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Experimental Studies on Impact of Dietary Supplementation of Single-strain versus Multi-strain Probiotic Consortia on Growth and Yield of Red Snapper, *Lutjanus argentimaculatus* in Marine Cage Farming

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Abstract

A study was undertaken on the effect of probiotic bacteria, belonging to genera *Bacillus* and *Streptococcus,* on survival, growth and production of wild-collected red snapper, *Lutjanus argentimaculatus* in marine cages for a period of 90 days. Wild-collected juvenile fish with an initial biomass of 0.79 kg/m^3 were stocked in marine cages at Karwar, India. All the fish (control and probiotic treated) were fed with pellet feed at 10% fish biomass. The feed was supplemented with multi-strain probiotic combination (T_1) and with single-strain probiotics (T_2 and T_3). Growth, survival of fish, and microbiological parameters were monitored at fortnightly intervals. After 90 days of culture, the final mean weight (g), Absolute Growth Rate (AGR g/day), and Specific Growth Rate (SGR%/day) of fish were recorded as 250 ± 0.5 g; 2.23 ± 0.012 g/day; 0.79 ± 0.052%/day, respectively, in T_1. A significant difference ($P < 0.05$) among the different treatments was recorded, and T_1 showed a higher growth rate. The highest survival rate was recorded in T_1 and T_2 and varied significantly among the treatments ($P < 0.05$). Microbiological analysis of fish gut revealed the presence of *Bacillus* species as the dominant group in T_1, followed by T_2. A significant variation ($P < 0.05$) was recorded in total bacterial and *Bacillus* loads among all the treatments. The results of the study indicated that the application of 0.1% of multi-strain probiotics as a supplementary diet is economically viable and also enhances the survival, growth, and production of cage-cultured *L. argentimaculatus*.

Keywords: Cage Culture, Efficacy, Growth, Multi-strain Probiotics, Single-strain Probiotics, Red Snapper, Yield

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INTRODUCTION

Cage farming of marine finfish in India is expanding very rapidly in the mariculture sector as an additional livelihood for the upliftment of the economic status of coastal communities. Increasing the demand for intensification and commercialization of mariculture production has led to the emergence of environmental issues and disease occurrences. To manage the culture systems for sustainable production, applying probiotics is the best tool to achieve better growth rate, survival, and disease resistance of fish. Marine microorganisms have a greater capacity for adhesion to gastrointestinal mucus and tissues, which may enhance host digestion, and are identified as a potential source of enzymes.[1] Probiotics in aquaculture have gained considerable attention due to their ability to enhance survival, feed efficacy, and growth performance.[2-4] Probiotic bacteria, which can produce inhibitory substances, can suppress the growth of pathogenic microorganisms and improves stress tolerance in fish under high stocking density.[5-11] Probiotic bacteria, like the *Bacillus* group, secrete various exogenous enzymes such as amylase, lipase, protease, and cellulose and also stimulate endogenous enzyme production that helps digestion, absorption, and assimilation. A wide range of bacterial species as probiotics, including *Bacillus* sp., *Pseudomonas* sp., *Pseudoalteromonas* sp., *Alteromonas macleodii, Enterococcus faecium,* and *Lactobacillus* sp., *Bifidobacterium* spp., *Lactococcus* spp., *Shewanella* spp., *Paenibacillus* spp., *Alteromonas* spp., *Saccharomyces cerevisiae, Streptococcus phocae,* and *Pediococcus acidilactici* are in the application in aquaculture systems.[10,12,13] Among *Bacillus* spp. The most widely used species in aquaculture include *Bacillus subtilis*, *B. cereus, B. pumilis*, *B. coagulans*, *B. clausii, B. megaterium, B. licheniformis, B. amyloliquifaciens*, *B. circulans, B. aerius* and *B. polymyxa*. Several reports on the effect of *Bacillus* species on the growth and feeding performance of fish indicated application of probiotics in aquaculture is the best alternative to antibiotics.[2,11,14-28] Improved growth with minimum cost, improvement in reproduction, hematology, immune response, disease and stress resistance, and better proximate composition was reported in different fish species on the application of *Bacillus* spp. as probiotics.[29] The application of probiotic bacteria also improves the reproductive performance of fish in terms of spawning female percentage, egg production, fry, fingerling production, and larval rearing.[12,29,30] The performance of multi-strain probiotics is better than specific probiotic strains, and each could increase the effect of the other strains.[23,31-33] Probiotic application in cage culture could be a dynamic approach to promote cost-effective culture. Few studies proved that dietary probiotic supplementation is economically feasible, as it augments survival, growth, health, production, and yield for a profitable cage culture.[34-37] Probiotic supplementation also improves intestinal health, with the abundance of supplemented probiotic bacteria, and reduction of pathogens, and this enhances the immunity and health of the fish.[19,32,38-43] Although several commercial probiotic bacteria are being used in fish culture, the role of its application in marine cage farming, especially the host specificity of strains and their dosage at different stages of the culture period, needs to be investigated in detail. The present study was carried out to understand the efficacy and the economic viability of multi-strain probiotic bacteria on survival, growth, production, and health status of the gut of cage-cultured red snapper, *Lutjanus argentimaculatus*.

MATERIALS AND METHODS

The present work was undertaken to study the effect of probiotic bacteria belonging to two different genera *Bacillus and Streptococci* on the survival, growth, and production of *L. argentimaculatus.*

The experiment was carried out in four 3m dia. cages. Experimental cages (3m diameter GI cages) were fabricated with galvanized iron (GI) pipe (1.5-inch diameter) frame and were floated in offshore of Karwar waters off the Arabian sea. The submerged volume of each cage was 35 m^3. Six thousand wild red snapper fish (*L. argentimaculatus*) were collected from Karwar and Udupi waters. All the fish were acclimatized for fifteen days in hapas before stocking into the cages. Acclimatized fishes were stocked randomly in cages at a density of 0.79 kg m^{-3} with an average initial body weight of 50 ± 0.06 g.

