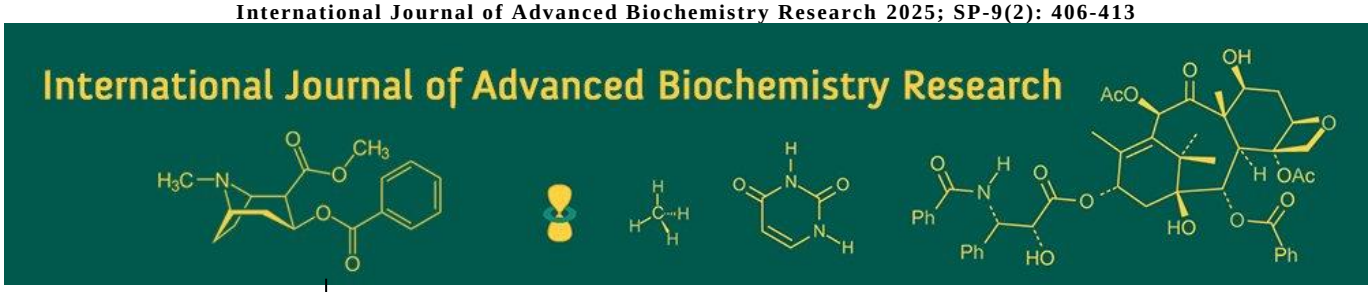

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Effect of *Bacillus amyloliquefaciens* on water quality and growth performance of Amur common carp (*Cyprinus carpio haematopterus*)

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Abstract
The increase in fish demand for human consumption has pushed the aquaculture industry towards intensified culture systems, but has increased the risk of infectious diseases. However, use of antibiotics has led to negative impact on the environment. This has spurred several researchers and biotechnology companies to develop an eco-friendly feed additive. The current experiment was carried in 250 l capacity FRP tanks for 90 days. Amur common crap (*Cyprinus carpio haematopterus*) fingerlings of 7.50g average weight were taken in triplicate with a stocking density of 15 fish/tank. The fish were fed at 5% of body weight with diets T_1 (0.5% *Bacillus amyloliquefaciens*), T_2 (1% *Bacillus amyloliquefaciens*), T_3 (1.5% *Bacillus amyloliquefaciens*) and T_0 (control without any additives). The growth parameters such as weight gain, weight gain percentage, SGR and PER were highest in fish fed with T_1 diet. Also, the lowest FCR was observed in fish fed with T_1 diet. The highest survival rate was recorded in fish fed with T_1 and T_2 diet and lowest was found in T_0. The water quality parameters like temperature, pH, dissolved oxygen, free carbon dioxide, total alkalinity, NH_3-N, NO_2-N, NO_3-N and total hardness were in a normal range. Thus, the present findings indicate positive impact on the dietary *Bacillus amyloliquefaciens* on growth performance and water quality of Amur common carp (*Cyprinus carpio haematopterus*).

Keywords: Growth performance, water quality, Amur common carp, *Bacillus amyloliquefaciens*, *Citrullus lanatus*

Introduction
Aquatic foods are regarded as one of the healthiest options available with their consumption being linked to improved public health outcomes. The significance of fisheries and aquaculture is increasingly recognized on global platforms, highlighting the potential of aquatic food systems to contribute solutions for enhancing food security, promoting healthy diets, fostering economic development and protecting the environment (FAO, 2024) [1]. Aquaculture has emerged as one of the sustainable approaches for global food diversification, providing people with healthy food options and profitable business opportunities (FAO, 2020) [2]. It plays a pivotal role in the global protein supply and helps to alleviate fishing pressure on natural aquatic resources (Lieke *et al*., 2020) [3].

Fish constitute a primary source of protein in human nutrition. The demand for fish protein sources is rising with the ever-increasing human population, while harvests from capture fisheries (both marine and freshwater) continue to decline (Biermann and Geist, 2019) [4]. This growing demand for fish has driven the aquaculture industry toward more intensive culture systems, which in turn has heightened the possibility of infectious diseases (Chen *et al*., 2012; Gallage *et al*., 2017) [5, 6].

In the last two decades, intensive and semi-intensive aquaculture systems have rapidly developed to meet the protein requirements in human diets (FAO, 2020) [2]. Maintaining efficient and sustainable production processes in intensive aquaculture necessitates attention to animal welfare. The health of fish is directly influenced by water quality, handling practices, stocking densities, disease management and nutrition (Oliva-Teles, 2012) [7]. Cutting-edge technologies have enabled increased fish production by maintaining high stocking densities (Bilen *et al*., 2013; Bilen *et al*., 2015) [8, 9].

However, these new technologies can also result in higher mortality rates due to prevalence of diseases in aquaculture (Elsheshtawy *et al*., 2019) [10].

Antibiotics and chemotherapeutics are the most widely used strategies for preventing and managing microbial infections in aquaculture (Corum *et al*., 2020; Terzi *et al*., 2020) [11, 12]. Nevertheless, these therapeutic agents may cause toxic residual effects in fish and pose health risks to humans (Capkin *et al*., 2015; Hoque *et al*., 2018) [13, 14]. Additionally, they can negatively impact on water quality and contribute to environmental pollution (Zoral *et al*., 2018) [15]. Over the years, numerous alternate techniques have been explored to minimize residual risks and develop environmentally friendly treatments.

