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Dietary Herbal Supplementation Improves Growth Performance and Haematological Responses in *Clarias gariepinus*

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Abstract Dietary composition can influence both the growth performance and haematological parameters of aquaculture species. This study evaluated the welfare benefits of dietary supplementation with ginger (*Zingiber officinale*), garlic (*Allium sativum*), and neem (*Azadirachta indica*) powder on behavioural responses, growth performance, and haematological profile of African catfish juveniles (*Clarias gariepinus*). The use of plant-based phytogetic additives in aquaculture feeds is increasingly recognized as a sustainable alternative to synthetic growth promoters and chemotherapeutic agents, with potential benefits for fish health. Four experimental diets were formulated: a control diet (0%) and three treatment diets supplemented with 2% ginger, 2% garlic, and 2% neem powder respectively. Juvenile *C. gariepinus* were fed these diets for a three-month period (12weeks). Growth parameters, length–weight relationship, and haematological indices were measured, while behavioural indicators such as feeding activity, behaviour, and general vitality were observed as indicators of welfare status. Fish fed the garlic-supplemented diet recorded the highest values of Packed Cell Volume (PCV), haemoglobin (Hb), and Red Blood Cells (RBC), while the neem-supplemented diet produced the highest White Blood Cell (WBC) counts, suggesting improved immune response. Behavioural observations also indicated stronger feeding responses and greater activity in garlic and neem treatments. Overall, phytogetic supplements improved physiological condition and behavioural welfare of *C. gariepinus*.

Keywords Supplemented; Phytogetic additives haematological; Synthetic growth promoters; Chemotherapeutic

1 Introduction

African catfish (*Clarias gariepinus*) is one of the most commercially important freshwater species cultured in Africa. Its widespread cultivation is attributed to its rapid growth, efficient feed utilization, high fecundity, and strong tolerance to a wide range of environmental conditions (Langi et al., 2024; Klimuk et al., 2024). These characteristics have supported the expansion of catfish farming across sub-Saharan Africa, where increasing demand for affordable animal protein continues to drive aquaculture development (Kwikiriza et al., 2025). In addition, selective breeding programmes have improved the growth potential and productivity of cultured stocks, further enhancing the economic value of the species in intensive production systems (Imron et al., 2020; Srimai et al., 2020; Ovie et al., 2025).

Despite these advantages, the rapid expansion and intensification of catfish farming have introduced several health and management challenges. Intensive production practices frequently expose fish to stress arising from fluctuations in water quality, temperature, salinity, and other environmental factors (Khieokhajonkhet et al., 2022; Zidan et al., 2022). Such conditions can disrupt physiological processes, impair growth performance, and compromise fish homeostasis, ultimately reducing production efficiency (Khieokhajonkhet et al., 2022; Klimuk et al., 2024).

In recent years, fish welfare has received considerable attention because it is closely associated with productivity, survival, and resistance to disease (Ojelade et al., 2022). Modern approaches to welfare assessment extend beyond growth measurements and incorporate indicators of physiological balance, immune function, and adaptive capacity

(Okomoda et al., 2024). Among these indicators, haematological parameters are particularly useful because changes in blood characteristics provide valuable information on fish health status on the health status and stress responses of fish (Melefa et al., 2020; Okomoda et al., 2024). Previous studies have shown that environmental changes can significantly affect erythrocyte and leukocyte profiles, highlighting the sensitivity of blood indices as indicators of fish health (Oluah et al., 2020; Mangkunegara et al., 2023).

Disease outbreaks remain a major constraint to sustainable aquaculture, particularly under intensive culture conditions where fish are exposed to multiple stressors (Mohammed et al., 2025). Antibiotics have traditionally been used to control disease problems; however, concerns over antimicrobial resistance have increased interest in alternative approaches to fish health management. Evidence suggests that excessive use of antibiotics in aquaculture may promote the emergence and spread of resistant microorganisms within aquatic environments (Mohammed et al., 2025). Consequently, attention has shifted towards sustainable dietary strategies that improve fish health without posing ecological risks (Akpomughe et al., 2024; Le Xuan, 2026).

One area that has attracted growing interest is the use of phytobiotics as functional feed additives. These plant-derived products contain bioactive compounds capable of influencing growth, metabolism, immune responses, and disease resistance in cultured fish (Le Xuan, 2026). Several studies have shown that phytobiotics can enhance digestive processes, improve nutrient utilization, and stimulate innate immunity. Their incorporation into aquafeeds is therefore increasingly regarded as an important component of sustainable aquaculture development because they offer health benefits while reducing dependence on synthetic drugs and chemotherapeutic agents (Le Xuan, 2026).

Among the various phytobiotics investigated in catfish culture, garlic (*Allium sativum*) and ginger (*Zingiber officinale*) have received considerable attention because of their beneficial effects on growth and health (Megbowon et al., 2024). Dietary supplementation with these herbs has been associated with improved growth performance, feed utilization, and body composition in African catfish fry and juveniles (Nyadjeu, 2021; Nyadjeu et al., 2021). Studies have also shown that the combined use of garlic and ginger can enhance physiological performance and growth responses in juvenile fish (Nyadjeu et al., 2023). Similarly, ginger supplementation has been reported to improve nutrient utilization and promote improved health status in catfish, supporting its potential as a functional feed ingredient (Wei et al., 2023; Uddin et al., 2025).

Neem (*Azadirachta indica*) has also gained recognition as a promising natural additive because of its antimicrobial, immunomodulatory, and prophylactic properties (Yusuff and Ibidapo Obe, 2024). Previous studies have demonstrated that neem-based products can contribute to disease prevention and improve health management in African catfish production systems (Oyebola et al., 2020). Therapeutic applications of neem have been linked with enhanced physiological resilience and improved health status in catfish species (Parvin et al., 2023). More recently, nano-formulated neem products have been reported to stimulate immune responses and increase resistance to disease, further emphasizing the biological potential of neem-derived compounds in aquaculture (Ibrahim et al., 2023).

The effects of dietary phytobiotics are often reflected in haematological characteristics because blood parameters respond readily to changes in nutritional and physiological conditions (Melefa et al., 2020). Parameters such as red blood cell count, haemoglobin concentration, and packed cell volume are commonly used to assess oxygen transport capacity and metabolic efficiency in fish (Okomoda et al., 2024). Likewise, white blood cell counts and differential leukocyte profiles provide useful information on immune responses and disease resistance mechanisms (Oluah et al., 2020; Sayed et al., 2022). For this reason, haematological analysis has become an important tool for evaluating the influence of dietary interventions on fish health and welfare.

Although garlic, ginger, and neem have each been reported to exert beneficial effects in fish, information comparing their relative contributions to welfare-related responses in *Clarias gariepinus* remains limited (Megbowon et al., 2024). Most existing studies have concentrated on growth performance or immune responses separately, with relatively few investigations adopting a more integrated approach that considers both physiological wellbeing and

welfare indicators simultaneously (Nyadjeu et al., 2023; Megbowon et al., 2024). This limitation has hindered the development of evidence-based feeding strategies aimed at improving fish welfare while sustaining productivity under commercial farming conditions (Awhefeada et al., 2025; Le Xuan, 2026).

Against this background, a comparative evaluation of dietary supplementation with garlic, ginger, and neem in *Clarias gariepinus* is necessary. Understanding how these phytobiotics influence physiological wellbeing and welfare-related parameters will provide valuable information for the development of sustainable nutritional strategies. Such approaches have the potential to improve fish health and reduce dependence on conventional chemotherapeutic agents, thereby supporting more environmentally responsible aquaculture practices (Mohammed et al., 2025; Le Xuan, 2026).

2. Materials and Methods

2.1 Study area

The feeding trial was carried out at the Fisheries Research Farm and Fisheries Laboratory, Delta State University, Asaba Campus, Delta State, Nigeria. Laboratory analyses were conducted under controlled conditions following standard aquaculture and fish health procedures.

2.2 Experimental fish and rearing conditions

A total of 120 healthy hatchery-produced African catfish (*Clarias gariepinus*) fingerlings with an average body weight of 10.6 g and an average total length of approximately 9 cm were obtained from the Department of Fisheries and Aquaculture Research Farm, Delta State University, Abraka.

The fish were acclimatized to the experimental conditions for 24 h before the commencement of the feeding trial. After acclimatization, the fish were randomly assigned to twelve plastic tanks (150 L capacity) at a stocking density of 10 fish per tank. Each tank was covered with mosquito mesh to prevent escape and protect the fish from external predators. Water in the tanks was renewed every three days throughout the experimental period to maintain suitable water quality.

2.3 Experimental design and duration

The experiment was arranged in a Completely Randomized Design (CRD) consisting of four dietary treatments, each replicated three times, giving a total of twelve experimental units. The feeding trial lasted for three months, from May to July.

The dietary treatments were as follows:

- T1: Control diet without herbal supplementation (0%)
- T2: Diet containing 2% garlic (*Allium sativum*) powder
- T3: Diet containing 2% ginger (*Zingiber officinale*) powder
- T4: Diet containing 2% neem (*Azadirachta indica*) leaf powder

2.4 Preparation of herbal additives

Fresh garlic bulbs and ginger rhizomes were purchased from a local market in Abraka, Delta State, whereas fresh neem leaves were collected from mature trees within the university environment. The plant materials were washed thoroughly and air-dried before being milled into fine powder. The powdered samples were stored in airtight containers until they were incorporated into the experimental diets.