Preparation of the feed

Pure isolates of *Bacillus subtilis, B. licheniformes, B. cereus* and *Streptococcus faecium* were grown in LB broth using a shaking incubator at 30 °C for 48 hrs. The cultures were then centrifuged at 3000 g for 10 min at 4 °C. The supernatant was discarded and the pelleted bacteria were re-suspended and washed thrice in sterile Normal Saline Solution (NSS, 0.9% NaCl). The cell densities of the suspensions were estimated by using spectrophotometer at 600 nm and also correlated to the colony-forming unit (CFU) using the spread-plate technique. These suspensions were kept at 4 °C until used.

Experimental fishes (Control, T_1, T_2 and T_3) were fed with a pelleted diet (Growel Fish Feed Pvt. Ltd. Andhra Pradesh, India) with 45% protein and 10% fat. Fish in all the treatments were fed with pellet feed @ 10% of fish biomass. In experimental treatments (T_1, T_2, and T_3), the feed was supplemented with multi-strain probiotics (T_1: *Bacillus subtilis, Bacillus cereus, B. licheniformes,* and *Streptococcus faecium*) and single strain probiotics (T_2*: Bacillus subtilis* and T_3*: Streptococcus faecium*). The probiotics, incorporated with vitamins, were given at a concentration of 0.5% of the feed for the first 30 days of culture and 0.2% and 0.1% of the feed for the next two-month culture period, respectively, in all the treatments (Table 1). The required quantity of probiotics was diluted, sprayed over the commercial pelleted feed, mixed uniformly, and air-dried. Triplicates were maintained for all the treatments and the fish were fed twice daily as per the experimental design. All the experimental cages were maintained in good condition with regular cleaning of nets and frames.

Growth and survival analysis

Fishes in all the cages were regularly monitored and observed visually for their external behaviour, colorations, movement, and presence of any infections or external damage. Sampling was done at fortnight intervals to measure growth parameters and survival of fish. A random sample of fish from each cage was done, and fishes were weighed with digital balance. Experimental fishes were harvested after three months of culture from each cage. Harvested fishes were counted and weighed individually to determine the mean final weight (g), survival (%), and production (kg). Percent Survival (%) and growth parameters viz., Mean Final weight (g), Average Weight gain (g), Average Daily Growth Rate (AGR g/day), % Weight gain, Specific Growth Rate (SGR% /day), and FCR were calculated by using standard formulas.[15,16]

The following formulas were used:

Weight gain (g): Final weight - Initial weight

% Weight gain WG (g) = 100 x (W2 - W1) / W1

Average Daily Growth Rate: (W2 - W1)/T

Specific Growth Rate (SGR) (%) = 100 x (In W2 - In W1) / T

FCR = Feed intake/Weight gain

where W1 and W2 are the initial and final weights, respectively, and T is the number of feeding days.

Total Biomass Increase (kg) = number of fishes harvested / average final weight increase of fishes

Water quality analysis

Water quality parameters (Temperature, Salinity, Dissolved Oxygen, Ammonia, Nitrate, and Nitrite) were analyzed at fortnightly intervals by using standard methods given in American Public Health Association.

Microbiological analysis

Microbiological parameters, such as Total Viable Heterotrophic Bacterial Count (TVHC), and Total presumptive counts of *Vibrios*, *Bacillus*, and *Streptococcus,* were estimated from the fish gut of all the treatments and control. The isolated colonies were identified by morphological and biochemical tests using standard methods.[44]

The experiment was conducted with the approval of Ethical Committee of the Institute and all the guidelines and protocols were followed as per International standards.

Yield and economic analysis

Economic analysis of the different treatments was evaluated on the basis of fixed and variable costs such as cage fabrication cost, snapper seed cost, feed cost, maintenance and labor cost, and revenue from the sale of harvested red snapper.

The following equations were used to calculate costs and returns.

Gross Revenue (GR) = Quantity Produced (Kg) × Price realised (Rs/Kg)
Net Profit = Gross revenue – Total cost of production
Rate of return on operating investment (RROI) = Gross revenue / Variable cost
Rate of return on capital investment (RRCI) = Gross revenue / Fixed cost
Benefit-cost ratio (BCR) = Gross revenue / Total cost of production

Statistical analysis

The data generated from the present work was analyzed using SPSS (Statistical Package for Social Science) version 2.0. The survival rate, growth, and yield variables were analyzed using analysis of variance (ANOVA) to compare the treatment mean at a 5% level of significance. Correlation, linear regression, and coefficient of determination (r^2) were conducted to predict and interpret the data at different probiotic concentrations.

RESULTS

Growth and survival analysis

Experiments on the efficacy of multi-strain (T_1) and single-strain (T_2 and T_3) probiotics revealed probiotic supplementation had a significant impact on the survival and growth parameters of red snapper. Variations in mean final weight (g) between the treatments were found significant, with the highest value recorded in T_1 (250 ± 0.5 g), followed by T_2 (185 ± 2.8 g) and T_3 (152 ± 3.3 g), and the lowest was recorded in control diet (135 ± 4.1 g). Average Daily Growth

Table 1. Details of concentrations of probiotics and additives used in the experiment

No.	Probiotic	Concentration	Additives	Concentration (mg of probiotic/ g of feed)
1.	*Bacillus subtilis* + *Bacillus licheniformis* + *Bacillus cereus* + *Streptococcus faecium*	1 x 10^{10} CFU/g	Vitamin C + Vitamin B6	5 mg + 5 mg
2.	*Bacillus subtilis*	1 x 10^{10} CFU/g	Vitamin C + Vitamin B6	5 mg + 5 mg
3.	*Streptococcus faecium*	1 x 10^{10} CFU/g	Vitamin C + Vitamin B6	5 mg + 5 mg