However, the overuse of chemotherapies and antibiotics has numerous drawbacks. These include compromising the natural immunity of fish and their ecosystems and increasing the resistance of pathogens to antibiotics (Lulijwa *et al*., 2020) [16]. Additionally, chemotherapies can indirectly impact on human health, prompting several countries to restrict antibiotic use in aquaculture (Dawood *et al*., 2018) [17]. Biotherapeutics such as prebiotics, probiotics and synbiotics have recently gained attention as potential alternatives to chemical and antibiotic agents (Nikoskelainen *et al*., 2001; Van Doan *et al*., 2016; Widanarni and Tanbiyaskur, 2015; Mehrabi *et al*., 2011) [18, 19, 20, 21].

Materials and Methods

The study was conducted in Fiber reinforced plastic (FRP) tanks of 250 l capacity at the Research and Instructional Fish Farm, College of Fisheries, Mangaluru to study the effect of *Bacillus amyloliquefaciens* on water quality and growth performance of Amur common carp (*Cyprinus carpio haematopterus*). The study was carried out in triplicate for a period of 90 days.

Feed ingredients, Formulation and Analysis

The ingredients used in the formulation of different experimental diets were fishmeal, rice bran, groundnut oil cake, tapioca flour, probiotic (*Bacillus amyloliquefaciens*) and vitamin-mineral premix. All the dry ingredients were purchased from the local market except probiotic and watermelon rind powder. The probiotic powder was procured from an online platform namely Mark nature. All the ingredients except probiotic was ground and sieved to get particles of uniform size. The sieved ingredients were packed in high density polythene bags and stored at room temperature. Vitamin and mineral premix in the form of Chelated Agrimin Forte, a product of Virbac Animal Health Pvt. Ltd, India was procured locally and added.

Formulation and Preparation of Experimental Diets

Table 1: The different inclusion level of feed additives for 90 days of experiment.

Treatments	Feed additives	Incorporation level
T_0	Without any additive	-
T_1	*Bacillus amyloliquefaciens* (10^8 CFU g^{-1})	0.5%
T_2	*Bacillus amyloliquefaciens* (10^8 CFUg^{-1})	1%
T_3	*Bacillus amyloliquefaciens* (10^8 CFUg^{-1})	1.5%

The required quantities of ingredients were weighed accurately, mixed and hand kneaded to required consistency with just enough water (1: 0.8) to get smooth dough. The dough so obtained was cooked under steam in a pressure cooker at 105 °C for 20 to 30 min. The cooked feed was cooled to room temperature rapidly by spreading in an enamel tray and required dose of feed additive was diluted with water properly and vitamin-mineral premix was added, mixed and blended. The dough was extruded through a pelletizer having 3 mm dia. Pellets were dried in a hot air oven at 60 °C till the moisture content was reduced to less than 10%. Diets were packed separately in high density polythene bags, labelled and stored air tight for further use.

Growth Studies

Experimental Animals

Fry of Amur common carp brought from Fisheries Research and Information Centre, Hesarghatta, Bengaluru were reared to fingerling size in Research and Instructional Fish Farm, College of Fisheries, Mangaluru. Prior to start of the experiment, fish were acclimatized to experimental condition and fed with dry pellet diet.

Experimental Set up

The procured fry of Amur common carp was further reared in the Research and Instructional Fish Farm, College of Fisheries, Mangaluru for about 2 months to attain fingerling size and used for the present study. Prior to start of the experiment, the fish were acclimatized to experimental condition and fed with dry pelleted diet. The study was conducted in 12 indoor FRP tanks of 250 l capacity. The experiment was carried out in triplicates under each treatment group and exchange of water was performed every week to maintain the water quality.

Stocking and Rearing

Amur common carp fingerlings with an average weight and length of 7.5 g and 7.0 cm respectively were stocked at the rate of 15 fish/tank. The experiment was carried out for a period of 90 days. Faecal matter and uneaten food were removed daily in the morning hours.

Feeding

Fish were fed at the rate of 5% of their body weight till the end of the experiment. The feed was broadcasted over the surface of water twice daily in the morning (10.00 a.m.) and evening (3.00 p.m.). After each sampling, the quantity of feed given was re-adjusted based on the increased weight of fish.

Fish Sampling and Growth Assessment

The fishes were sampled fortnightly to assess the growth. The stocked fish were collected during each sampling and measured individually for length and weight. After a rearing period of 90 days, all the survived fishes were collected at once and their growth assessment such as total weight gain, weight gain percentage, specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and survival were carried out using standard formulae as mentioned below.

Total weight gain (g) = Mean final weight gain (g) ₋ Mean initial weight gain (g)

% Weight gain = [(Mean initial weight – Mean final weight)/(Mean initial weight)] ×100

SGR (%/day) = [ln final weight – ln initial weight/days] × 100

FCR = Dry weight of the feed given (g)/Gain in weight of fish (g)

PER = weight gain/feed consumed × percentage of protein in feed

Survival was calculated as the difference between the number of live animals at the beginning and at the end of the experiment and expressed in percentage.