2.5 Diet formulation

Experimental diets were formulated using Pearson's square method. The major feed ingredients consisted of maize, fishmeal, groundnut cake, palm kernel cake, wheat meal, bone meal, vitamin-mineral premix, palm oil, and cassava starch. Depending on the treatment, garlic, ginger, or neem powder was incorporated into the diets at an inclusion level of 2%.

The diets were pelleted, air-dried, and stored under dry conditions until use.

2.6 Proximate analysis of experimental diets

Samples of the experimental diets were analysed for moisture content, crude protein, crude fibre, ether extract, ash content, and nitrogen-free extract according to the standard procedures of the Association of Official Analytical Chemists (AOAC, 2000). Nitrogen-free extract was determined by difference after subtracting the percentages of moisture, crude protein, crude lipid, crude fibre, and ash from 100.

2.7 Feeding trial and growth monitoring

Following acclimatization, the fish were weighed and measured before the start of the feeding experiment. Feeding was carried out twice daily between 08:00-09:00 h and 17:00-18:00 h at a rate equivalent to 3% of body weight. Feed ration was adjusted periodically in line with changes in fish weight.

Throughout the study, feeding behaviour and feed acceptance were observed and recorded. Body weight and total length measurements were taken monthly using a digital weighing balance and a graduated measuring board, respectively.

2.8 Water quality monitoring

Water quality parameters were monitored throughout the experiment to ensure favourable conditions for fish growth and survival. Water temperature was measured using a calibrated thermometer, dissolved oxygen concentration was determined following APHA (1998) procedures, and pH was measured with a digital pH meter.

2.9 Length-weight relationship

The length–weight relationship was determined using the equation:

$$W = aL^b$$

where W represents body weight (g), L represents total length (cm), a is the intercept, and b is the growth coefficient.

Length-weight relationships are widely used to evaluate growth performance, biomass accumulation, and population condition in cultured and natural fish populations (Musa et al., 2021; Ekpemikoghene et al., 2024). Regression analysis was used to estimate the growth coefficient following established fisheries procedures.

2.10 Condition factor

The condition factor (K) was calculated as:

$$K = \frac{1000W}{L^3}$$

where W is body weight (g) and L is total length (cm).

Condition factor provides an integrated measure of fish well-being because it reflects the relationship between body weight and length under specific environmental and nutritional conditions (Isibor et al., 2023; Sadauki et al., 2024).

2.11 Haematological analysis

At the end of the feeding trial, fish were randomly selected from each treatment and transported alive to the laboratory for blood collection. Blood samples were collected using sterile syringes and transferred into ethylenediaminetetraacetic acid (EDTA) tubes for subsequent analysis.

The haematological parameters determined were packed cell volume (PCV), haemoglobin concentration (Hb), red blood cell count (RBC), white blood cell count (WBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC).

2.12 Statistical analysis

Data obtained from the experiment were expressed as mean $\pm$ standard deviation (SD). Treatment means were subjected to one-way analysis of variance (ANOVA), and where significant differences were observed, Duncan's Multiple Range Test was used to separate the means. Statistical significance was established at $P < 0.05$.

3 Results

3.1 Proximate composition of experimental diets

The proximate composition of the control and herbal-supplemented diets is presented in Table 1. Differences in nutrient composition were observed among the experimental diets following the inclusion of garlic, ginger, and neem.

Moisture content varied from 9.07% in the ginger-supplemented diet to 10.18% in the neem diet. Ash content was relatively higher in the neem (4.65%) and ginger (4.38%) diets than in the control (3.08%) and garlic (3.04%) diets.

The inclusion of the herbal additives resulted in slightly higher crude protein levels, with the neem-based diet recording the highest value (23.40%). Crude protein content in both the garlic and ginger diets was 22.00%, compared with the control diet. A similar trend was observed for crude fibre content, which increased from 7.20% in the control diet to 11.00% in the ginger-supplemented diet.

Ether extract ranged from 12.00% in the control diet to 15.00% in the ginger diet. In contrast, nitrogen-free extract decreased following herbal supplementation and was highest in the control diet (47.85%).

Table 1 Proximate composition (%) of the experimental diets

Parameter (%)	Control	Garlic (2%)	Neem (2%)	Ginger (2%)
Moisture	9.57	9.24	10.18	9.07
Ash	3.08	3.04	4.65	4.38
Crude fibre	7.20	7.47	8.80	11.00
Crude protein	20.30	22.00	23.40	22.00
Ether extract	12.00	12.41	14.29	15.00
Nitrogen-free extract	47.85	45.84	38.68	38.55

3.2 Haematological responses of *Clarias gariepinus*

The haematological characteristics of *Clarias gariepinus* fed the experimental diets are presented in Table 2. Dietary supplementation with garlic, ginger, and neem influenced several erythrocytic and leucocytic parameters.

Table 2 Haematological parameters of *Clarias gariepinus* fed experimental diets

Parameter	Control	Garlic (2%)	Ginger (2%)	Neem (2%)
PCV (%)	32.20 $\pm$ 2.96 ^{ab}	37.80 $\pm$ 2.69 ^a	30.20 $\pm$ 0.66 ^b	34.40 $\pm$ 1.91 ^{ab}
Plasma protein (g dL ⁻¹)	6.56 $\pm$ 0.45	5.96 $\pm$ 0.30	5.84 $\pm$ 0.24	6.02 $\pm$ 0.34
Hb (g dL ⁻¹)	10.72 $\pm$ 0.93 ^{ab}	12.48 $\pm$ 0.77 ^a	10.04 $\pm$ 0.28 ^b	11.36 $\pm$ 0.67 ^{ab}
WBC (cells μ L ⁻¹)	4890 $\pm$ 302 ^b	5690 $\pm$ 93 ^{ab}	5660 $\pm$ 529 ^{ab}	6750 $\pm$ 469 ^a
RBC ($\times 10^6$ cells μ L ⁻¹)	23.60 $\pm$ 4.10 ^b	51.80 $\pm$ 3.38 ^a	20.00 $\pm$ 2.30 ^b	48.00 $\pm$ 6.36 ^a
Neutrophils (%)	41.20 $\pm$ 0.58 ^b	46.40 $\pm$ 4.99 ^{ab}	44.80 $\pm$ 0.37 ^{ab}	50.00 $\pm$ 0.71 ^a
Lymphocytes (%)	50.80 $\pm$ 1.74	49.80 $\pm$ 5.02	48.80 $\pm$ 0.58	44.80 $\pm$ 0.80
Monocytes (%)	2.80 $\pm$ 0.37 ^a	2.00 $\pm$ 0.00 ^b	1.60 $\pm$ 0.24 ^b	2.00 $\pm$ 0.00 ^b
Eosinophils (%)	5.00 $\pm$ 1.00 ^a	2.60 $\pm$ 0.40 ^b	4.40 $\pm$ 0.24 ^a	4.00 $\pm$ 0.00 ^{ab}
Basophils (%)	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00

Table notes: Values are presented as mean $\pm$ SD. Means within a row bearing different superscripts differ significantly at $P < 0.05$.

Fish fed the garlic-supplemented diet exhibited the highest packed cell volume (37.80 $\pm$ 2.69%) and haemoglobin concentration (12.48 $\pm$ 0.77 g dL⁻¹), whereas the lowest values were recorded in fish fed the ginger diet. Red

blood cell counts were significantly higher ($P < 0.05$) in fish fed garlic and neem diets than in the control and ginger-fed groups, indicating enhanced erythropoietic activity in these treatments.

White blood cell counts increased across all herbal-supplemented groups relative to the control treatment, with the highest count observed in fish fed the neem diet (6750 ± 469 cells μL^{-1}). Similarly, neutrophil percentages increased significantly from $41.20 \pm 0.58\%$ in the control group to $50.00 \pm 0.71\%$ in the neem-fed fish. These changes suggest enhanced cellular defence responses in fish receiving herbal supplementation.

Lymphocyte percentages ranged from $44.80 \pm 0.80\%$ to $50.80 \pm 1.74\%$ and did not differ significantly among treatments. Monocyte values were significantly lower in the supplemented groups than in the control treatment, while eosinophil counts were lowest in fish fed the garlic diet. Basophil values remained constant across all treatments.

4 Discussion

The proximate composition of the experimental diets showed that the inclusion of garlic, ginger, and neem modified the nutrient profile of the feeds. Compared with the control diet, the supplemented diets contained higher levels of crude protein, crude fibre, ash, and ether extract, while nitrogen-free extract decreased. These changes indicate that the herbal materials contributed additional nutrients alongside their phytochemical constituents. Since feed composition plays a major role in nutrient utilization and physiological processes, such variations may influence the overall health and performance of cultured fish. Similar observations have been reported for African catfish, where dietary nutrient composition has been shown to affect digestibility and metabolic efficiency (Langi et al., 2024; Oluyemi et al., 2024).

Among the diets, the neem-supplemented feed recorded the highest crude protein content (23.40%), whereas the garlic and ginger diets contained 22.00% crude protein. Protein is essential for tissue growth, enzyme synthesis, haemoglobin formation, and immune function. Previous studies have shown that adequate dietary protein improves nutrient utilization and physiological performance in African catfish (Fregene et al., 2024). The relatively higher protein content of the neem diet may have contributed to the favourable haematological responses observed in fish exposed to this treatment.

Mineral content, as reflected by ash values, was also higher in the herbal diets, particularly in the neem and ginger treatments. Ash represents the total mineral fraction of the feed and provides an indication of the availability of essential elements involved in cellular metabolism and blood formation. Earlier studies have shown that adequate mineral supply supports normal physiological activities and promotes efficient nutrient utilization in cultured fish (Oluyemi et al., 2024). Thus, the elevated ash content observed in the supplemented diets may have supported the maintenance of physiological stability throughout the feeding period.

