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Research article

Do Multispecies Probiotics Modulate Microbiota in Finfish Polyculture System?

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ABSTRACT

The present research aimed to find out role of multispecies probiotics on water and gut microbiota modulation, water quality parameters, growth rate and total production of Indian major carp species in a polyculture system. Two treatments (T1 and T2) and a control (C) with three replications were stocked with *Labeo rohita*, *Catla catla*, and *Cirrihinus mrigala* at a ratio of 2:1:1.06 for this purpose in FMRT discipline pond complex of Khulna University in June 2022. The results showed that the mean hardness, ammonia, nitrate and nitrite were significantly improved by water probiotics compared to control. The water and gut bacterial analysis disclosed that total heterotropic bacterial loads were significantly higher in control than water and feed probiotics respectively. Additionally, results also depicted that feed and water probiotics significantly reduced *Vibrio* spp. and eliminated *Pseudomonas* spp. though no difference between two types of probiotics. However, water and feed probiotics could not able to lessen total load of *Bacillus cereus* in water. Average load of *Nitrosomonas spp.* and *Nitrobacter spp.* in water sample were significantly ($p<0.05$) higher in T1 and T2 than C. Moreover it also explicitized that feed probiotics could able to significantly improve the mean relative weight gain ($p<0.001$) and specific growth rate ($p<0.001$) of Indian major carp species as well as mean total production ($p<0.001$) at 5% level of significance.

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Introduction

Globally, aquaculture provides 56.7% of aquatic product. However, increasing productivity also emerged pathogens variety as well as bacterial resistance. Probiotics are live microbial feed supplement which advantageously promote the host animal by ameliorating gut microbial composition through different mechanisms like competitive exclusion for adhesion sites, upgrading intestinal mucosal barrier and boost up of gut immunomodulation system as well as environment by recycling nutrients and reducing organic matter accumulation. Hence, probiotics are better environment friendly alternative to antibiotics treatment, as application of antibiotics associated with problems like emergence of variety of pathogens as well as antibacterial resistance. Use of probiotics in human and animal nutrition as well as shrimp or prawn is well reported though a little work found in case of finfish (Fuller, 1992; Gatesoupe, 1999; Verschuere *et al*., 2000; Irianto and Austin, 2002a; Irianto and Austin, 2002b). Considering the on above fact, the potential of addition of probiotic in finfish culture needs to investigate. Therefore the aim of the research was to evaluate the role of multispecies probiotics on water and gut microbiota modulation, water quality parameters, growth rate and production of Indian major carp polyculture system.

Methodology

Experimental site and design

This experiment was performed in pond complex and different laboratories of FMRT discipline, Khulna University during June 2022 to September 2023. A completely randomized design was outlined (two different treatments and a control) to accomplish the field based and lab based research (Table 1).

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Table 1: Experimental design

Parameter	Control (C)	Treatment 1 (T1)	Treatment 2 (T2)
Pond size	109.184 m^2	109.184 m^2	109.184 m^2
Species (mean weight)	*Labeo rohita*, (20.06g) *Catla catla* (20.86g) and *Cirrhinus mrigala* (10.82g)	*L. rohita*, (20.06g) *C. catla* (20.86g) and *C. mrigala* (10.82g)	*L. rohita*, (20.06g) *C. catla* (20.86g) and *C. mrigala* (10.82g)
Stocking ratio	2:1: 1.06	2:1: 1.06	2:1: 1.06
Fertilization application	Urea 2.45, TSP 2.45, and Molasses-yeast mixture 3.09 (g/m^2/week)	Urea 2.45, TSP 2.45, and Molasses-yeast mixture 3.09 (g/m^2/week)	Urea 2.45, TSP 2.45, and Molasses-yeast mixture 3.09 (g/m^2/week)
Probiotic type	No	Feed probiotic	Water probiotic
Probiotic composition	Not Applicable	*Pseudomonas striata, Pseudomonas putida, Trichoderma harzianum, Aspergillus niger, Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus mesentricus, Streptococcus faecium, Clostridium sp., Yeast, Nitrosomonas* sp., *Nitrobacter* sp..	*Nitrosomonas* sp., *Nitrobacter* sp., *Pseudomonas denitrificans, Bacillus subtilis.*
Replication	3	3	3