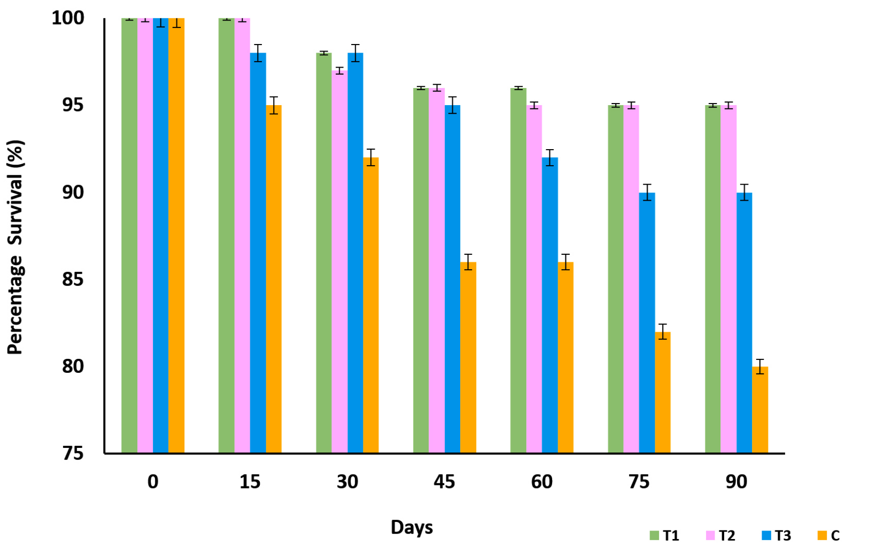


Figure 1. Percentage survival (%) of *L. argentimaculatus* supplemented with single and multi-strain probiotics

Table 2. Details of growth parameters of *L. argentimaculatus* supplemented with multi-strain and single-strain probiotics at 90 days of culture

Parameter	T_1	T_2	T_3	C
Initial biomass (kg/m^3)	0.79 ± 0.01	0.79 ± 0.01	0.79 ± 0.01	0.79 ± 0.03
Final biomass (kg/m^3)	3.39 ± 0.18	2.51 ± 0.14	1.95 ± 0.12	1.54 ± 0.11
Percent Survival (%)	95 ± 3	95 ± 2	90 ± 1	80 ± 2
Mean initial weight (g)	50 ± 0.06	50 ± 0.06	50 ± 0.06	50 ± 0.06
Mean final weight (g)	250 ± 0.5	185 ± 2.8	152 ± 3.3	135 ± 4.1
Average Daily Growth Rate (g/day)	2.23 ± 0.012	1.5 ± 0.08	1.14 ± 0.05	0.94 ± 0.12
Weight gain (%)	400	270	204	170
Specific Growth Rate (%/day)	0.79 ± 0.052	0.63 ± 0.06	0.54 ± 0.07	0.48 ± 0.18
FCR	1.04 ± 0.015	1.24 ± 0.05	1.58 ± 0.04	1.97 ± 0.14

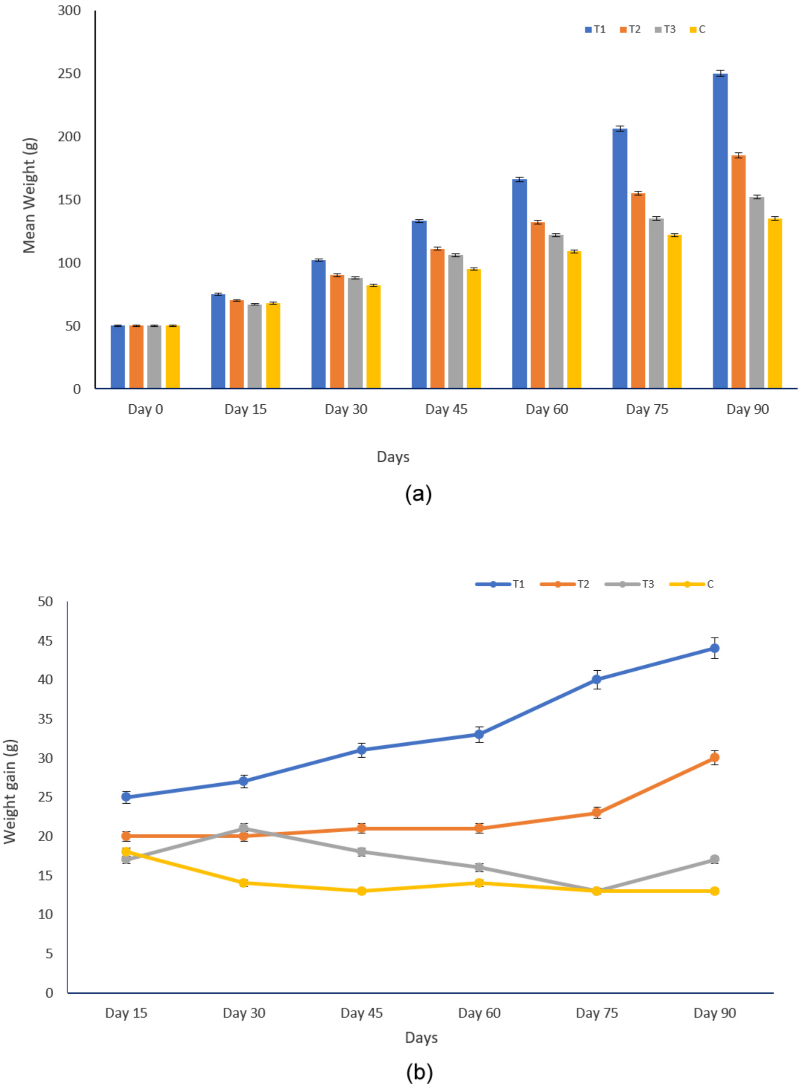


Figure 2. Variations in Mean weight (g) and weight gain (g) of *L. argentimaculatus* supplemented with probiotics (a & b)

Rate, specific growth rate, and % weight gain of fish were found to be significantly high in T_1. A significant increase was recorded in mean final weight (g), % weight gain, and specific growth rate (%SGR) of fish supplemented with multi-strain probiotics (T_1) compared to control and single-strain supplementations (T_2 and T_3). The feed conversion ratio of T_1 was observed as the lowest (1.04 ± 0.015), and there was a significant variation with control (1.97 ± 0.14) and other treatments at 90 days of the experimental culture period (Table 2).

Percentage survival of fish was significantly higher in T_1 (95%) and T_2 (95%), followed by T_3 (90%) and control (80%) at 90 days of culture (Figure 1). A positive correlation (r = 0.98, 0.99, 0.98 in T_1, T_2, T_3, respectively) was observed between % Survival and concentration of probiotic bacteria supplemented in all the three treatments. A significant variation in the percent survival of fish was recorded between the treatments (P = 0.063141) and also between days of culture (P = 0.00498).