Water Sampling

Water quality parameters were maintained within the normal range throughout the experimental period. Water samples collected weekly and were analyzed for water temperature, pH, dissolved oxygen, free carbon dioxide, total alkalinity, NH_3-N, NO_2-N, NO_3-N and total hardness. Digital pH meter model LI 613 was used to record pH. Water temperature was recorded by using standard thermometer. Dissolved oxygen was estimated by Winkler's method. Free carbon dioxide, total alkalinity, Ammonia-Nitrogen, Nitrite-Nitrogen, Nitrate-Nitrogen and total hardness were determined by standard methods (APHA, 1995) [22].

Statistical analysis

One-way ANOVA was used to determine significance of difference between treatments. Differences among means were tested at the 5% probability level using Duncan Multiple Range test.

Results and Discussion

The initial weight of the fish was 7.49-7.72 g. The values of initial weight of the fish showed no significant difference ($p>0.05$) between the different treatment groups (Table 2 and Fig 1). The final weight was observed highest with the value of 22.86±0.14 g in T_1 treatment group, 22.15±0.12 g in T_2, 21.73±0.15 g in T_3 and the lowest final weight was recorded in T_0 with a value of 19.65±0.08 g (Fig 2). The total weight gain was found to be highest with the value of 15.37±0.04 g in T_1, 14.44±0.18 g in T_2, 14.22±0.08 g in T_3 and lowest value of 11.93±0.06 g in T_0. It was observed that there was a significant difference ($p<0.05$) between control and treatment groups (Fig 3). The highest weight gain percentage was observed with the value of 205.21±0.15% in T_1, 189.35±0.23% in T_3, 187.29±0.31% in T_2 and 154.53±0.22% in T_0. It was noticed that there was a significant difference ($p<0.05$) between the control and treatment groups (Fig 4).

Table 2: Details of growth performance of Amur common carp recorded in different treatment groups.

Parameters	Treatments			
	T_0	T_1	T_2	T_3
Initial weight (g)	7.72±0.06	7.49±0.12	7.71±0.05	7.51±0.1
Final weight (g)	19.65±0.08^d	22.86±0.14^a	22.15±0.12^b	21.73±0.15^c
Total weight gain (g)	11.93±0.06^c	15.37±0.04^a	14.44±0.18^b	14.22±0.08^b
Weight gain (%)	154.53±0.22^d	205.21±0.15^a	187.29±0.31^c	189.35±0.23^b
SGR (%)	1.04±0.02^c	1.24±0.03^a	1.17±0.01^b	1.18±0.02^b
PER	1.19±0.05^d	1.54±0.08^a	1.45±0.02^b	1.42±0.04^c
FCR	2.79±0.09^a	2.17±0.06^d	2.31±0.04^c	2.34±0.08^b
Survival rate (%)	80.00±3.85^c	97.78±2.22^a	97.78±2.22^a	95.56±2.22^b

SGR: Specific growth rate; PER: Protein efficiency ratio; FCR: Feed conversion ratio. Results are presented as Mean ± SE of triplicate observation. Means in the same row with different superscripts are significantly different ($p<0.05$).

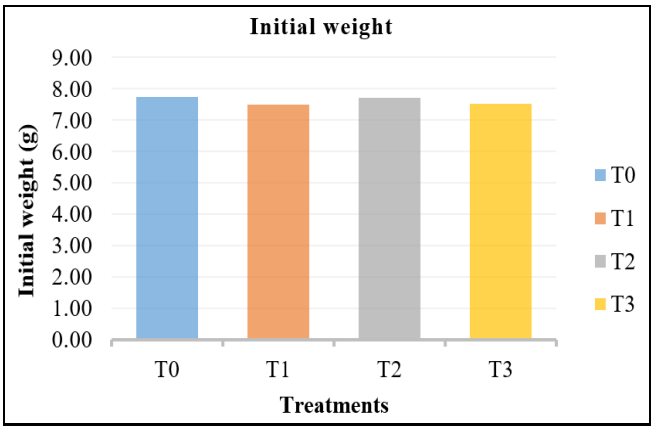

Fig 1: Initial weight of fish recorded in different treatment groups and control.

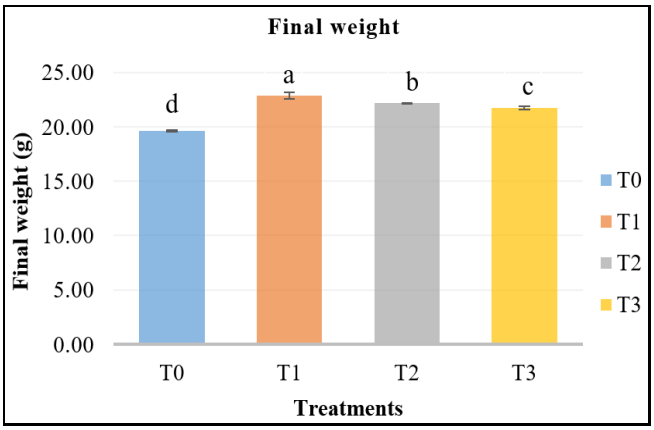

Fig 2: Final weight of fish recorded in different treatment groups and control.