Pre-stocking to post-stocking management

Rectangular earthen ponds fenced by nylon net with an average depth of 1m each were used. Eradication of unwanted species, liming (25g/m^2), fertilization (Table 1) etc. were conducted following standard aquaculture method. T1 and T2 were treated with commercial feed probiotic namely “BIOVET-SP” (marketed by Vetoquinol India Animal Health Pvt. Ltd. Hiranandani Business Park, Hiranandani Estate) and water probiotic namely “Nitrid” (manufactured by Symbiome organics Pvt. Ltd, India) respectively. The fingerlings were stocked in June 2022 after two weeks of fertilization. Stocking detailed was showed in Table 1. Feed was provided at rate of 3% body weight, half at the morning and rest half at the evening. Feed probiotic was added to feed in case of T1, water probiotic was added to water in T2 while no probiotic was added to C. Ponds were fertilized at every 7 days interval to hasten primary production while water parameters were checked monthly. Five individuals from each treatment are sampled monthly to check the growth and health condition as well as feed adjustment.

The following parameters were used to evaluate the growth of fishes:

Relative weight gain (%) = 100 × (Final weight − Initial weight)/Initial weight

Specific growth rate (%) = 100 × ln (Final weight/Initial weight)/days

Bacteriological analysis

The water samples were collected from ponds of C, T1 and T2 in well cleaned, derived and sterile bottles for bacteriological analysis while some fishes of different species were sacrificed for gut microbial analysis.

Bacteriological analysis was carried out for the isolation and enumeration of total heterotrophic bacteria (THB), *Nitrosomonas* spp., *Nitrobacter* spp., *Bacillus cereus*, *Pseudomonas* spp. and *Vibrio* spp. from water samples while total heterotrophic bacteria (THB), *B. cereus*., *Pseudomonas* spp. and *Vibrio* spp. from gut of fin fishes were analysed in three ponds of C, T1 and T2 (Figure 1 and Table 2).

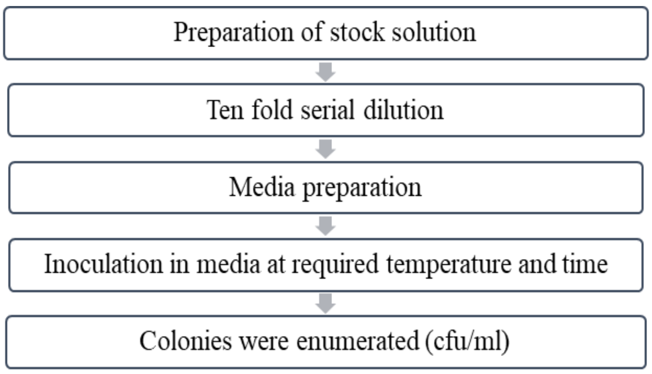

Figure.1. General scheme for bacterial culture and colony counting

Bacteriological analysis for water

Column water samples were used for the analysis of total heterotrophic bacteria (THB), *B. cereus*, *Pseudomonas* spp., *Vibrio* spp whereas bottom waters for *Nitrosomonas* spp. and *Nitrobacter* spp. Ten fold serial dilution was performed. Then media were prepared for bacterial colony counting. 0.1 ml of the sample was inoculated into the media, incubated and bacterial load was counted (Figure 1 & Table 2).

Table 2: Media details

Type	Media	Company name	Incubation temperature and time
Total heterotrophic bacteria (THB)	Plate count agar (Standard Methods)	Hi-Media, Mumbai	37ºC for 24h
B. cereus	Bacillus Cereus Agar Base		37ºC for 24h
Pseudomonas spp.	Pseudomonas Agar Base		37ºC for 24h
Vibrio spp.	TCBS Agar		37ºC for 24h
Nitrosomonas spp.	Winogradsky Phase-1 Medium		28±2ºC for 48 h.
Nitrobacter spp.	Winogradsky Phase-2 Medium		28±2ºC for 48 h.