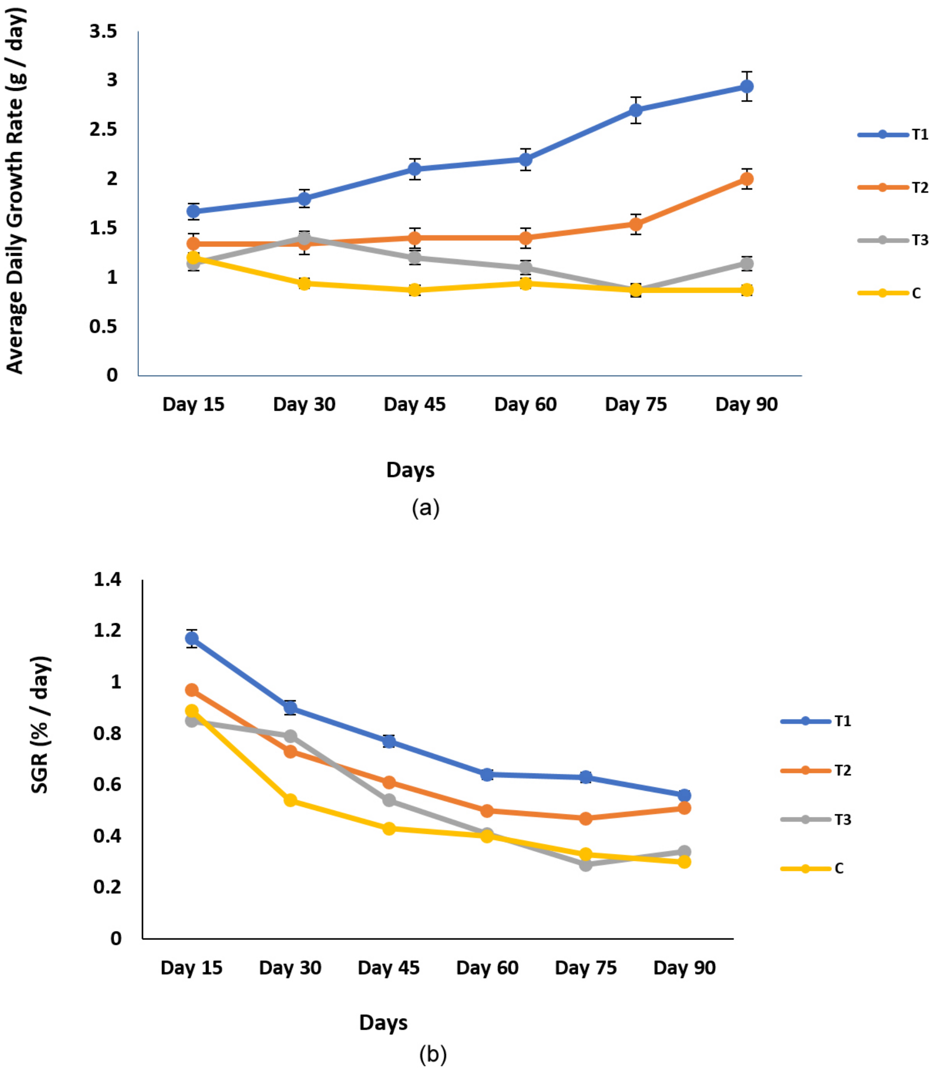
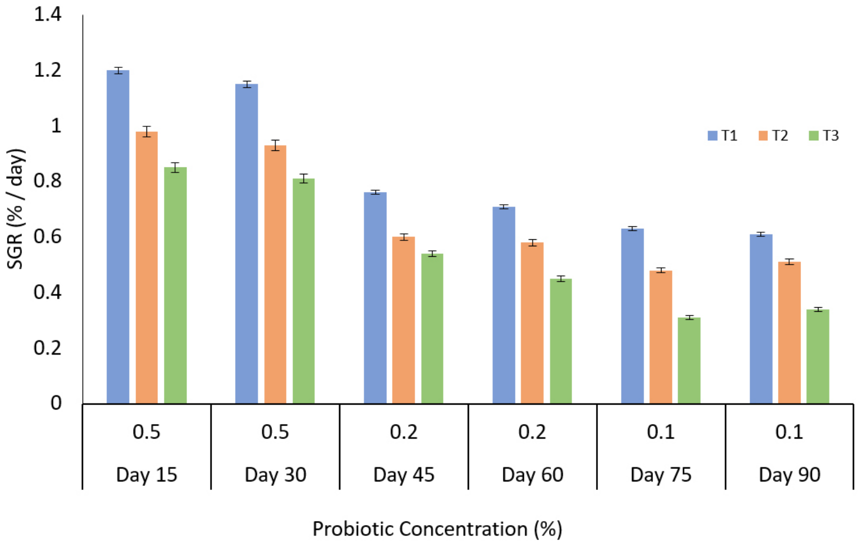


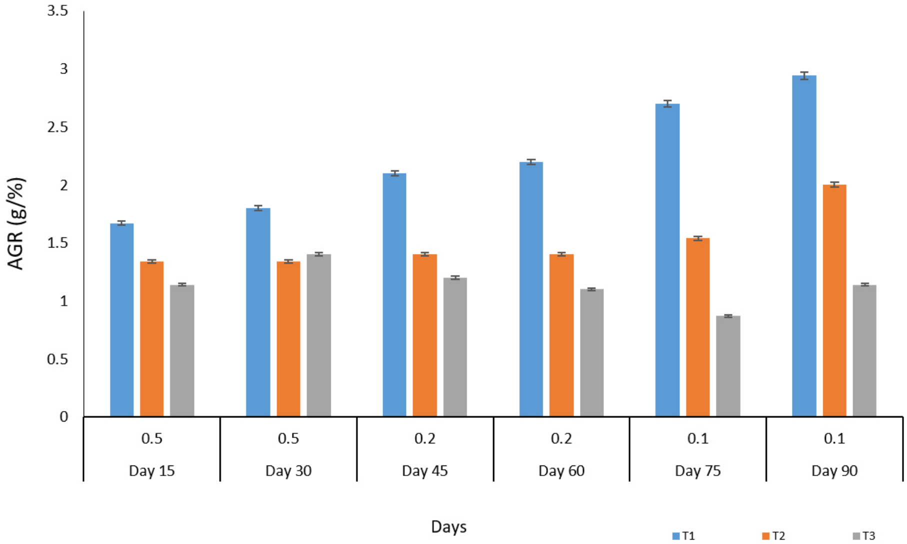
Figure 3. Variations in Average Daily Growth Rate (AGR g /day) and SGR (% /day) of *L. argentimaculatus* fed with three different probiotics (a & b)

The mean final weight (g) of red snapper supplemented with probiotics varied significantly between treatments and days of culture ($P < 0.05$). A gradual increasing trend in mean weight (g) and weight gain (g /day) of fish was observed in T_1 and T_2. In T_3, the weight gain was initially increased up to 30 days of culture and later showed a decline up to 75 days of the culture period (Figure 2). In control, no consistent trend was recorded between days of culture.