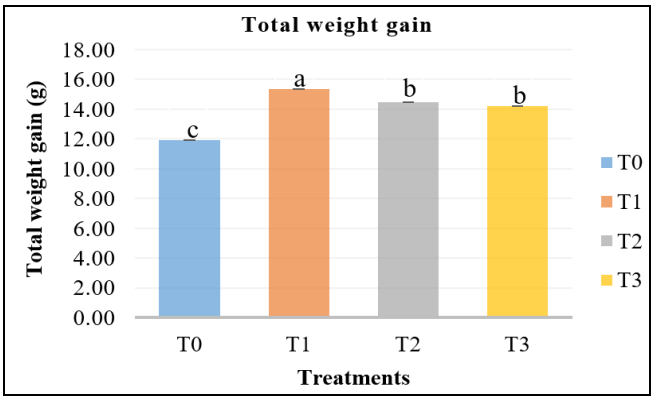

Fig 3: Total weight gain of fish recorded in different treatment groups and control.

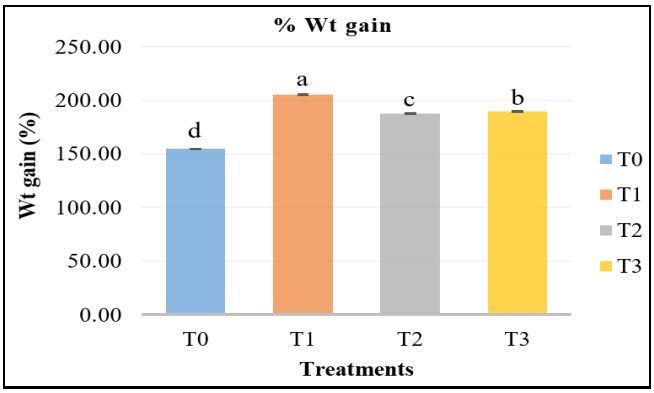

Fig 4: Weight gain percentage of fish recorded in different treatment groups and control.

The SGR was recorded highest with the value of 1.24±0.03% in T_1, 1.18±0.02% in T_3, 1.17±0.01% in T_2 and 1.04±0.02% in T_0. There was a significant difference (p<0.05) between the treatment groups and control (Fig. 5). The lowest value of FCR was observed in T_1 with the value of 2.17±0.06 followed by T_2, T_3 and T_0 with the values of 2.31±0.04, 2.34±0.08 and 2.79±0.09 respectively. There observed a higher significant difference (p<0.05) in fish fed with test diet T_1 compared to rest of the treatment groups and control (Fig 6). The value of PER was recorded highest in T_1 (1.54±0.08) followed by T_2 (1.45±0.02), T_3 (1.42±0.04) and T_0 (1.19±0.05). There was a significant difference (p<0.05) between treatment groups and control (Fig 7). The highest survival rate was observed in T_1 and T_2 with the value of 97.78±2.22%, followed by T_3 with the value of 95.56±2.22% and lowest survival rate was observed in T_0 with 80.00±3.85% (Fig 8).

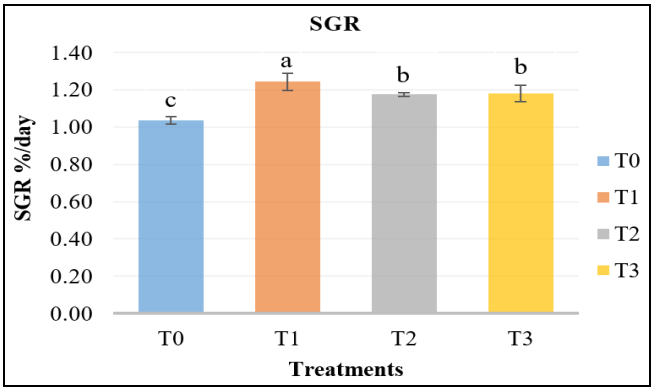

Fig 5: Specific growth rate (SGR) of fish recorded in different treatment groups and control.

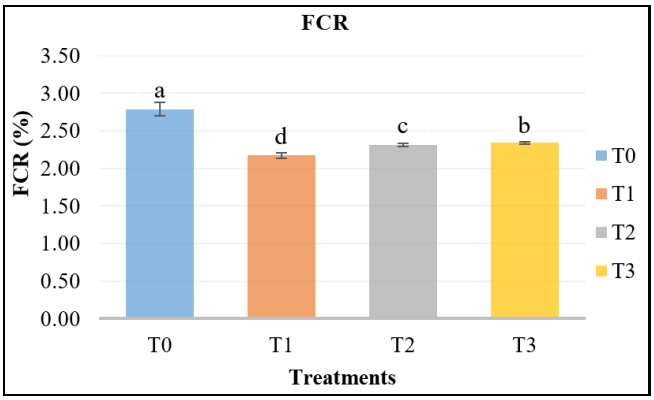

Fig 6: Feed conversion ratio (FCR) of fish recorded in different treatment groups and control.