Crude fibre increased with the inclusion of the herbal additives, with the highest value recorded in the ginger-supplemented diet. This increase was expected because plant materials naturally contain appreciable amounts of fibre. Although excessive dietary fibre may reduce digestibility in carnivorous fish species, moderate inclusion levels are generally tolerated. The absence of adverse changes in the measured blood parameters suggests that the 2% inclusion level used in the present study did not negatively affect fish health.

A decline in nitrogen-free extract was observed in the supplemented diets compared with the control. This reduction appears to be a consequence of the corresponding increases in protein, fibre, lipid, and mineral fractions. Since African catfish rely largely on protein and lipid metabolism for growth and maintenance, moderate changes in carbohydrate content are unlikely to compromise performance provided that energy requirements are adequately met. Similar observations have been reported by Langi et al. (2024), who emphasized the greater importance of protein quality in determining the performance of *Clarias gariepinus*.

The haematological responses observed in this study indicate that dietary supplementation with garlic, ginger, and neem influenced the physiological condition of the fish. Blood parameters are widely used in fish health assessment

because they respond to nutritional status, stress, and disease conditions. Previous studies have demonstrated that haematological variables serve as sensitive indicators of physiological changes in African catfish (Melefa et al., 2020; Okomoda et al., 2024). The differences recorded among the dietary treatments suggest that the herbal supplements elicited distinct physiological responses.

Fish fed the garlic-supplemented diet recorded the highest packed cell volume and haemoglobin concentration. These parameters are closely associated with oxygen transport and are often used to assess the physiological status of fish. Reduced values have been linked with stress and impaired metabolic activity, whereas elevated values are generally associated with improved oxygen-carrying capacity and better physiological condition (Dawood et al., 2022; Okomoda et al., 2024). The results obtained in the present study suggest that garlic promoted erythropoietic activity and enhanced blood oxygen transport.

This pattern was further reflected in the red blood cell counts, which were considerably higher in fish fed garlic and neem diets than in those receiving the control diet. Red blood cells are responsible for transporting oxygen to tissues and are therefore important for metabolic processes. The beneficial effects of garlic observed in the present study agree with earlier reports indicating that garlic supplementation improves physiological condition and innate immune responses in African catfish (Nyadjeu et al., 2023; Megbowon et al., 2024). These findings highlight the potential of garlic as a functional feed additive capable of improving blood characteristics in cultured fish.

Among the herbal treatments, neem produced the strongest response in terms of immune-related parameters. Fish fed the neem diet recorded the highest white blood cell counts and neutrophil percentages. White blood cells and neutrophils are important components of the innate immune system and are involved in defence against invading pathogens. Previous studies have shown that neem-derived compounds possess immunostimulatory properties and can enhance disease resistance in cultured fish (Oyebola et al., 2020; Ibrahim et al., 2023). The elevated leukocyte values observed in this study are therefore indicative of enhanced immune competence rather than pathological stress.

Although the erythrocytic responses obtained with ginger were less pronounced than those observed with garlic and neem, the measured blood parameters remained within normal physiological limits. This suggests that ginger supported normal physiological function throughout the feeding period. Earlier studies have consistently reported beneficial effects of ginger on growth, antioxidant status, and overall health in African catfish (Wei et al., 2023; Uddin et al., 2025). Differences in inclusion level, feeding duration, and phytochemical composition may explain the relatively moderate responses recorded in the present study.

Another notable finding was the increase in white blood cell counts across all herbal treatments compared with the control group. This trend indicates that the three phytobiotics stimulated aspects of innate immunity, although the magnitude of response varied among treatments. Similar observations have been reported in studies advocating the use of phytogenic additives as sustainable alternatives to antibiotics in aquaculture (Mohammed et al., 2025; Le Xuan, 2026). The results of the present study support the growing interest in plant-based feed additives as practical tools for improving fish health and reducing dependence on conventional chemotherapeutic agents.

5 Conclusion

This study examined the effects of dietary supplementation with garlic (*Allium sativum*), ginger (*Zingiber officinale*), and neem (*Azadirachta indica*) on the nutritional quality of formulated diets and the haematological responses of *Clarias gariepinus*. The inclusion of these herbal additives improved the nutrient composition of the diets through increases in crude protein, ash, crude fibre, and ether extract, accompanied by a reduction in nitrogen-free extract. These changes indicate that the herbs contributed not only bioactive compounds but also nutrients capable of enhancing feed quality.

The haematological results showed that the phytogenic supplements influenced fish health in different ways. Fish fed the garlic-supplemented diet recorded higher packed cell volume, haemoglobin concentration, and red blood cell counts, suggesting improved oxygen transport and better physiological condition. In contrast, neem

supplementation resulted in higher white blood cell and neutrophil counts, indicating a stronger stimulation of innate immune responses. Although the effects of ginger were less pronounced, the blood parameters remained within normal physiological ranges, suggesting that the additive supported normal body functions without adverse effects.

An important finding of this study is that the three phyto-genic supplements did not produce identical responses. Garlic showed greater influence on erythrocytic parameters, whereas neem exerted stronger effects on immune-related indices. These differences highlight the diverse biological roles of herbal feed additives and suggest that their use can be tailored to specific production goals, whether improving physiological performance, enhancing immunity, or promoting overall fish health.

The results also emphasize the importance of evaluating dietary interventions beyond growth performance alone. The relationship observed between dietary quality and haematological responses suggests that nutritional status and physiological wellbeing are closely linked. Consequently, blood-based indicators provide additional information that complements conventional growth measurements and contributes to a more comprehensive assessment of fish welfare.

Overall, the findings indicate that garlic, ginger, and neem have considerable potential as functional feed additives in African catfish culture. Their ability to support physiological condition and immune function without reliance on synthetic chemotherapeutic agents highlights their value in sustainable and environmentally responsible aquaculture practices.

The findings demonstrate the complementary physiological roles of garlic, ginger, and neem as phyto-genic feed additives, supporting their application in sustainable African catfish production. Future research should optimise dietary inclusion levels, evaluate combined herbal formulations, and integrate biochemical, molecular, and commercial performance indicators under long-term farming conditions to strengthen evidence-based nutritional strategies for aquaculture.

Conflict of Interest Disclosure

The author affirms that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

References

- Akpomughe E., Nwachi O.F., and Awhefeada O.K., 2024, Estimation of genetic diversity in silver catfish (*Chrysichthys nigrodigitatus*) using random amplified polymorphic DNA, FUDMA Journal of Sciences, 7(6): 110-119.
<https://doi.org/10.33003/fjs-2023-0706-2177>
- Awhefeada O.K., Bekederemo B.O., Akpomughe E., and Okpu P.N., 2025, Morphometric characterization, heterosis expression, and growth performance of genetically improved farmed tilapia and red tilapia hybrids for sustainable aquaculture development, Journal of Science Research and Reviews, 2(2): 145-155
<https://doi.org/10.70882/josrar.2025.v2i2.102>
- Dawood M.A., Noreldin A.E., and Sewilam H., 2022, Blood biochemical variables, antioxidative status, and histological features of tissues of African catfish exposed to stress, Environmental Science and Pollution Research, 29(37): 56357-56369.
<https://doi.org/10.1007/s11356-022-19702-0>
- Ekpemikoghene I.E., Arimiche N.A., and Kesena E.J., 2024, Length-weight relationship of *Clarias gariepinus* fed plant extracts, FUDMA Journal of Sciences, 8(2): 217-224.
<https://doi.org/10.33003/fjs-2024-0802-2348>
- Fregene B.T., Yossa R., Karisa H.C., Olaniyi A., and Bolorunduro P., 2024, Extension manual on quality low-cost fish feed formulation, Gates Open Research, 8(69): 69.
<https://doi.org/10.21955/gatesopenres.1117112.1>
- Ibrahim R.E., Elshopakey G.E., Abdelwarith A.A., Younis E.M., Ismail S.H., Ahmed A.I., et al., 2023, Chitosan neem nanocapsule enhances immunity in Nile tilapia, Heliyon, 9(9).
<https://doi.org/10.1016/j.heliyon.2023.e19354>
- Imron I., Iswanto B., Suparpto R., and Marnis H., 2020, Development of genetically improved farmed African catfish, *Clarias gariepinus*: A review and lessons learned from Indonesian fish breeding program, IOP Conference Series: Earth and Environmental Science, 593(1): 012032.
<https://doi.org/10.1088/1755-1315/593/1/012032>