Average Daily Growth Rate (2.23 ± 0.012 g /day), % Weight gain (400), and Specific Growth



(a)



(b)

Figure 4. Variations in Specific Growth Rate (% /day) and Average Daily Growth Rate (g /day) of *L. argentimaculatus* supplemented with three different probiotic concentrations between treatments

Table 3. Water quality parameters in different treatments during the experimental culture period

Parameter	T1	T2	T3	C
Temperature (°C)	29.1 ± 0.2 to 30 ± 0.2	29.3 ± 0.3 to 30 ± 0.4	29.0 ± 0.4 to 30 ± 0.6	29.5 ± 0.1 to 30 ± 0.4
Salinity (‰)	28.2 ± 2.2 to 29.4 ± 1.2	28.8 ± 1.2 to 29.3 ± 4.1	30.1 ± 3.2 to 31.1 ± 0.2	30.2 ± 2.4 to 31.4 ± 1.1
Dissolved Oxygen (mg/l)	5.4 ± 0.3 to 5.5 ± 0.8	5.2 ± 0.2 to 5.6 ± 0.1	4.8 ± 0.1 to 5.1 ± 0.2	4.2 ± 0.1 to 4.6 ± 0.2
pH	8.2 ± 0.01 to 8.2 ± 0.02	8.1 ± 0.02 to 8.2 ± 0.03	8.1 ± 0.01 to 8.1 ± 0.03	8.1 ± 0.02 to 8.1 ± 0.03
Ammonia) (mg /l)	0.01 ± 0.001 to 0.014 ± 0.003	0.02 ± 0.003 to 0.028 ± 0.001	0.05 ± 0.005 to 0.052 ± 0.001	0.06 ± 0.002 to 0.064 ± 0.002
Nitrate (mg /l)	1.1 ± 0.2 to 1.3 ± 0.1	1.4 ± 0.3 to 1.5 ± 0.2	1.8 ± 0.6 to 2.1 ± 0.18	2.8 ± 0.5 to 3.1 ± 0.23
Nitrite (mg /l)	0.2 ± 0.02 to 0.3 ± 0.011	0.4 ± 0.04 to 0.45 ± 0.022	0.4 ± 0.05 to 0.52 ± 0.06	0.6 ± 0.02 to 0.65 ± 0.03

Rate (0.79 ± 0.052% /day) of fish at 90 days of culture period were significantly higher in T_1, followed by T_2 and T_3, whereas, in control, the lowest growth rate was recorded (AGR = 0.94 ± 0.12 g/day, SGR = 0.48 ± 0.18%/day). The % weight gain in the control diet was significantly lower than in all other treatments supplemented with probiotics. AGR of fish varied significantly between the treatments and days of culture (P = 1.14759E-05). A gradual increase of AGR with days of culture was recorded in T_1 and T_2, whereas T_3 and Control showed a decline in AGR from 45 days of culture. The Specific Growth rate of the red snapper at 90 days of culture was significantly higher (0.79 ± 0.052%/day) in T_1 but was insignificant in T_2 (0.63 ± 0.06%/day). SGR was significantly low in the control (0.48 ± 0.18%/day) compared to T_1 (Figure 3) (P = 1.4038E-09; P = 4.65429E-07).

Variations in SGR ($\%day^{-1}$) and AGR ($gday^{-1}$) between the treatments of different probiotic supplementations: The variations in SGR ($\%day^{-1}$) and AGR (day^{-1}) with probiotic concentrations at different intervals of the experimental culture period were found highly significant ($P < 0.05$; P-value <0.01) between treatments and days of culture. In all the treatments, the SGR was found to decrease with a reduced probiotic concentration. Still, the growth rate in all concentrations was consistently higher in T_1 compared to other probiotics (Figure 4a). AGR of T_1 and T_2 increased with days of culture even in lower concentrations of probiotic supplements, whereas no consistent trend was recorded in AGR of T_3 (Figure 4b). Positive correlation was recorded between probiotic concentrations and SGR (%/day) in all the treatments. The linear regression between probiotic dose (x) and SGR (y) was: T_1: $y = 1.4058x + 0.4685$, $r^2 = 0.9902$; T_2: $y = 1.1654x + 0.3692$, $r^2 = 0.9899$; T_3: $y = 1.2288x + 0.2223$, $r^2 = 0.9716$.

Feed efficiency of snapper in all the treatments supplemented with probiotics showed a significantly lower FCR value in T_1 (1.02 ± 0.015) compared to T_2 (1.24 ± 0.05), T_3 (1.58 ± 0.04) and control (1.97 ± 0.14) at 90 days of the experiment. A significant variation was observed in FCR values between the treatments ($P < 0.01$; P-value = 0.0023) and also between the days of culture ($P < 0.01$; P-value = 0.0089). FCR was found to be high in low concentrations (0.1%) of

Table 4. Cost and returns from probiotic-supplemented cage culture after 90 days experimental period