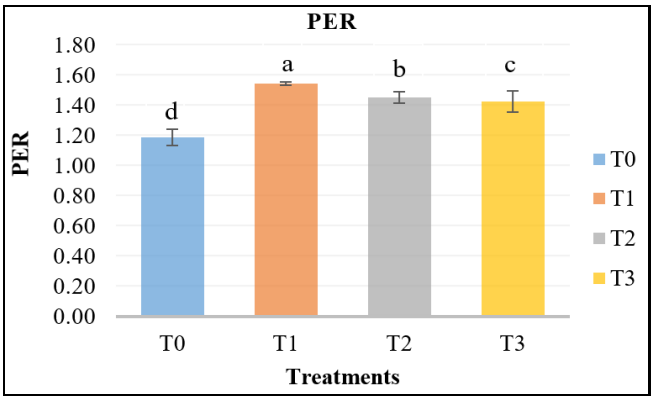

Fig 7: Protein efficiency ratio (PER) of fish recorded in different treatment groups and control.

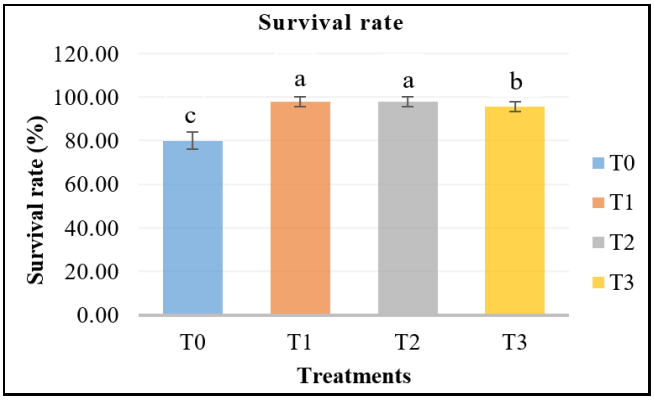

Fig 8: Survival rate (%) of Amur common carp after 90 days feeding in different treatment groups and control.

Similarly, incorporating *B. amyloliquefaciens* spores at a concentration of 1.4×10^6 colony-forming units per gram ($CFUg^{-1}$) of feed in Rock Bream (*Oplegnathus fasciatus*) after 90 days led to significant improvements in body weight (BW), weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR) (Subramanian *et al.*, 2017) [23]. An oral administration of probiotic *B. subtilis* at 10^8 $CFUg^{-1}$ diet was found to be the most effective dose enhancing the growth performance in freshwater fish Japanese eel, *Anguilla japonica* (Lee *et al.*, 2017) [24]. Putri *et al.* (2012) [25] indicated that administering EM4 probiotics to *O. niloticus* at doses of 5, 10, 15 and 20 ml kg^{-1} feed enhanced growth rates, with the highest growth observed at 15 ml kg^{-1}. Researchers investigated the impact of *Lactobacillus plantarum* on the growth of Nile tilapia fed with *L. plantarum* at concentrations of 10^7 and 10^9 $CFUg^{-1}$ of feed reported significant improvements in weight gain and specific growth rate (SGR) in the groups receiving the probiotic-supplemented diets compared to the control group (Hasan and Banerjee, 2020) [26].

The effect of *Bacillus subtilis* supplementation on the growth performance of common carp supplemented with *B. subtilis* at a concentration of 10^8 $CFUg^{-1}$ of feed exhibited enhanced growth performance including increased final weight and improved feed conversion ratio (FCR) compared to the control group (Rosidah, 2021) [27]. A study assessed the impact of *Bacillus amyloliquefaciens* on the growth and health of largemouth bass administered diets containing *B. amyloliquefaciens* at a concentration of 10^6 $CFUg^{-1}$ of feed showed significant improvements in growth parameters including higher weight gain and better FCR compared to the control group (Ariyanto and Anika, 2024) [28]. The effects of *Enterococcus faecium* on the growth performance of rainbow trout supplemented at a concentration of 10^8 $CFUg^{-1}$ of feed reported significant enhancements in growth metrics including increased final weight and improved SGR in the probiotic-treated group compared to the control (Hasan and Banerjee, 2020) [26].

The water temperature during the study period ranged from 26.15 to 29.13 °C in T_0, 26.16 to 29.11 °C in T_1, 26.18 to 29.13 °C in T_2 and 26.14 to 29.18 °C in T_3 (Table 3 and Fig 9). Water temperature is a crucial factor for all aquatic life influencing all biological and chemical processes in an aquaculture operation. It is one of the most significant external factors affecting fish production. Boyd (1982) [29] reported that a water temperature ranges from 26.06 to 31.97 °C is suitable for warm water fish culture. The pH range during the study period was around neutral to alkaline ranged from 7.59 to 7.94 in T_0, 7.56 to 8.00 in T_1, 7.63 to 8.02 in T_2, 7.57 to 7.94 in T_3 (Fig 10). A pH range between 6.4 and 8.3 is favourable for fish growth (Robert *et al.*, 1940) [30] with a protective limit for aquatic life being 6.0 to 8.5 (ISI, 1974) [31]. Hepher and Pruginin (1981) [32] suggested that a pH range of 6.5 to 9.0 is suitable for fish culture.

Table 3: Details of water quality parameters recorded during different sampling days of the experimental period.