- Isibor P.O., Ogochukwu O.O., Valerie I.E., Samson T.O., Akinduti P., and Yemisi O., 2023, Comparative analysis of the trophic status, length-weight relationship, gastro-somatic index and bioaccumulation of trace metals in wild and captive *Clarias gariepinus*, in Biotechnological Approaches to Sustainable Development Goals, pp. 85-101, Springer Nature Switzerland, Cham.
https://doi.org/10.1007/978-3-031-33370-5_6
- Khieokhajonkhet A., Sangphrom S., Aeksiri N., Tatsapong P., Wuthijaree K., and Kaneko G., 2022, Effects of long-term exposure to high temperature on growth performance, chemical composition, haematological and histological changes, and physiological responses in hybrid catfish [σ *Clarias gariepinus* (Burchell, 1822) $\times$ ϕ *C. macrocephalus* (Günther, 1864)], Journal of Thermal Biology, 105: 103226.
<https://doi.org/10.1016/j.jtherbio.2022.103226>
- Klimuk A.A., Beketov S.V., and Kalita T.L., 2024, Physiological and ecological features of cultivation African catfish *Clarias gariepinus*, Biology Bulletin Reviews, 14(Suppl. 3): S326-S335.
<https://doi.org/10.1134/S2079086424600747>
- Kwikiriza G., Tebitendwa S.M., Rwezawula P., Mwanja W.W., Abaho I., and Meimberg H., 2025, Enhancing African catfish (*Clarias gariepinus*) aquaculture in Uganda: Insights into hatchery propagation, population suitability, and broodstock management, Fishes, 10(6): 290.
<https://doi.org/10.3390/fishes10060290>
- Langi S., Maulu S., Hasimuna O.J., Kaleinasho Kapula V., and Tjipute M., 2024, Nutritional requirements and effect of culture conditions on the performance of the African catfish (*Clarias gariepinus*): A review, Cogent Food & Agriculture, 10(1): 2302642.
<https://doi.org/10.1080/23311932.2024.2302642>
- Le Xuan C., 2026, Phytobiotics in aquafeeds: Challenges, opportunities, and sustainable practices, Phytobiotics for Sustainable Aquaculture, pp. 205-219.
<https://doi.org/10.1201/9781003646945-18>
- Mangkunegara A.A., Fadjar M., and Iranawati F., 2023, Dumbo catfish (*Clarias gariepinus*) microplastic exposure on abnormalities and level of blood component using a completely randomized design, Jurnal Teknologi (Sciences & Engineering), 85(6): 133-143.
<https://doi.org/10.11113/jurnalteknologi.v85.19764>
- Megbowon I., Ukenye E.A., Oguntade O.R., Akinwale M.M.A., Joseph J.B., Edah B., et al., 2024, Individual and combined effects of moringa leaf, ginger and garlic powder on growth and innate immune response of *Clarias gariepinus* juveniles, Cogent Food & Agriculture, 10(1): 2328427.
<https://doi.org/10.1080/23311932.2024.2328427>
- Melefa T.D., Mgbenka B.O., Aguzie I.O., Andong F.A., Nwakor U., and Nwani C.D., 2020, Morphological, haematological and biochemical changes in African catfish *Clarias gariepinus* (Burchell 1822) juveniles exposed to clotrimazole, Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 236: 108815.
<https://doi.org/10.1016/j.cbpc.2020.108815>
- Mohammed E.A.H., Kovács B., Kuunya R., Mustafa E.O.A., Abbo A.S.H., and Pál K., 2025, Antibiotic resistance in aquaculture: Challenges, trends analysis, and alternative approaches, Antibiotics, 14(6): 598.
<https://doi.org/10.3390/antibiotics14060598>
- Musa B.O., Hernández-Flores A., Adeogun O.A., and Oresogun A., 2021, Determination of a predictive growth model for cultivated African catfish *Clarias gariepinus* (Burchell, 1882), Aquaculture Research, 52(9): 4434-4444.
<https://doi.org/10.1111/are.15282>
- Nyadjeu P., 2021, Effect of *Zingiber officinale* and *Allium sativum* powders as natural feed additives promoting growth, feed utilization and whole-body composition in *Clarias gariepinus* fry, Food and Nutrition Sciences.
- Nyadjeu P., Arreytabe D.J., Nkongho G.O., and Tomedi-Tabi M.E., 2023, Growth and physiological responses of juvenile African catfish (*Clarias gariepinus*) fed with dietary supplementation of garlic-ginger mixture, International Journal of Fisheries.
<https://doi.org/10.37745/ijfar.15/vol9n21832>
- Nyadjeu P., Tamko Ndjuiisi N.A., Mane Yemdjie D.D., Chamsy Dedou N.Y., and Fonkwa G., 2021, Dietary inclusion effect of ginger and garlic blend on growth in African catfish, International Journal of Aquaculture and Fishery Sciences, 7(2): 014-023.
- Ojelade O.C., Durosaro S.O., Akinde A.O., Abdulraheem I., Oladepo M.B., Sopein C.A., et al., 2022, Environmental enrichment improves growth and behavior of juveniles of *Clarias gariepinus*, Frontiers in Veterinary Science, 9: 980364.
<https://doi.org/10.3389/fvets.2022.980364>
- Okomoda V.T., Isah S., Solomon S.G., and Ikhwannuddin M., 2024, Salinity tolerance in *Clarias gariepinus*: Insight on blood parameter variations, Fish Physiology and Biochemistry, 50(2): 605-616.
<https://doi.org/10.1007/s10695-023-01293-3>
- Oluah N.S., Aguzie I.O., Ekechukwu N.E., Madu J.C., Ngene C.I., and Oluah C., 2020, haematological and immunological responses in African catfish exposed to herbicide, Ecotoxicology and Environmental Safety, 201: 110824.
<https://doi.org/10.1016/j.ecoenv.2020.110824>
- Oluayemi G., Oluchukwu A., and Alabi V., 2024, Nutrient digestibility of non-conventional protein sources in African catfish, Asian Journal of Fisheries and Aquatic Research, 26(4): 100-106.
<https://doi.org/10.9734/ajfar/2024/v26i4760>
- Ovie K.A., Okpu P., Oster F.N., and Emuesiri A., 2025, Evaluation of proximate composition of varying black soldier fly (BSF) larvae as an alternative protein source, Journal of Science Research and Reviews, 2(4): 1-8.
<https://doi.org/10.70882/josrar.2025.v2i4.95>

- Oyebola O.O., Adebayo J.O., and Oshinowo A.J., 2020, Piscicidal and prophylaxis potentials of neem extract in African catfish, *Journal of Experimental Agriculture International*, 42(9): 250-261.
<https://doi.org/10.9734/jeai/2020/v42i930607>
- Parvin A., Shukla U., and Singh R., 2023, Therapeutic impact of neem oil on walking catfish health, *World Journal of Biology, Pharmacy and Health Sciences*, 14(3): 83-91.
- Sadauki M.A., Bichi A.H., and Umar J., 2024, Length-weight relationship and condition factor of *Clarias gariepinus* from Ajiwa Reservoir, *Life Sciences Journal*, 1(1): 143-150.
- Sayed A.E.D.H., Taher H., Soliman H.A., and Salah El-Din A.E.D., 2022, Immunological and hemato-biochemical effects on catfish exposed to dexamethasone, *Frontiers in Physiology*, 13: 1018795.
<https://doi.org/10.3389/fphys.2022.1018795>
- Srimai W., Koonawootrittriron S., Chaivichoo P., Manee-aphai W., Phu-onnim A., Koolboon U., and Na-Nakorn U., 2020, Selection response and genetic parameters for growth in African catfish, *Aquaculture*, 518: 734843.
<https://doi.org/10.1016/j.aquaculture.2019.734843>
- Uddin N., Shahriar F., Ibasina A.A., Hasan M.T., Kawsar M.A., and Munny F.J., 2025, Efficacy of *Zingiber officinale* for growth and health of catfish, *Aquaculture, Fish and Fisheries*, 5(3): e70087.
<https://doi.org/10.1002/aff2.70087>
- Wei L.S., Kari Z.A., Kabir M.A., Khoo M.I., Azra M.N., and Wee W., 2023, Promoting growth and health of African catfish using ginger leaf powder, *Aquaculture Studies*, 24(4).
<https://doi.org/10.4194/AQUAST1719>
- Yusuff K., and Ibidapo-Obe E., 2024, Insights in the use of neem in aquaculture industry, *Conference Proceedings*.
- Zidan E.M., Goma A.A., Tohamy H.G., Soliman M.M., and Shukry M., 2022, Impact of salinity on behavioural and biochemical responses of African catfish, *Aquaculture Research*, 53(7): 2750-2759.
<https://doi.org/10.1111/arc.15790>



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Review and Progress

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Application Mechanisms and Practical Pathways of Ecological Food Chain Closure in Freshwater Pond Aquaculture

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Abstract Freshwater pond aquaculture is an important component of China's aquaculture system. However, long-term high-density feeding and intensive management can easily lead to the accumulation of residual feed, feces, organic detritus, and nutrients in the water column and sediment, resulting in water quality deterioration, sediment aging, eutrophication, and increased pressure from tailwater discharge. Ecological food chain closure is based on material cycling, energy flow, and trophic complementarity. By rationally configuring producers, consumers, and decomposers, aquaculture wastes can be reintroduced into the internal circulation of pond ecosystems, thereby achieving resource reuse and pollution reduction. This study explores the application mechanisms and practical pathways of ecological food chain closure in freshwater pond aquaculture, elaborates its roles in nutrient recycling, water self-purification regulation, and multi-trophic synergistic enhancement, and summarizes typical models including fish-shrimp-shellfish-algae coordinated aquaculture, fish-aquatic plant-microorganism ecological regulation, and pond-ecological ditch-constructed wetland recycling purification. The study suggests that ecological food chain closure can improve pond resource utilization efficiency, enhance water self-purification capacity, reduce pressure from tailwater treatment, and promote the transformation of aquaculture systems from high-input and high-discharge models toward ecological, circular, and efficient development. However, its promotion still faces challenges such as unreasonable biological configuration, fluctuations in environmental conditions, insufficient technical management capacity, and incomplete economic evaluation systems. In the future, green and sustainable development of freshwater pond aquaculture should be promoted through site-specific model selection, optimization of aquaculture species and trophic-level configuration, improvement of water quality monitoring and tailwater recycling systems, and strengthening of standardized and digital management.