Parameters	T_1	T_2	T_3	C
Variable cost				
Fingerlings cost ($)	91.83	91.83	91.83	91.83
Feed cost ($)	131.65	106.96	93.25	78.16
Probiotic cost ($)	4.29	2.32	1.24	0
Miscellaneous (Fuel cost, Labour cost for maintenance of cage, etc.) ($)	6.12	6.12	6.12	6.12
Total Variable Cost ($)	233.89	207.25	192.46	176.12
Initial Investment				
Cage-making cost (GI pipes, fabrications, nets, anchors, barrels, etc.) ($)	183.67	183.67	183.67	183.67
The life span of cages and nets	5 years	5 years	5 years	5 years
Fixed Cost				
Depreciation ($)	9.18	9.18	9.18	9.18
Interest on Fixed Capital @7% per annum ($)	6.43	6.43	6.43	6.43
Total Fixed cost ($)	15.61	15.61	15.61	15.61
Total Cost of Production ($)	249.50	222.86	208.06	191.74
Production				
Total fish production (kg/Cage)	118.8	87.9	68.4	54
Price realised ($/Kg)	4.29	4.29	4.29	4.29
Gross Revenue ($)	509.12	376.7	293.13	231.41
Net Profit ($)	259.62	153.84	85.06	39.68
Rate of Return on operating Investment (RROI)	2.18	1.82	1.52	1.31
Rate of Return on Capital Investment (RRCI)	32.61	24.13	18.78	14.82
Beneficial Cost Ratio (BCR)	2.04	1.69	1.41	1.21

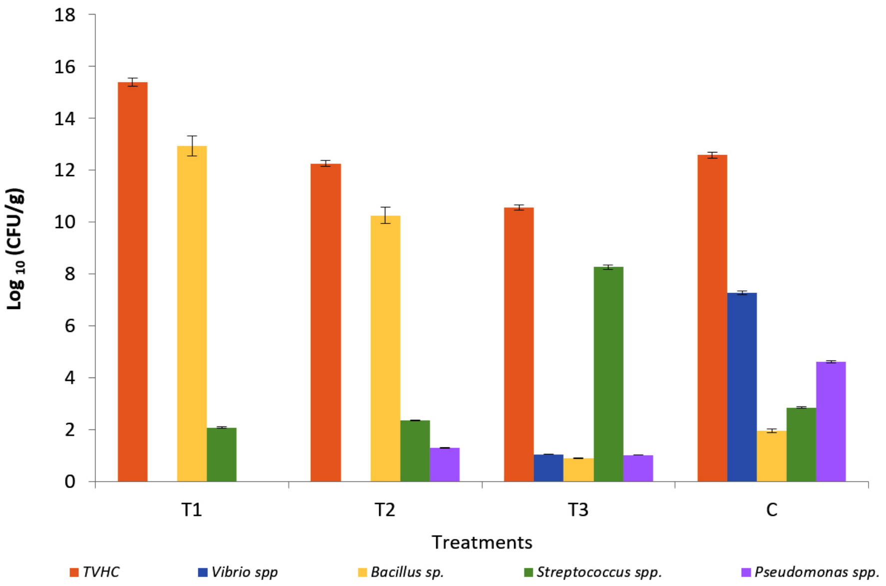


Figure 5. Variations in average log colony forming units per gram tissue of *L. argentimaculatus* gut between treatments at the end of the experiment

probiotic supplementation in all the treatments. The linear regression between probiotic dose and FCR was: y = -0.9577x +1.0054; R^2 = 0.9961 in T_1; y = -1.2962x +1.2923; R^2 = 0.9968; y = -3.1937x + 2.246; R^2 = 0.9968.

Water quality analysis

The Mean monthly values of the water quality parameters studied are shown in Table 3. No significant variation in water quality parameters was recorded between the treatments during the experimental period. Water Temperature (°C) and salinity (‰) are almost similar in all the treatments.

Microbiological analysis

A positive correlation was recorded between SGR and gut bacterial loads (r = 0.95, 0.92, and 0.86 for T_1, T_2, and T_3, respectively). Microbiological analysis of fish gut revealed the presence of *Bacillus* species as the dominant group in T_1, followed by T_2. A significant variation (P <

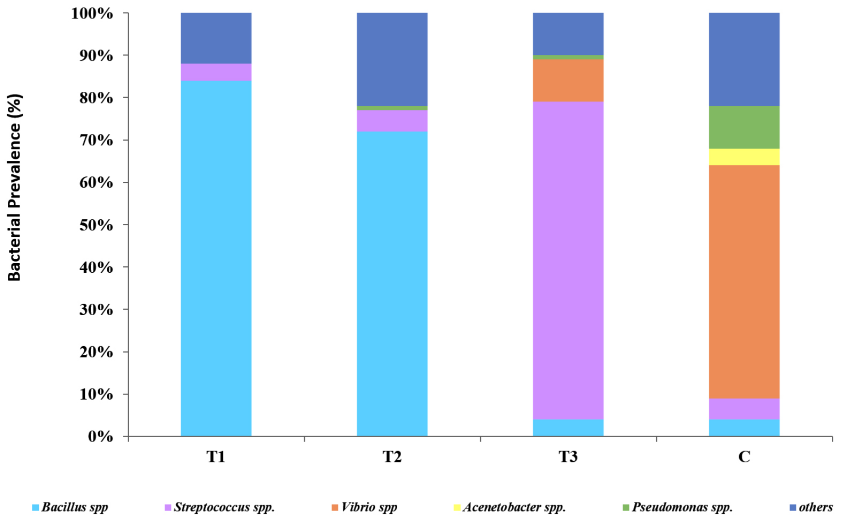


Figure 6. Prevalence of different bacteria isolated from the gut of *L. argentimaculatus* supplemented with probiotics at the end of the experiment

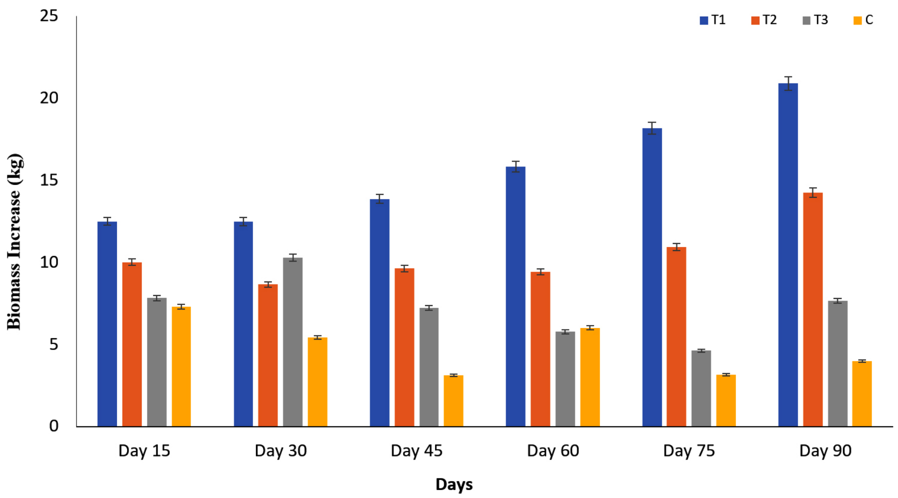


Figure 7. Variations in Biomass Increase (kg) of *L. argentimaculatus* supplemented with probiotic strains between treatments