Bacteriological analysis for gut

One (1) g gut content was mashed and alkaline peptone water was added at a ratio of 1:1 and was mixed homogenously using vortex. Then the solution was taken in an eppendorf tube and centrifuged at 4000 rpm for 5 minutes. After centrifugation the supernatant liquid portion was collected with micropipette and taken into falcon tubes. Stock solution was being stored in -20°C for further uses. Then ten fold serial dilution was conducted. 0.1 ml of the sample was inoculated into the prepared media, incubated and bacterial load enumerated (Table 2 & Figure 1).

Final harvesting

Ponds were completely harvested by seine net on September 2023. All fishes were counted and weighed separately to assess survival rate and production.

a) Survival rate (%) = No of fish harvested/ No of fish stocked x 100
b) Biomass production (kg/ha)= [(Number of fish harvested x Individual weight of fish) x 10000] / 109.184

Statistical analysis

The regular recorded data on sampling, water quality and final harvest were gathered and analyzed by using IBM SPSS (version 25.0). Analysis of variance (one-way and two-way ANOVA) and the least significant difference (LSD) test were performed as appropriate.

Results and Discussion

Water quality

The results of water quality parameters during culture period are presented in Table 3. However, It observed that some parameters were deviated from standard range and there were no significant difference ($p>0.05$) among T1, T2 and C in case of dissolve oxygen, pH, temperature, and alkalinity. The mean of the other parameters like hardness, turbidity, ammonia and nitrite were significantly different ($p<0.05$) among two treatments and control (Table 3). Result of the present study showed that water probiotic was able to reduce nitrite level in water, while feed probiotic was responsible for increased level of nitrite in water. Table 3 shows that treated probiotics had played significant role in maintaining optimum level of different water quality parameters. This finding also supported by Sunitha and Krishna (2016). Though the effectiveness of probiotics is controlled by method of application like water medication or in feed medication.

Table 3: Role of probiotics on water quality parameters in different treatments

Parameters	C		T1		T2		P value
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	
pH	7.41 ± 0.34	7.00 - 8.00	7.38 ± 0.61	6.60 - 8.20	7.56±0.20	7.20 - 7.90	0.168
DO (ppm)	5.85 ± 0.76	5.00 - 7.00	5.66 ± 0.70	4.80 - 7.00	5.70±0.61	4.20 - 6.40	0.516
Temperature (˚C)	25.43± 6.45	13 - 33	25.43 ± 6.45	13 - 33	24.43±6.44	13 - 32	0.775
Alkalinity (mg/L)	223.75± 38.26	150 - 165	237.65 ± 50.86	155 - 320	243.28±46.06	175 - 320	0.213
Hardness (mg/L)	291.87±55.02	120 - 330	364.37 ± 29.58	280 - 395	232.34±78.31	148 - 320	0.001
Turbidity (inch)	22.31±6.08	12.20 - 29.00	23.77 ± 3.93	20.00-30.57	17.48±5.78	6.90 - 22.50	0.001
Conductivity (µS /cm)	1497.22 ± 197.78	1030 - 1740	1963.40 ± 424.59	1240 - 2625	1586.71±258.06	1230 - 2180	0.001
NH_3 (ppm)	1.34 ± 1.19	0.19 - 2.89	0.26 ± 0.12	0.04 - 0.79	0.40±0.11	0.16 - 0.88	0.001
Nitrate (ppm)	1.07 ± 5.86	0.07 - 11.81	0.77 ± 4.35	0.08 - 9.82	0.75±2.85	0.09 - 5.69	0.045
Nitrite (ppm)	0.63 ± 0.62	0.02 - 1.67	1.80 ± 2.35	0.02 - 5.60	0.59±0.52	0.01 - 1.40	0.001