Keywords Ecological food chain closure; Freshwater pond aquaculture; Nutrient cycling; Water self-purification; Green aquaculture

1 Introduction

Freshwater pond aquaculture is an important component of China's aquaculture system and also a major supporting form of global aquatic food production. It plays an important role in ensuring the supply of aquatic products, increasing farmers' income, and promoting the development of rural industries. In countries with large-scale freshwater aquaculture, such as China, pond aquaculture accounts for a substantial proportion of total aquatic product output. However, with the rapid intensification of pond aquaculture, traditional production methods that rely on manual feeding, mechanical aeration, frequent water exchange, and pharmaceutical disease control have increased yield per unit area to some extent, but they have also intensified the dependence of aquaculture systems on external inputs such as feed, water resources, energy, and drugs. As stocking density and feed input increase, residual feed, feces, dead algae, and other organic matter continuously accumulate in the water column and sediment, easily leading to increases in ammonia nitrogen, nitrite, total nitrogen, and total phosphorus. This, in turn, results in water quality deterioration, sediment aging, eutrophication, frequent disease outbreaks, and increased pressure from tailwater discharge. These problems not only constrain the sustainable development of freshwater pond aquaculture but may also adversely affect surrounding water bodies and ecosystems (Boyd et al., 2020; Liu et al., 2020; Duan et al., 2024). Meanwhile, the growing demand for green, low-carbon, and environmentally friendly food systems is driving pond aquaculture to shift from a high-input,

high-consumption, and pollution-emitting model toward a resource-saving, ecologically efficient, and circular utilization model (Henares et al., 2020; Zhong et al., 2022; Dong et al., 2025).

The green transformation of freshwater pond aquaculture is both a practical need for environmental protection and an important opportunity for high-quality industrial development. Relevant studies have shown that, during the long-term intensive expansion of pond aquaculture in China, pond water quality once showed a declining trend, whereas the promotion of ecological engineering, ecological aquaculture practices, and green policy measures has helped improve pond water environments (Liu et al., 2020; Duan et al., 2024). Eco-efficiency assessments also indicate that freshwater aquaculture still faces redundant inputs and pressure from pollutant emissions, suggesting considerable room for improving resource use efficiency and reducing pollution discharge while maintaining economic benefits (Zhong et al., 2022; Dong et al., 2023). Against this background, ecological engineering pathways such as integrated multi-trophic aquaculture, pond cascade systems, planted oxidation ponds, bio-integrated aquaponic systems, and aquaculture-agriculture integrated systems have gradually attracted attention and are regarded as important technical directions for coordinating aquaculture production with environmental protection (Henares et al., 2020; Thomas et al., 2020; ong et al., 2022; Lothmann and Sewilam, 2022; D Kajgrová et al., 2023; Goda et al., 2024).

In the process of green transformation of freshwater pond aquaculture, the concept of ecological food chain closure provides a new approach for optimizing aquaculture systems. Ecological food chain closure refers to the establishment of relatively stable material cycling and energy flow relationships within pond ecosystems through the rational allocation of producers, consumers, and decomposers. In this process, residual feed, feces, and organic detritus generated after feed input can be decomposed by microorganisms, absorbed by algae and aquatic plants, and further reused by filter-feeding fish, omnivorous fish, benthic animals, and other organisms at different trophic levels. Existing studies have shown that combining fed fish with detritivores, filter feeders, macrophytes, and vegetables can improve feed conversion efficiency, increase nitrogen and phosphorus retention, and transform aquaculture waste into additional biomass (Thomas et al., 2020; Dong et al., 2022; Kajgrová et al., 2023; Goda et al., 2024). Therefore, ecological food chain closure does not simply mean increasing the number of cultured species; rather, it emphasizes the complementarity among different biological groups in terms of ecological niches, feeding targets, spatial distribution, and functional roles. Integrated multi-trophic aquaculture in freshwater and marine systems, pond cascade systems, and aquaculture-agriculture integrated models all indicate that strengthening internal food webs, promoting nutrient recycling, and reducing dependence on external water exchange can improve resource use efficiency and generate diversified products (Henares et al., 2020; Lothmann and Sewilam, 2022). Ecological food chain closure not only helps improve nutrient recycling efficiency by converting residual feed and metabolic wastes that might otherwise become pollution sources into ecological resources that can be reused within the system, but also enhances pond self-purification capacity through nitrogen and phosphorus uptake by aquatic plants, organic matter degradation by microorganisms, phytoplankton regulation by filter feeders, and sediment improvement by benthic animals, thereby reducing disease risks and pressure from tailwater treatment.

This study will explore the application mechanisms and practical pathways of ecological food chain closure in freshwater pond aquaculture. In response to the demand for green transformation in freshwater pond aquaculture, it systematically analyzes the basic connotations, system composition, and operational logic of ecological food chain closure; focuses on its mechanisms in nutrient cycling, water self-purification regulation, and multi-trophic synergistic enhancement; and further examines its implementation pathways in production practice by considering typical models such as fish-shrimp-shellfish-algae coordinated aquaculture, fish-aquatic plant-microorganism ecological regulation, and pond-ecological ditch-constructed wetland recycling purification. Through an analysis of ecological structure design, aquaculture species configuration, water quality monitoring, tailwater recycling, and standardized management, this paper aims to provide theoretical references and practical guidance for the green, ecological, and efficient development of freshwater pond aquaculture.

2 Basic Connotations and System Composition of Ecological Food Chain Closure

2.1 Conceptual definition of ecological food chain closure

Ecological food chain closure refers to the rational configuration of different functional organisms, such as producers, consumers, and decomposers, within a defined aquaculture space according to the principles of material cycling, energy flow, and trophic complementarity in ecosystems. Through this configuration, major outputs generated during aquaculture, including residual feed, feces, organic detritus, sludge, tailwater, and dead organisms, can be collected, transformed, and reintegrated into production pathways within the system, thereby minimizing direct discharge, landfill disposal, and nutrient loss. Compared with traditional pond aquaculture characterized by single-species culture, high feed input, and high discharge, ecological food chain closure places greater emphasis on functional complementarity among biological groups within the aquaculture system. By constructing relatively complete food chains, decomposition chains, and purification chains, wastes that might otherwise become pollution sources can be transformed into reusable ecological resources, thereby reducing waste accumulation and discharge pressure. Relevant studies have shown that zero-waste and circular aquaculture models can integrate closed ponds, composting treatment, and plant production, allowing nutrients in fish aquaculture wastewater and sludge to be valorized, thereby improving productivity and reducing operating costs (Lothmann and Sewilam, 2022; Nenciu et al., 2022). Closed ecological recirculating aquaculture systems also couple biological components such as phytoplankton, zooplankton, and fish to achieve internal CO₂- O₂ circulation and nutrient reuse within the system (Takeuchi, 2017).

From the perspective of operational logic, ecological food chain closure is not simply about increasing the number of polyculture species; rather, it establishes systematic connections around the process of "resource input-biological utilization-waste transformation-nutrient reuse-product output." After feed input, part of the nutrients is directly consumed by the main cultured animals and converted into aquaculture products, while residual feed, feces, and metabolic wastes that are not fully utilized can re-enter the food chain through microbial decomposition, aquatic plant uptake, plankton transformation, and re-feeding by filter-feeding or omnivorous animals. In this way, pond aquaculture systems can gradually shift from a one-way consumption structure to a circular utilization structure. In aquaculture practice, recirculating aquaculture systems (RAS), aquaponic systems, biofloc technology, and integrated multi-trophic aquaculture (IMTA) all demonstrate closed-loop characteristics by strengthening nutrient cycling, reducing tailwater discharge, and improving resource utilization efficiency. At the ecosystem level, integrated pond and IMTA models show that the introduction of filter feeders, macroalgae, or other functional cultured species can enhance the utilization of primary productivity and detrital resources, improve energy transfer efficiency, and increase system maturity and stability (Dong et al., 2022; Zhang et al., 2024). Therefore, "closure" is both a physical attribute, referring to reduced discharge and external losses, and a functional attribute, referring to improved internal recycling efficiency and energy utilization.

2.2 Main functional units of freshwater pond ecosystems

Freshwater pond ecosystems can be regarded as relatively small and simplified artificial-natural composite ecosystems. Although their food chains are relatively short, they contain structured functional groups. The main functional units include producers, consumers, and decomposers. Producers mainly include phytoplankton, periphyton, submerged plants, emergent plants, macrophytes, and plants on artificial ecological floating beds. They fix energy through photosynthesis and absorb nutrients such as nitrogen and phosphorus from the water, serving as an important foundation for maintaining pond ecological balance. Consumers include the main cultured fish, shrimp and crabs, shellfish, filter-feeding fish, omnivorous fish, benthic invertebrates, and zooplankton. These organisms occupy different water layers and ecological niches and utilize formulated feed, plankton, organic detritus, and benthic resources in different ways. Ecopath-based pond studies generally divide pond systems into components such as primary producers, zooplankton of different size classes, benthic invertebrates, bacteria in water and sediment, detritus pools, and cultured fish and invertebrates at lower trophic levels (Zhang et al., 2024). In integrated aquaculture ponds, clams or other filter feeders usually occupy the second trophic level and can directly utilize phytoplankton and detritus, thereby altering the overall energy flow pathway of the system (Dong et al., 2022).