0.05) was recorded in total bacterial and *Bacillus* loads among all the treatments. Bacterial strains belonging to the species *B. subtilis, B. cereus, B. licheniformis, B. pumilis,* and *Streptococcus* sp. were isolated and identified from the gut of red snapper in all the treatments (Figures 5 and 6) after 90 days of culture. *Bacillus* loads in T_1 and T_2 treatments ranged between 0.2 x 10^8 CFU^{-g} to 1.8 x 10^7 CFU^{-g}, whereas, *Streptococcus* was isolated from fish in T_3 treatment and the load ranged between 2.6 x 10^5 to 1.2 x 10^4 CFU^{-g}. Total gut heterotrophic bacteria of T_1, T_2, T_3 and control fish were recorded as 2.1 x 10^{12} CFU^{-g}, 0.1 x 10^{10} CFU^{-g}, 2.1 x 10^9 CFU^{-g} and 0.2 x 10^9 CFU^{-g} respectively. Major percentage of bacteria was dominated by *Bacillus* spp. (84% and 72% in T_1 and T_2, respectively), whereas *Streptococcus* spp. was the dominant species in the gut of T_3. A significant variation in the probiotic bacterial concentrations between the treatments ($P < 0.05$) was recorded. A positive correlation was recorded between probiotic bacterial concentration and gut microbes ($r = 0.96$, 0.88, 0.82 in T_1, T_2 and T_3, respectively). Also, a significant variation was observed between the *Bacillus* and *Streptococcus* counts of T_1 and T_2 before and after treatments ($P < 0.05$). A significant decrease in *Vibrio* loads was observed in T_1 and T_2 during the culture period, and the *Vibrio* loads became zero at 90 days of culture. In control, significantly higher *Vibrio* loads were recorded at 90 days of culture.

Production and yield analysis

Biomass of red snapper supplemented with different probiotic strains varied significantly between treatments and days of culture ($P = 0.00101$) with a maximum production of 118.8 ± 2.5 kg in T_1, followed by 87.9 ± 4.1 kg in T_2, 68.4 ± 3.7 kg in T_3 and 54 ± 2.2 kg in control at 90 days of culture. The average production of red snapper at 90 days culture period was significantly influenced by the probiotic application (Figure 7).

The total variable cost ($176.12-$ 233.89), the total cost of production ($191.74-$249.5), and the net return ($39.68-$259.62) were different among treatments (Table 4). A substantially higher value was found in T_1 (*Bacillus* + *Streptococci*) probiotic-supplemented red snapper fish. The rate of return on operating investment (RROI) was higher in T_1 (2.18), followed by T_2 (1.82), than in T_3 (1.52) and control (1.31). The rate of return on capital investment (RRCI) for control was 14.82, for T_1 was 32.61, for T_2 was 24.13 and for T_3 was 18.78. The benefit-cost ratio (BCR) analysis indicated higher BCR in T_1 (2.04) and T_2 (1.69) than in T_3 (1.41) and control (1.21).

DISCUSSION

The findings of the present study indicated that multi-strain probiotic bacteria as feed supplementation enhance the growth and production of cage-cultured red snapper. The present study on dietary supplementation of multi-strain probiotics (T1: *Bacillus subtilis, B. licheniformes, B. cereus*, and *Streptococcus faecium*) indicates a higher growth rate and survival of red snapper compared to single-strain supplementation (T_2 and T_3). Several researchers reported *Bacillus* sp. as antagonistic to pathogenic bacteria.[16,28,45-49] Supplementation of *Bacillus* spp. in fish diets, which are antagonistic to pathogenic bacteria, is known for improving the digestive absorption of cultured fish and significantly increasing some digestive enzymes, survival rate, and growth.[10,24,25,28,31,37,40,50-55] In the present study, mean final weight (g), Average Daily Growth Rate (AGR g /day), specific growth rate (SGR %/ day), and final biomass (kg^{-1} m^3) are found to be higher in T_1, where the feed supplementation was with multi-strain probiotics. In contrast, in other treatments, lower growth rates and biomass were recorded, where the supplementation was with single-strain probiotics. The final biomass of fish in T_1 (multi-strain probiotics) was significantly higher than in control and other treatments (T_2 and T_3). The dosage of probiotics is an important aspect of the supplementation of fish diets.[10,56,57] A strong relationship was reported between probiotic dose and weight gain, survival rate, relative growth rate, specific growth rate, feed conversion ratio, and protein efficiency ratio of tilapia.[58,59] The addition of probiotics in tilapia fry diets improved animal growth and mitigated the effects of stress factors and stated that the supplementation of probiotics is the most viable option for optimizing growth and feed utilization in intensive tilapia culture.[60] An increase in weight gain, survival rate, relative growth rate, specific growth rate, and protein efficiency ratio and a decrease in feed conversion ratio was recorded with the increasing probiotic dose.[22] The present study reveals that the multi-strain probiotic concentration at 0.1%, which was cost-effective, yielded a better growth rate and production of red snapper. Higher concentrations of multi-strain probiotics as a supplementary diet might affect the health of intestinal gut microbiota due to the supplementation of multiple bacterial strains in abundance and their active competition for colonization niches in the gut of red snapper. 0.2% dietary probiotics might be the optimum supplementation level for improved physiological and hematological status and more than 0.2% probiotics in the diet are responsible for the lower growth of *O. pabda* juveniles.[61] Similar results were observed in the present study, and higher growth rates of red snapper were recorded at the lower probiotic concentration (0.1%).