$p < 0.05$ indicates significant difference at 5% level of significance

Bacteriological analysis

The total heterotrophic bacterial load of water of T1 and T2 was significantly lower ($p<0.05$) than control (Table 4 and 5). Additionally, results also depicted that feed and water probiotics significantly effectively reduced pathogenic bacteria *Vibrio* spp. and eliminated *Pseudomonas* spp. though no difference between two types of probiotics bacteria at 5% level of significance, which was supported by Subharanjani *et al.* (2015). Moreover, feed and water probiotics boosted nitrifying bacterial loads of water than control at 5% level of significance which indicated that the presence of lower concentrations of ammonia and nitrite in T1 and T2 (accept nitrate) which might suppressed the pathogenic bacterial load which was supported by Sunitha and Krishna (2016). Liu *et al.* (2020) stated that microbial nitrification produces nitrate, which is used by phytoplankton and recycled. *Nitrosomonas* spp. oxidizes ammonia into nitrite while *Nitrobacter* spp. oxidized to nitrate by nitrite oxidoreductase. Hence nitrifying bacteria like *Nitrosomonas* spp. and *Nitrobacter* spp. helps to prevent exposing farmed aquatic animals to high concentrations of ammonia and nitrite which ultimately lead to good water quality. Above literature supported the recent findings (Liu *et al.*, 2020). However, the present study showed that water and feed probiotics could not able to lessen total load of *B. cereus* in water.

Table 4: Microbiota in water sample in different treatments

Types	C	T1	T2	P value
Total heterotrophic bacteria (THB)	247.67±13.61 × 10^8	148.67±26.54 × 10^6	168.00±61.44 × 10^6	0.001
B. cereus	65±8.71×10^2	150.67±36.5×10^5	104.67±27.53×10^4	0.001
Nitrosomonas spp.	139.67±7.77 × 10^3	82.00±9.17 ×10^5	55.00±13.53 × 10^5	0.002
Nitrobacter spp.	51.33±10.41 × 10^2	145.67±12.06 ×10^4	165.00±51.57 × 10^4	0.029
Vibrio spp.	208.67±9.71 × 10^6	148.33±44.86×10^3	62.67±12.10×10^3	0.001
Pseudomonas spp.	85.33±20.74 × 10^5	00	00	0.001

p < 0.05 indicates significant difference

Table 5: Microbiota in gut sample in different treatments

Types	Species	C	Total	T1	Total	T2	Total	P value
Total heterotrophic bacteria (THB)	*L. rohita*	106.67 ± 25.70×10^8	139.89 ± 43.96×10^8	72.67 ± 17.79×10^5	88.89 ± 29.28×10^3	176 ± 53.84×10^6	126.11 ± 41.73×10^6	0.001
	C. catla	153 ± 36.43×10^6		141 ± 32.79×10		124.33 ± 24.54×10^6		0.569
	C. mrigala	160 ± 69.74×10^6		53 ± 37.27×10		78 ± 46.81×10^6		0.026
B. cereus	*L. rohita*	67 ± 20.07×10^2	123.11± 26.40×10^2	63.67 ± 27.47×10^4	79.89 ± 19.0×10^4	168 ± 75.36×10^5	144.57 ± 42.4×10^5	0.005
	C. catla	197 ± 13.08×10^2		117 ± 21.00×10^4		212.67 ± 14.57×10^4		0.001
	C. mrigala	105.33 ± 46.06×10^2		59 ± 8.54×10^4		53 ± 37.27×10^4		0.034
Pseudomonas spp.	*L. rohita*	74 ± 42.67×10^6	71.33 ± 35.12×10^6	00	00	00	00	0.016
	C. catla	86.33 ± 57.01×10^5		00		00		0.028
	C. mrigala	53.67 ± 5.69×10^5		00		00		0.001
Vibrio spp.	*L. rohita*	37.33 ± 6.66×10^6	92.22 ± 32.02 ×10^6	189.33 ± 85.24×10^3	116 ± 41.31×10^3	36.67 ± 7.37×10^3	264 ± 21.76×10^3	0.001
	C. catla	204 ± 54.08×10^5		78 ± 22.54×10^3		90.33 ± 25.66×10^3		0.001
	C. mrigala	35.33 ± 4.17×10^5		80.67 ± 16.17×10^3		137 ± 32.25×10^3		0.001

p < 0.05 indicates significantly different at 5% level of significance

Table 6: Posthoc analysis of gut microbiota in different treatments

Bacterial category	*L. rohita*			*C. catla*			*C. mrigala*		
	CT1	CT2	T1T2	CT1	CT2	T1T2	CT1	CT2	T1T2
Total heterotrophic bacteria (THB)	0.001	0.001	0.894	0.659	0.310	0.543	0.009	0.084	0.144
B. cereus	0.865	0.003	0.004	0.001	0.001	0.001	0.018	0.028	0.751
Pseudomonas spp.	0.010	0.010	1.000	0.018	0.018	1.000	0.001	0.001	1.000
Vibrio spp.	0.001	0.001	0.963	0.001	0.001	0.996	0.001	0.001	0.782