Parameters	Treatments			
	T_0	T_1	T_2	T_3
Temperature (°C)	26.15-29.13	26.16-29.11	26.18-29.13	26.14-29.18
pH	7.59-7.94	7.56-8	7.63-8.02	7.57-7.94
DO (mg l^{-1})	5.25-5.54	5.13-5.62	5.17-5.64	5.22-5.62
Free CO_2 (mg l^{-1})	1.11-1.75	1.02-1.89	1.17-1.98	1.17-1.88
Total Alkalinity (mg l^{-1})	63.07-104.03	62.12-102.18	62.07-104.11	63.17-103.04
Ammonia-nitrogen (µg l^{-1})	0.12-0.39	0.09-0.38	0.11-0.38	0.16-0.35
Nitrite-nitrogen (µg l^{-1})	0.04-0.27	0.06-0.28	0.01-0.26	0.06-0.26
Nitrate-nitrogen (µg l^{-1})	0.18-0.39	0.17-0.4	0.12-0.39	0.15-0.42
Hardness (mg l^{-1})	79.15-105.12	78.83-102.09	79.63-101.75	77.75-104.12

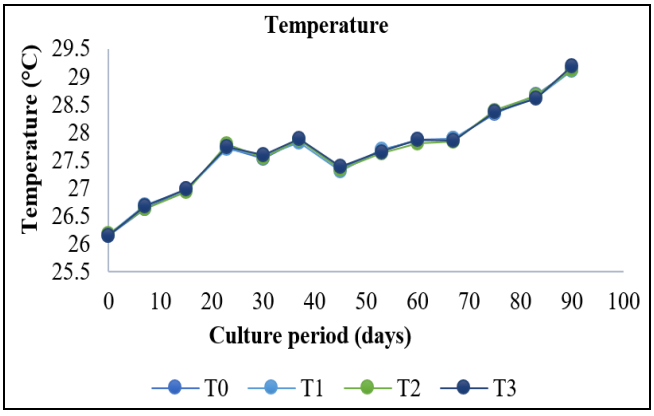

Fig 9: Profile of water temperature recorded during the experimental period.

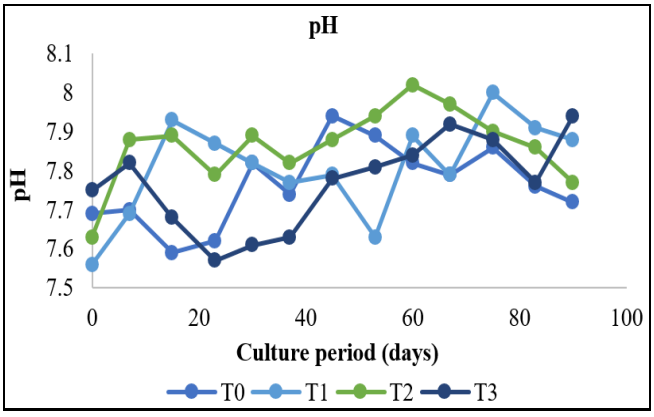

Fig 10: Profile of the pH of water recorded during the experimental period.

The values of dissolved oxygen throughout the study period ranged from 5.25-5.54 mg l^{-1} in T_0, 5.13 to 5.62 mg l^{-1}in T_1, 5.17 to 5.64 mg l^{-1} in T_2 and 5.22 to 5.62 mg l^{-1} in T_3 (Fig. 11). Dissolved oxygen (DO) is a crucial parameter in water quality assessment reflecting the physical and biological processes in the water. The DO concentration of 5 mg l^{-1} throughout the year is productive for fish culture (Tarzwell, 1957; Banerjea, 1967) [33, 34]. The free carbon dioxide values were 1.11 to 1.75 mg l^{-1} in T_0, 1.02 to 1.89 mg l^{-1} in T_1, 1.17 to 1.98 mg l^{-1} in T_2 and 1.17 to 1.88 mg l^{-1} in T_3 (Fig. 12). Free carbon dioxide (CO_2) is a highly soluble gas in water and plays a key role in the carbon cycle. Fish generally avoid free CO_2 levels as low as 5 mg l^{-1} (Boyd and Lichtkoppler, 1979) [35]. Ekubo and Abowei (2011) [36] reported that the tropical fish can tolerate CO_2 levels over 100 mg l^{-1}, but the ideal level in fish ponds is less than 10 mg l^{-1}.

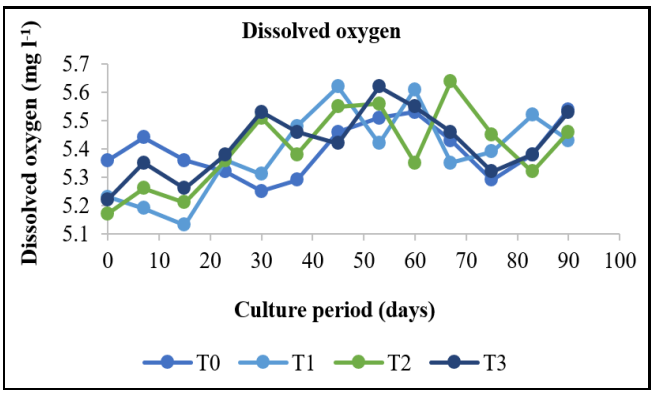

Fig 11: Profile of the dissolved oxygen (mg l^{-1}) recorded during the experimental period.