In the present study, maximum growth and production of red snapper were observed in T_1 supplemented with multi-strain probiotics. Linear regression analysis indicated a significant improvement in the growth and survival of red snapper by feed supplementation with multi-strain probiotics. The lowest FCR from T_1 indicated the highest feed utilization by the cultured red snapper. These lower values in probiotic-supplemented treatments (T_1, T_2, and T_3) indicate better feed utilization by fish, which might be due to the impact of probiotic bacteria on stimulating the digestive capacity of consumed food. Few researchers also reported similar results indicating colonization rate in the gut, higher nutrient digestibility, and enhancement of growth of fish with probiotic supplementation.[58,62-69] Multi-strain probiotic diets may offer greater advantages than single-strain applications, as they can combine a broader spectrum of beneficial effects into one formulation.[70] Recent studies have highlighted the various functional roles of multi-species probiotics and their components in aquaculture, particularly in fish farming. These probiotics have been effectively utilized to enhance biometric indices, improve carcass composition, increase feed conversion rates, and boost survival rates among fish populations.[32,70] The introduction of a probiotic bacterial consortium, administered at a concentration of 1.15×10^9 to $2 \times 1.15 \times 10^9$ CFU/kg feed, significantly enhanced the growth of Asian stinging catfish over a 60-day feeding trial when compared to the untreated control group.[33] It was also reported that five probiotic bacterial strains, used single or in combination, displayed different levels of susceptibility to thirteen tested antibiotics. The probiotic consortium, showed antagonistic effects against *Aeromonas hydrophila*, with *Bacillus* strain demonstrating the highest inhibition efficiency.[33]

Probiotics act as a biocontrol agent, which can eliminate pathogens either through the production of antimicrobial agents or by inhibition of pathogen growth, which ultimately reduces the stress, and increases survival and growth.[71-79] In the present study, a positive correlation was recorded between probiotic bacterial concentration and gut microbes, and the majority of bacteria were dominated by *Bacillus* spp. (84% and 72% in T_1 and T_2 respectively), whereas *Streptococcus* spp. was the dominant species in the gut of T_3. A significant variation was observed between the *Bacillus* and *Streptococcus* counts of T_1 and T_2 before and after treatments ($P < 0.05$), indicating that the supplemented bacterial strains effectively colonized the fish gut. The present study also revealed the complete elimination of *Vibrio* loads with the application of probiotic bacteria in T_1 (multi-strain) and T_2 (Single strain). In control, significantly higher *Vibrio* loads were recorded at 90 days of culture. An improvement in intestinal health with a relatively high abundance of *Pediococcus* in the intestinal microbiota of red drum was recorded as a result of supplementation with probiotic and prebiotic combinations (*Pediococcus acidilactic* and yeast) in low fishmeal-based diets.[42] The present study also indicated an improvement in intestine health with a high abundance of *Bacillus* spp. and *Streptococcus* spp. and complete elimination of pathogenic *Vibrios* in the gut of red snapper, supplemented with multi-strain probiotics. The incorporation of probiotics could reduce the number of pathogens, which further improves the growth response, feed utilization, and survival of fish. Since bacterial adhesion to tissue surface is important during the initial stages of pathogenic infection, competition for adhesion receptors with pathogens might be the first probiotic effect.[80-82] The composition of gut microbiota demonstrated a dose-dependent enhancement of beneficial lactic acid bacteria (LAB), alongside a decrease in total aerobic plate count (TAPC), pathogenic coliforms (TFCC), and *Vibrio* species (TVC).[33,70,80-84] The present results also indicate that the abundance of probiotic bacteria in the gut of red snapper might have eliminated pathogenic *Vibrios*, which were recorded at high concentrations in control fish. Similar results were earlier reported that the probiotic bacteria inhibit gut colonization and prevent infection of pathogens in the fish.[11,83,84]

Total production of red snapper in different treatments varied significantly, with the lowest production of 54 ± 2.2 kg in control and the highest production of 118.8 ± 2.5 kg in T_1 at 90 days of experimental culture period and indicated supplementation of multi-strain probiotic bacteria to yield more production. Similar results were reported in striped catfish supplemented with multi-strain probiotics and reported significantly higher production and gross revenue.[37] The supplementation of *Bacillus* sp. and *Lactobacillus* sp. in feed for Nile Tilapia fingerlings has shown an improvement in the growth performance, survival rate, feed conversion ratio, and benefit-cost ratio without negatively influencing the sex reversal of the fingerling.[36] They also stated that probiotic supplementation could optimize protein usage for growth and reduce the cost of feed and of production. An economic evaluation of the present study indicated total variable cost and total production cost to be more in probiotic-supplemented treatments compared to control, which could be due to the high feed cost and similar results were reported by earlier researchers.[37,71,82] Significantly higher revenue generation was recorded in T_1 supplemented with multi-strain probiotics with a gross revenue of $509.12, whereas the gross revenue ($231.41) was significantly low in control ($P < 0.05$). Rate of Return on operating Investment (RROI), Rate of Return on Capital Investment (RRCI), and Benefit Cost Ratio were also significantly high in T_1. The probiotic inclusion (0.2%) in fish feed exhibited significantly higher yield, growth performance, survival, and better feed usage with a profitable economic return.[37] It is always suggested that minimum usage of probiotic concentration would be profitable from an economic point of view. In the present study, the application of multi-strain probiotics in lower concentrations exhibited higher production and gross revenue, indicating that the probiotic supplementation at lower concentrations would give more production and yield. It could be due to the efficiency of those probiotics in the enhancement of colonization of these bacteria and further improvement of digestibility and nutritional efficiency of fish to enhance the growth rate and production.

CONCLUSION

The present study indicates multi-strain probiotics to be highly potential, effective, and economically viable. The study also shows promising results in the elimination of pathogenic *Vibrios* and enhancing survival and growth rates for sustainable cage farming of red snapper, *L. argentimaculatus*. It is suggested that farmers can use multi-strain probiotics (*Bacillus* spp. and *Streptococcus* spp.) at a 0.1% concentration for higher yield and profit in marine cage farming of red snapper, *L. argentimaculatus*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

JL conceptualized the study. JL and KRR developed the methodology. JL, KRR, PD and SMS performed experimental design and execution. JL, PD, KRR and SSR performed formal analysis and investigation. JL wrote the manuscript. JL, PPSB, AA, KRR, SSR, IJ, BI and JKK reviewed and edited the manuscript. All authors read and approved the final manuscript for publication.

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None.

DATA AVAILABILITY

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

This study was approved by the Ethical Committee, ICAR-Central Marine Fisheries Research Institute, Kochi, Kerala, India.

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