Decomposers are mainly composed of bacteria, fungi, and other microbial communities and serve as indispensable transformation links in ecological food chain closure in ponds. They decompose residual feed, feces, dead organisms, and organic matter in sediment, transforming complex organic matter into inorganic nutrients that can be absorbed and utilized by plants and algae, and they play key roles in carbon, nitrogen, and phosphorus cycling. Meanwhile, sediment, water bodies, ecological ditches, constructed wetlands, biofilm substrates, and aeration facilities can also be regarded as important supporting units for system operation. Together, these units affect dissolved oxygen, pH, transparency, sediment condition, nutrient concentration, and microbial community structure in the water, thereby determining whether the ecological closed-loop system can operate stably. It should be noted that the functional units in freshwater pond ecosystems are not static; rather, they are strongly influenced by aquaculture management. Intensive or integrated aquaculture ponds are usually under a high degree of artificial control, and feed input, mechanical aeration, water exchange regulation, and water quality management can alter trophic relationships and internal system structure (Zhang et al., 2024). Studies have also shown that zooplankton and macrozooplankton are often important functional groups affecting energy transfer, whereas detritus and associated bacteria in the water and sediment are important nodes of system throughput, frequently receiving large amounts of unused feed, feces, and dead biomass. These materials may become pollution loads, but they can also be reintroduced into the food chain through appropriate species or ecological substrates (Dong et al., 2022).

2.3 Material cycling and energy flow in closed-loop systems

In ecological food chain closure in freshwater ponds, material cycling mainly involves the transformation of carbon, nitrogen, phosphorus, and organic matter. Aquaculture feed is the main source of external material input into the system. After the main cultured animals consume feed, part of the nutrients is converted into biomass, while the remaining portion enters the water column and sediment in the form of residual feed, feces, and metabolic products. Microorganisms decompose these organic substances and release nutrients such as ammonia nitrogen and phosphate, which are then absorbed and utilized by algae, aquatic plants, and constructed wetland plants. Some of these nutrients are further converted into new aquaculture products through zooplankton, filter-feeding fish, shellfish, and benthic animals. The goal of closed-loop aquaculture is to maximize the internal cycling of nutrients and energy while minimizing their loss to the environment. Ecopath analyses of integrated ponds show that most primary production and energy usually flow into detritus pools, but the introduction of additional species such as clams and filter-feeding fish can significantly increase the proportion of primary productivity and detritus flowing to the second trophic level and harvestable biomass. Ecosystem indicators such as Finn's cycling index and mean path length usually increase in integrated aquaculture systems, indicating that, compared with monoculture systems, integrated systems have higher internal recycling levels, greater maturity, and stronger resistance to disturbance (Figure 1) (Dong et al., 2022; Zhang et al., 2024).

Energy flow begins from primary energy sources such as feed, algae, and aquatic plants and is transferred stepwise along different trophic levels. Because energy is continuously lost during transfer, the key objective of closed-loop systems is not to achieve complete energy recovery, but to maximize utilization efficiency among different trophic levels and reduce resource waste and pollution accumulation. For example, filter-feeding fish and shellfish can utilize plankton and suspended particles, benthic animals can utilize deposited organic detritus, aquatic plants can absorb dissolved nutrients, and microorganisms and biofilms can promote organic matter degradation and nutrient regeneration. Technological and management innovations further strengthen these biological cycles. Studies have shown that ecological substrates that support biofilm formation can increase food-web pathways by more than 25%, improve detritus utilization by approximately 15%, and enhance energy transfer efficiency and system stability (Zhang et al., 2024). Recirculating aquaculture systems and aquaponic systems achieve water circulation through mechanical and biological filtration, sometimes with the addition of wetland or algae units. In such systems, nutrient-rich tailwater can be used to irrigate crops or cultivate macroalgae, thereby closing water and nutrient cycles and reducing eutrophication potential (Lothmann and Sewilam, 2022; Nenciu et al., 2022). Life cycle assessments also indicate that when feed, energy use, and by-product utilization are optimized, closed-loop systems can reduce water consumption, nutrient emissions, and overall environmental burdens per unit of food produced. Therefore, material cycling and energy flow in

closed-loop freshwater ponds essentially integrate pond ecological structure, trophic configuration, and circular economy principles, providing a basis for water quality improvement, yield enhancement, and green aquaculture.

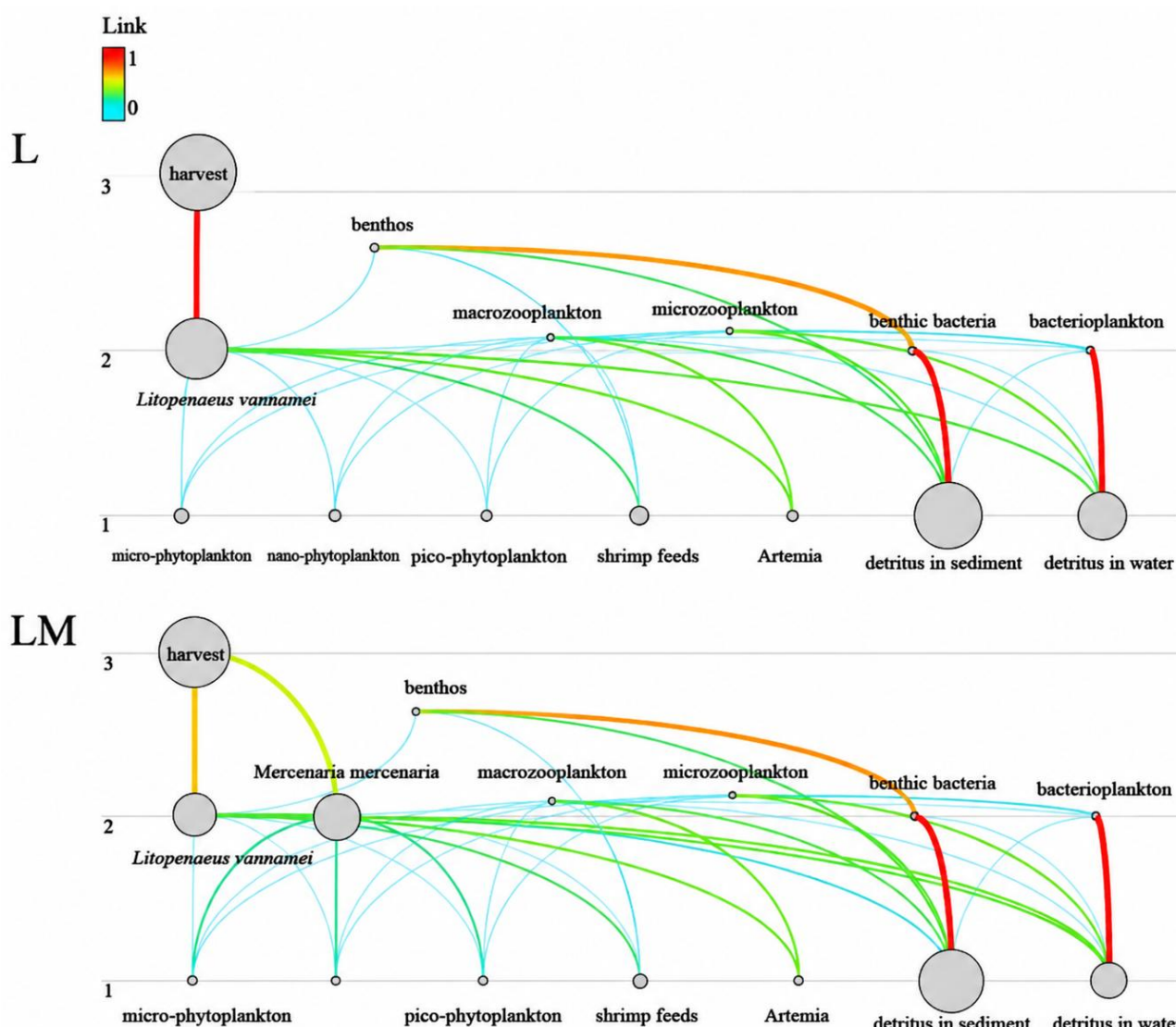


Figure 1 Trophic relationships between functional groups in the two aquaculture ecosystems (Adopted from Dong et al., 2022)
Image caption: L-a monoculture system of *L. vannamei*; LM-an integrated aquaculture system of *L. vannamei* with *M. mercenaria*. Each circle represents the corresponding functional group, the connecting line represents the energy transfer process, and the circle size represents the biomass volume of each group (Adopted from Dong et al., 2022)

3 Application Mechanisms of Ecological Food Chain Closure in Freshwater Pond Aquaculture

3.1 Nutrient recycling and utilization mechanism

In freshwater pond aquaculture, feed input is the main source through which nutrients such as nitrogen (N) and phosphorus (P) enter the system. After cultured organisms such as main cultured fish, shrimp, and crabs consume feed, part of the nutrients is converted into body biomass and eventually forms aquatic products; the remaining part enters the water column and sediment in the form of residual feed, feces, metabolic products, and dead organisms. If these materials cannot be transformed and utilized in a timely manner, they will continuously accumulate in the pond, causing increases in ammonium nitrogen, nitrite, total nitrogen, and total phosphorus, which further leads to water quality deterioration, eutrophication, and increased disease risk. Relevant studies have shown that a considerable proportion of input nitrogen and phosphorus in traditional ponds accumulates in sediments or is discharged with tailwater, resulting in low nutrient utilization efficiency and increasing the risk of

ammonium nitrogen and nitrite accumulation (Zhang et al., 2024). Enzyme activity in sediments and coupled nitrogen-phosphorus cycling can regenerate large amounts of dissolved inorganic nutrients; if these nutrients cannot be effectively absorbed and utilized by biota, they will further aggravate water eutrophication and aquaculture disease risks (Bai et al., 2022). Therefore, one of the core functions of ecological food chain closure is to reincorporate these potentially polluting nutrients into the internal cycling of the pond-farm system, transforming them from "wastes" into reusable ecological resources.