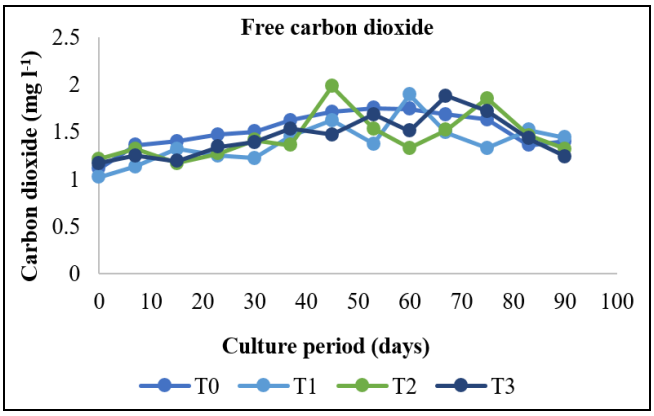

Fig 12: Profile of the free carbon dioxide (mg l^{-1}) recorded during the experimental period.

The total alkalinity value recorded during the study period ranged from 63.07-104.03 mg l^{-1}in T_0, 62.12 to 102.18 mg l^{-1} in T_1, 62.07 to 104.11 mg l^{-1} in T_2 and 63.17 to 103.04 mg l^{-1}in T_3 (Fig. 13). Total alkalinity, the sum of carbonate and bicarbonate alkalinity is crucial in aquaculture. Natural water with a total alkalinity of 40 mg l^{-1} or higher is more productive, while ponds with alkalinity over 300 mg l^{-1} may be unproductive due to carbon dioxide limitations (Mairs, 1966; Adhikari, 2000) [37, 38]. Boyd (1982) [29] suggested that a total alkalinity range between 20 and 300 mg mg l^{-1} is ideal for fish culture with values less than 20 mg l^{-1} causing stress in fish.

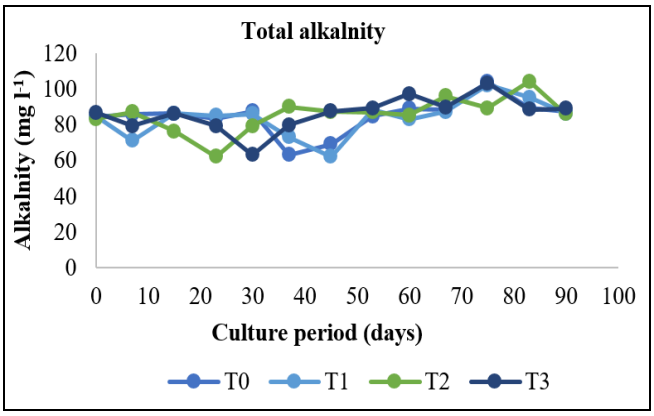

Fig 13: Profile of the total alkalinity (mg l^{-1}) recorded during the experimental period.

The mean values of ammonia-nitrogen during the study period ranged from 0.12 to 0.39 µg l^{-1} in T_0, 0.09 to 0.38 µg l^{-1} in T_1, 0.11 to 0.38 µg l^{-1} in T_2 and 0.16 to 0.35 µg l^{-1} in T_3 (Fig. 14). Ammonia is the primary nitrogenous waste produced by aquatic animals through metabolism and is excreted across the gills (Cao *et al.*, 2007) [39]. Chen (1988) [40] noted that the ammonia levels below 1 mg l^{-1} in pond water are acceptable for fish culture, while Boyd (1998) [41] recommended values below 0.1 mg l^{-1}. The optimal concentration for warmwater fish culture is 0.02 mg l^{-1} (EPA, 1973; Jhingran, 1988) [42, 43]. The values of nitrite-nitrogen ranged from 0.04 to 0.27 µg l^{-1} in T_0, 0.06 to 0.28 µg l^{-1} in T_1, 0.01 to 0.26 µg l^{-1} in T_2 and 0.06 to 0.26 µg l^{-1} in T_3 (Fig. 15). Nitrite is an intermediary by-product in the conversion of NH_3 or NH_4^+ into NO_2 through nitrification by highly aerobic, gram-negative, chemoautotrophic bacteria. Boyd (1998) [41] suggested that the desired nitrite concentration is less than 0.3 mg l^{-1} in aquaculture. The nitrate-nitrogen values throughout the study period ranged from 0.18 to 0.39 µg l^{-1} in T_0, 0.17 to 0.4 µg l^{-1} in T_1, 0.12 to 0.39 µg l^{-1} in T_2 and 0.15 to 0.42 µg l^{-1} in T_3 (Fig 16). Nitrate is formed through the oxidation of NO_2 to NO_3 by aerobic bacteria, a process known as nitrification. Boyd (1998) [41] reported that the desired nitrate concentration for aquaculture is between 0.2 and 10 mg l^{-1}.

