04±0.005
	P2	28.7±0.05	26±0	7.8 ± 0.01	0.01±0.002
	P3	28.8±0.05	26±1	7.9 ± 0.02	0.02±0.002
4	C	29.2±0.2	26±1	7.8 ± 0.02	0.04±0.002
	P1	29.1±0.05	26±1	7.8 ± 0.04	0.02±0.002
	P2	29.1±0.2	26±0	7.8 ± 0.02	0.01±0.006
	P3	29.2±0.05	26±1	7.8 ± 0.05	0.01±0.006
5	C	28.9±0.2	26±1	7.8 ± 0.03	0.06±0.008
	P1	28.8±0.1	26±1	7.8 ± 0.05	0.03±0.004
	P2	28.8±0.05	26±1	7.8 ± 0.04	0.01±0.001
	P3	28.9±0.1	26±1	7.8 ± 0.04	0
6	C	29.2±0.1	26±1	7.8 ± 0.04	0.08±0.002
	P1	29.3±0.05	26±0	7.8 ± 0.01	0.01±0.001
	P2	29.2±0.06	26±0	7.8 ± 0.01	0
	P3	29.3±0.05	26±0	7.8 ± 0.01	0
7	C	29.2±0.05	26±0	7.9 ± 0.02	0.08±0.005
	P1	29.1±0.1	26±0	7.9 ± 0.008	0.02±0.004
	P2	29.1±0.2	26±0	7.9 ± 0.01	0
	P3	29.1±0.05	26±0	7.9 ± 0.01	0

(Schulter and Groeneweg, 1985; Chen and Chen, 2001). Zink *et al*. (2013) reported significant decrease in DO levels in probiotic treated tanks owing to higher rotifer populations. The high bacterial loads and ammonia levels were the major factors contributing to reduction in the density of rotifers in control tanks. Total bacterial loads in treatment tanks were high in water due to additional bacterial inocula from probiotic bacterial supplementation, which might have led to elimination of *Vibrio* spp. from probiotic treated tanks.

Results of the present study, clearly indicate that application of probiotic bacteria could be used as an alternative for antibiotic treatment in elimination of *Vibrios* and also for enhancement of rotifer density in outdoor mass culture systems. However, further studies are needed to characterise the compounds that are responsible for the antagonistic activity of these probiotics against vibrios

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