Ecological food chain closure realizes nutrient cycling through the multi-stage process of "microbial decomposition-plant uptake-animal reuse." Microbial communities first decompose residual feed, feces, and organic detritus, converting complex organic matter into inorganic nutrients such as ammonia nitrogen and phosphate. Phytoplankton, aquatic plants, and artificial floating-bed plants further absorb nitrogen, phosphorus, and other nutrients in the water, thereby reducing the risk of eutrophication. Zooplankton, filter-feeding fish, shellfish, and benthic animals can then utilize algae, suspended particles, and deposited organic matter, converting low-level resources into harvestable biomass. Model analyses and budget assessments show that macrophytes and other primary producers can contribute more than half of nitrogen and phosphorus retention, indicating that plant and microbial components play key roles in closed nutrient cycling (Campanati et al., 2021; Zhang et al., 2024).

Ecological engineering and integrated multi-trophic aquaculture (IMTA) further restructure nutrient cycling pathways, allowing feed residues and excreta to be gradually transformed and absorbed by multiple biological sinks. For example, in freshwater IMTA-aquaponic systems, fed fish convert part of dietary nitrogen and phosphorus into biomass, detritivores and filter feeders recover nutrients from particulate matter, and macrophytes and hydroponic vegetables assimilate dissolved nutrients, increasing overall nitrogen and phosphorus utilization efficiency to more than 80%~90% and significantly reducing discharge (Figure 2) (Campanati et al., 2021; Goda et al., 2024). Microalgae-bacteria consortia, biofilms, and functional microbial groups involved in nitrification, denitrification, and organic phosphorus hydrolysis are important foundations supporting this cycling process. They can transform toxic forms of nitrogen into biomass or inert end products while providing material support for organisms at higher trophic levels (Biswas et al., 2020).

3.2 Water self-purification and ecological regulation mechanism

Ecological food chain closure can enhance the water self-purification capacity of freshwater ponds. The key lies in the coordinated configuration of hydraulic processes, habitat structures, and biological components, which enables pollutants to be intercepted, settled, absorbed, and transformed along the water flow pathway. Aquatic plants absorb nutrients such as nitrogen and phosphorus through their roots, stems, leaves, and associated microorganisms, while also providing attachment substrates and habitats for microorganisms and small aquatic animals, thereby helping stabilize the ecological structure of the water body. Phytoplankton can release oxygen through photosynthesis, but excessive proliferation may cause algal blooms and diel fluctuations in dissolved oxygen; therefore, filter-feeding fish, shellfish, and zooplankton are needed for moderate regulation to maintain algal abundance within a relatively reasonable range. Cascade pond systems, ecological engineering pond aquaculture systems, and recirculating pond systems equipped with ecological ponds usually employ sequential structures such as ditches, settling units, vegetated zones, biofilm-rich purification ponds, and culture ponds to progressively remove ammonia nitrogen, total nitrogen, total phosphorus, organic matter, and phytoplankton (Liu et al., 2014). Relevant measurements show that the removal rates of total nitrogen and total phosphorus in such systems often exceed 40%~60%, and chlorophyll a and chemical oxygen demand are also significantly reduced, allowing culture ponds to maintain lower nutrient and algal levels than conventional ponds under similar or even higher production conditions (Biswas et al., 2020).

Microorganisms play a fundamental role in water purification and ecological regulation. Beneficial microbial communities can promote organic matter degradation, participate in nitrification, denitrification, and sediment remediation, and reduce the accumulation of harmful substances such as ammonia nitrogen, nitrite, and hydrogen sulfide. High-throughput sequencing studies have shown that Proteobacteria, Actinobacteria, Bacteroidetes,

Cyanobacteria, and specialized denitrifying genera are dominant in engineering ponds, IMTA systems, and recirculating ponds. As system operation time increases, improvements in microbial diversity and functional gene abundance are usually associated with water quality improvement. Meanwhile, seagrass or macrophyte reservoirs and constructed wetlands can filter suspended solids, store nutrients in plant biomass, regulate dissolved oxygen through photosynthesis, and reduce pathogen loads, thereby pretreating and purifying pond inflow or tailwater (Liu et al., 2014; Biswas et al., 2020). While feeding on deposited organic detritus, benthic animals can disturb sediment through their activities, improving bottom-water exchange and sediment permeability. Engineering units such as aeration facilities, ecological ditches, and constructed wetlands further strengthen water circulation, sedimentation filtration, and plant uptake functions. As a result, pond systems no longer rely solely on frequent water exchange and chemical regulation, but instead maintain water quality stability and ecological balance through the integration of biological purification, microbial transformation, and engineering regulation.

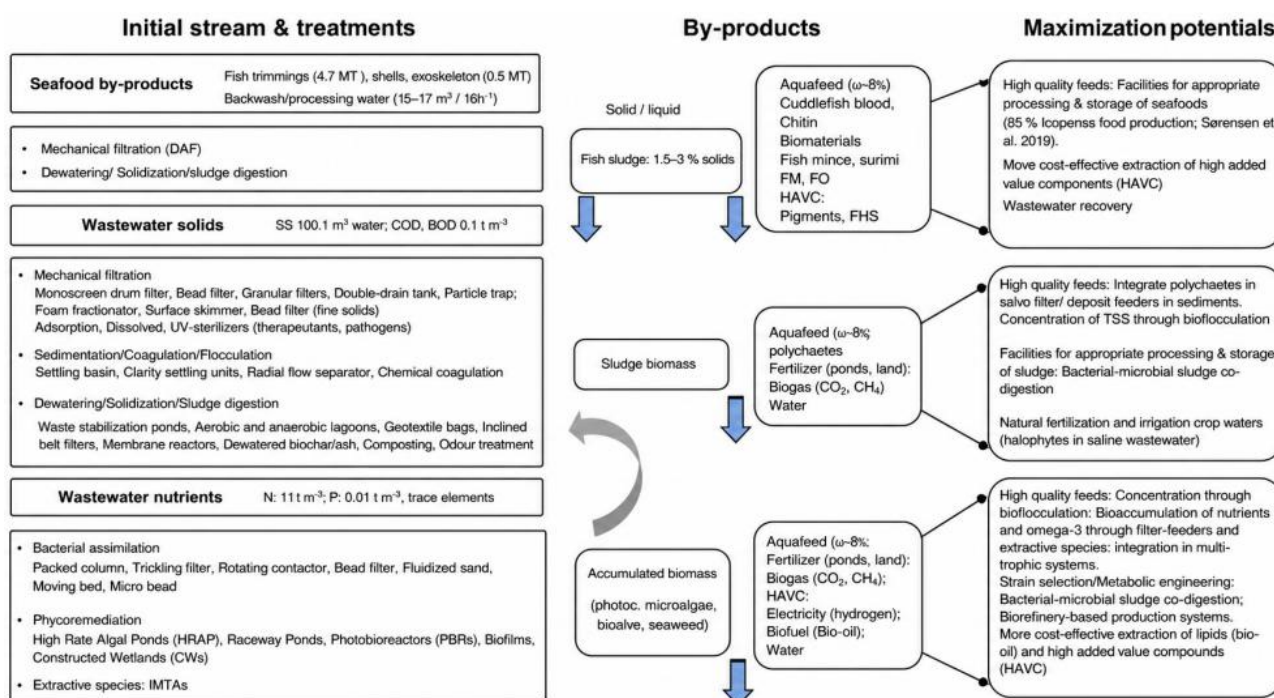


Figure 2 Potential co-products derived from the processing of seafood and wastewater treatments (Adopted from Campanati et al., 2021)

Image caption: Solid material from wastewaters is retained through mechanical filtration and/or sedimentation. Dissolved nutrients are retrievable from wastewater through bacterial assimilation and phycoremediation and can be upcycled (i.e. through increased yield in biomass) throughout the food web (e.g. IMTAs). Following removal of large solids and accumulated suspended material (i.e. TSS, biofloc, microalgae) in concentrated sludge form, dewatering and thickening then permit by-products to be obtained through different recovery paths and allow subsequent applications. Through this process of solid-liquid separation, the water discarded or generated from the processing (light-blue arrows) can be reused. Improvements that can maximize production (e.g. cost-effective methods for higher biomass yield) from recycled materials are highlighted on the right side of the diagram. DAF: Dissolved air floatation; FM: Fish meal; FO: Fish oil; HAVC: High added value components; PHs: Protein hydrolysate (Adopted from Campanati et al., 2021)

3.3 Multi-trophic synergistic enhancement mechanism

Multi-trophic synergy is an important basis for the comprehensive benefits of ecological food chain closure and is also the key mechanism distinguishing it from traditional monoculture or simple polyculture. Traditional single-species aquaculture often focuses on the utilization of a particular type of feed resource or water-layer space, which easily leads to insufficient resource use, unused ecological niches, and pollutant accumulation. In contrast, ecological food chain closure combines organisms according to their feeding habits, activity layers, and ecological functions, enabling effective use of different spaces, including the upper, middle, and bottom water

layers, as well as the water column, sediment, and plant zones. For example, herbivorous or omnivorous fish can utilize some plant-based feed and organic detritus; filter-feeding fish and shellfish can utilize plankton and suspended particles; detritivores can process benthic wastes; aquatic plants and macroalgae can absorb dissolved nutrients and improve habitat conditions; and microbial consortia support the transformation of carbon, nitrogen, phosphorus, and other elements (Biswas et al., 2020; Tang et al., 2024). This niche complementarity enables materials and energy in ponds to cascade through multiple trophic levels, thereby improving system resource utilization efficiency.