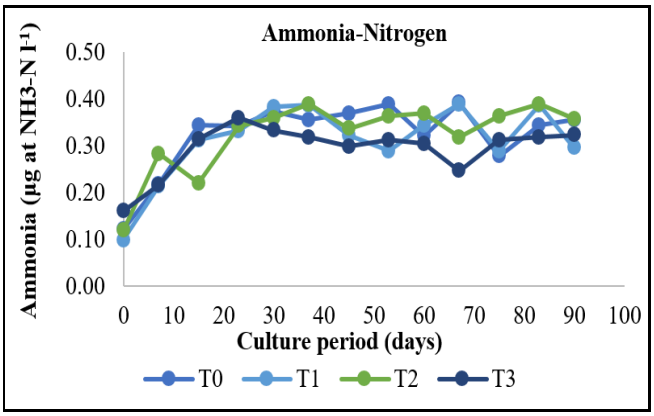

Fig 14: Profile of the Ammonia (µg-at NH_3-N l^{-1}) recorded during the experimental period.

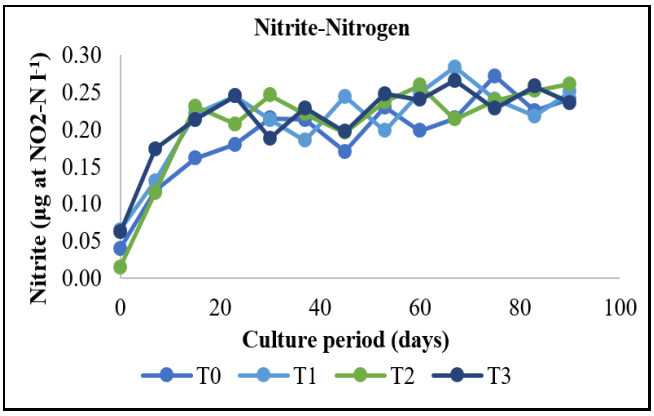

Fig 15: Profile of the Nitrite (µg-at NO_2-N l^{-1}) recorded during the experimental period.

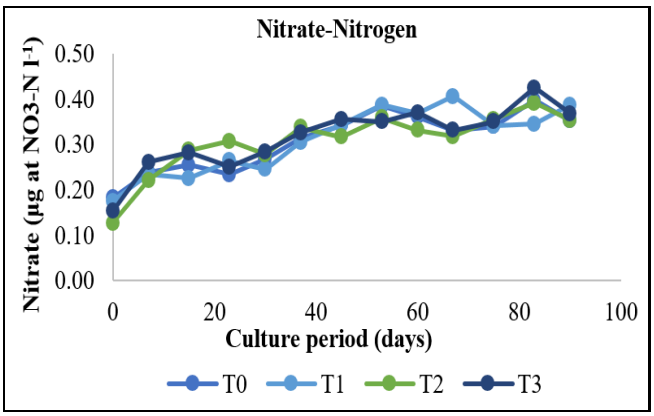

Fig 16: Profile of the Nitrate (µg-at NO_3-N l^{-1}) recorded during the experimental period.

The observed hardness value during the study period fell in moderately hard range. And the ranges for T_0, T_1, T_2 and T_3 were 79.15 to 105.12 mg l^{-1}, 78.83 to 102.09 mg l^{-1}, 79.63 to 101.75 mg l^{-1} and 77.75 to 104.12 mg l^{-1}respectively (Fig. 17). Water hardness is determined by calcium and magnesium salts that affects fish health. Soft water (0-75 ppm $CaCO_3$) has low buffering capacity, moderately hard water (75-150 ppm $CaCO_3$) is better and hard water (150-300 ppm $CaCO_3$) is even more buffered. Very hard

water (>300 ppm $CaCO_3$) has the highest buffering capacity (Boyd, 1990; 1998) [44, 41]. For optimal health of warm water fishes, hardness over 15 mg l^{-1} is required (EPA, 1973; Jhingran, 1988) [42, 43].

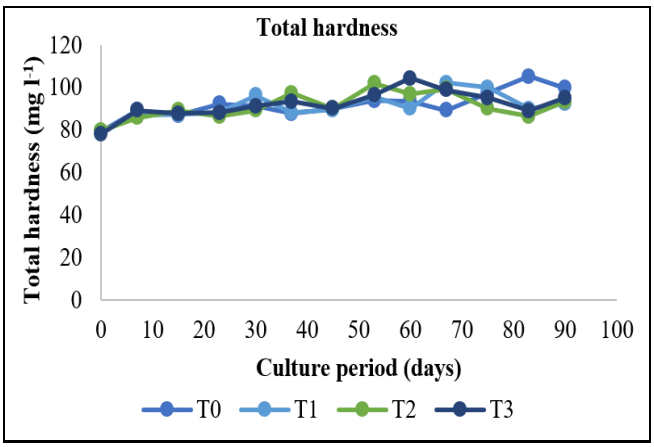

Fig 17: Profile of the total hardness (mg l^{-1}) recorded during different sampling days of the experimental period.

Conclusion

The present study demonstrates that the dietary supplementation with *Bacillus amyloliquefaciens* positively influences on the growth performance, feed utilization and survival of Amur common carp (*Cyprinus carpio haematopterus*). *Bacillus amyloliquefaciens* incorporated diet at the rate of 0.5% resulted in the highest weight gain, specific growth rate (SGR), protein efficiency ratio (PER) and the lowest feed conversion ratio (FCR). Additionally, water quality parameters remained within the optimal range indicating no adverse effects of the probiotic supplementation. These findings suggest that *Bacillus amyloliquefaciens* can serve as an eco-friendly alternative to antibiotics enhancing fish health and aquaculture sustainability.

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