p < 0.05 indicates significant difference

Survival, Growth and Production

Details of growth performance and production rate of fish are presented in Table 7. A two-way ANOVA was conducted that examined the effect of treatment and species on specific growth rate (% d^{-1}) and relative weight gain (%). There was a statistically significant interaction between the effects of treatment and species on relative weight gain (p = <0.001) and specific growth rate (p = <0.001). Relative weight gain (%) and specific growth rate (% d^{-1}) of catla were significantly higher than mrigal and rui (p= <0.001) in feed probiotics treated ponds than water probiotics treated pond (p= <0.001) and control ponds (p= <0.001) respectively.

Table 7: Effects of probiotics on growth performance of Indian major carps in polyculture system

Parameters	Species	C	T1	T2	P value
Relative weight gain (%)	Rui	1127.39±126.68	1411.64±217.23	1300.62±154.53	<0.001
	Catla	1132.51±324.80	2025.77±553.28	1750.99±375.11	<0.001
	Mrigal	1576.26±186.10	1840.90±255.98	1632.55±265.90	<0.001
	Total	1225.41±272.31	1648.3±424.84	1476.04±313.32	<0.001
Specific growth rate (% d^{-1})	Rui	0.60±0.03	0.64±0.04	0.63±0.03	<0.001
	Catla	0.60±0.07	0.72±0.07	0.69±0.05	<0.001
	Mrigal	0.67±0.03	0.70±0.03	0.68±0.04	<0.002
	Total	0.61±0.05	0.68±0.06	0.65±0.05	<0.001
Survival rate (%)	Rui	28.28±0.88	28.79±0.00	27.78±0.88	0.296
	Catla	22.86±0.00	22.86±0.00	20.95±1.65	0.079
	Mrigal	22.22±1.75	23.23±0.58	23.23±1.75	0.729
	Total	24.45±3.05	24.96±3.01	23.99±3.27	0.236
Production	Rui	420.94±20.37	527.67±16.53	473.58±14.28	<0.001
(kg/ha)	Catla	188.37±4.82	305.61±30.29	255.35±35.02	0.005
	Mrigal	124.86±15.52	147.45±13.71	132.74±11.63	0.204
	Total	244.73±135.62	326.91±166.45	287.22±150.81	<0.001

p< 0.05 refers significant difference among different treatments in each row

Results also showed that there were no significance difference of mean total survivality among C, T1 and T2. There was a statistically significant interaction between the effects of treatments and species on fish production.

The mean total production of T1 (p <0.001) was significantly higher than T2 and C respectively. Additionally it also showed that production of rui (p <0.001) was significantly higher in T1 than T2 and C respectively than catla and mrigal. Results also showed that there were no significance difference of mean production of mrigal among C, T1 and T2 (p=0.204).

A study by Ngan (2023) increases in farm productivity can be supported by using healthy stock, more effective use of inputs (e.g., feed, probiotics, fertilizers), and improved disease management, which is similar to this study. In this study, there also had higher production in different probiotics administration

A study by Ferdous (2023), the results showed that weight gain (WG) and specific growth rate (SGR) were decreased significantly in T1 (probiotics), while those substantially increased in T2 (probiotics) compared to C. On the other hand, WG and SGR were regained as compared to C in T2. The multi-species probiotics are recognized to improve the growth performance of fish and shellfish species through modification of microbial community, exclusions of the pathogen, upgradation of a non-specific immune response, and disease resistance (Li *et al.*, 2009). The present study was demonstrated that probiotic had significant effect on the growth of fish (Yaqin *et al*., 2020).

Conclusion

The feed and water probiotics played a major role in controlling pathogenic bacterial load, growth rate and total production rate by maintaining better water quality and gut microbial composition.

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Conflict of Interest

None of the authors present any conflicts of interest.

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