IMTA and polyculture studies consistently show that, compared with monoculture or simple polyculture, combining fed species such as fish and shrimp with detritivores, filter feeders, and primary producers can increase total productivity, improve individual growth and survival, and simultaneously reduce environmental impacts. For example, combinations such as shrimp-fish-bivalve-seaweed or giant freshwater prawn-carp-mussel can suppress cyanobacterial blooms, enhance beneficial microorganisms in sediments, reduce nitrogen and phosphorus emissions in tailwater by 50%~60%, and achieve higher productivity indices and profitability (Biswas et al., 2020; Tang et al., 2024). Ecosystem models and dynamic energy budget models of three-trophic-level IMTA systems further confirm that properly balanced stocking densities can reduce waste concentrations while maximizing multispecies yield, indicating that food-web design and density optimization are central to achieving trophic synergy. Therefore, by reasonably controlling the proportions of main cultured species, companion species, and purification organisms, freshwater ponds can form composite aquaculture systems that integrate production, purification, and ecological regulation functions, thereby achieving the coordinated goals of yield enhancement, environmental improvement, and risk reduction.

4 Typical Application Models of Ecological Food Chain Closure in Freshwater Ponds

4.1 Fish-shrimp-shellfish-algae coordinated aquaculture model

The fish-shrimp-shellfish-algae coordinated aquaculture model is a representative multi-trophic utilization model in ecological food chain closure in freshwater ponds. In essence, it is the pond-based application of integrated multi-trophic aquaculture (IMTA). This model combines fed species, such as fish and shrimp, with extractive species, such as bivalves, seaweeds, or macroalgae, so that feed, plankton, organic detritus, and dissolved nutrients in ponds can be utilized across different levels. In such systems, fish and shrimp consume formulated feed and form the main economic output, while also producing particulate and dissolved wastes. Shellfish reduce water turbidity and regulate plankton abundance by filtering phytoplankton, bacterial flocs, and suspended particles. Algae or macrophytes, as primary producers, can absorb dissolved nutrients such as nitrogen and phosphorus and also provide a natural food base for filter-feeding organisms. Existing studies have shown that combining fish and shrimp with extractive organisms such as shellfish and algae can transform waste nutrients into additional biomass and improve aquaculture water quality (Biswas et al., 2020; Chang et al., 2020; Thomas et al., 2020; Tang et al., 2024).

During the operation of this model, external feed is first consumed by fish and shrimp. Uneaten feed and excreta from cultured animals then enter the water column and can promote the growth of algae and microorganisms. An appropriate amount of algae can increase dissolved oxygen through photosynthesis and provide food for filter-feeding fish and shellfish, while filter-feeding organisms can in turn control excessive algal proliferation and reduce the risk of algal blooms. Shrimp and some benthic animals can utilize organic detritus deposited at the pond bottom, thereby reducing the organic load in sediment. Thus, a circular chain of "feed input-fish and shrimp growth-organic matter release-algal transformation-reuse by shellfish and filter feeders" is formed within the pond. In brackish or low-salinity pond trials, combinations of shrimp, fish, oysters, and water spinach or seaweeds significantly reduced inorganic nitrogen and phosphorus levels, improved dissolved organic carbon utilization, and enhanced shrimp growth performance (Biswas et al., 2020).

In experimental IMTA ponds, combinations of shrimp, milkfish, mussels, and *Gracilaria* showed significantly higher specific growth rates, survival rates, and combined biomass of shrimp and fish than monoculture or simple

polyculture systems, demonstrating the advantages of multi-trophic complementarity. From an economic perspective, combinations of shrimp, filter-feeding animals, and macroalgae can increase biomass yield and the survival rate of high-value shrimp, while reducing disease and stress through bioremediation, shading, and water quality stabilization, thereby improving overall returns (Thomas et al., 2020). Studies on shrimp IMTA in China have also emphasized that combinations such as shrimp + crab + clam + fish or shrimp + jellyfish + clam can be flexibly adjusted according to local species and resource conditions, making full use of pond carrying capacity and reducing tailwater discharge with little additional feed and labor input (Chang et al., 2020).

4.2 Fish-aquatic plant-microorganism ecological regulation model

The fish-aquatic plant-microorganism ecological regulation model emphasizes the synergistic effects among cultured fish, aquatic plants, and microbial communities to improve pond water quality and sediment conditions, control eutrophication risks, and enhance system self-purification capacity. Aquatic plants include submerged plants, emergent plants, floating-leaved plants, and plants on artificial ecological floating beds. They can absorb nutrients such as nitrogen and phosphorus from the water and reduce eutrophication pressure. Meanwhile, plant roots and surfaces provide substrates for microbial attachment, growth, and reproduction, forming rhizosphere microecosystems that enhance organic matter degradation and nutrient transformation. For fish culture, appropriate aquatic plant configuration can also provide shelter, buffer changes in water temperature, and improve the habitat environment of cultured animals. Taking largemouth bass ponds planted with the submerged macrophyte *Elodea nuttallii* as an example, studies have shown that integrating submerged plants into pond systems can significantly reduce total nitrogen, total phosphorus, and chemical oxygen demand, increase dissolved oxygen levels, and effectively prevent cyanobacterial blooms compared with conventional ponds without vegetation (Nie et al., 2023). Lotus-fish co-culture systems can also reduce nitrogen, phosphorus, nitrite, and COD in the water, as well as organic matter and total nitrogen in sediments, while supporting functionally beneficial archaeal and fungal networks that promote ammonia oxidation, organic matter decomposition, and pathogen suppression.

Microorganisms are an important link connecting aquaculture production and ecological purification in this model. Beneficial microbial communities in ponds can decompose residual feed, feces, and dead algae, promote the transformation of organic matter into inorganic nutrients, and participate in the transformation of substances such as ammonia nitrogen and nitrite. Aquatic plants absorb these transformed nutrients and reduce their accumulation in the water, while microorganisms in the plant rhizosphere can further improve pollutant degradation efficiency. Studies on pond microbiology have pointed out that algae and bacteria play central roles in maintaining pond productivity, regenerating nutrients, and influencing tailwater, and that management of microbial food webs is an important basis for optimizing pond production (Moriarty, 1997).

Recent studies have shown that, in recirculating ponds enhanced by bacteria-microalgae associations, targeted bioaugmentation using biofilm carriers can significantly reduce total nitrogen, ammonia nitrogen, and nitrite by altering nitrogen-cycling functional genes and microbial community composition, while increasing fish production by more than 13%. Studies on microbial and water quality dynamics in tilapia ponds have also shown that bacterial and microalgal communities are closely related to water quality parameters such as water temperature, dissolved oxygen, ammonia nitrogen, and nitrate, indicating that ecological regulation based on microbial processes is an important pathway for maintaining stable pond operation. Through the operational process of "nutrient load generated by fish culture-microbial decomposition and transformation-aquatic plant uptake and fixation-water quality improvement," this model can enhance the self-purification capacity and ecological stability of pond systems while maintaining aquaculture output (Nie et al., 2023).

4.3 Pond-ecological ditch-constructed wetland recycling purification model

The pond-ecological ditch-constructed wetland recycling purification model is an engineered closed-loop model that integrates pond aquaculture systems with external ecological purification facilities. It is particularly suitable for large-scale and contiguous freshwater pond aquaculture areas. By connecting aquaculture ponds with

multi-stage purification units such as ecological ditches, ecological ponds, settling zones, and constructed wetlands, this model extends ecological food chain closure from inside a single culture pond to areas beyond the pond, thereby achieving tailwater recycling and advanced purification. In this model, aquaculture tailwater is no longer directly discharged into external water bodies. Instead, it first enters a settling zone or ecological ditch, where reduced flow velocity allows suspended particles, residual feed, and part of the organic detritus to settle. The tailwater then flows through ecological ditches and constructed wetlands planted with aquatic vegetation, where plant uptake, substrate adsorption, microbial degradation, and sedimentation-filtration further reduce nitrogen, phosphorus, organic matter, and suspended solids. Studies on ecological engineering pond aquaculture recirculating systems (EPAS) have shown that, after integrating culture ponds with ecological ditches, ecological ponds, and subsurface-flow constructed wetlands, the average removal rates of ammonia nitrogen, total nitrogen, and total phosphorus in the treatment process can reach 44%~63%, CODMn removal can reach 62%, and chlorophyll a removal can reach 83%, making nutrient and pigment concentrations in culture ponds significantly lower than those in control ponds (Liu et al., 2014).

The advantage of this model lies in its ability to extend ecological regulation from individual ponds to the aquaculture area scale, forming a system pathway of "pond production-tailwater collection-ecological purification-recycling or compliant discharge." Ecological ditches serve as channels for water transport and primary purification, while constructed wetlands undertake advanced purification and ecological buffering functions. Treated water can be reused for pond replenishment according to water quality conditions or discharged into the external environment after meeting discharge requirements, thereby reducing the impact of aquaculture tailwater on surrounding rivers, lakes, and farmland ecosystems. Under the EPAS configuration, unit yield increased from 0.75 kg/m³ to 0.82 kg/m³, and the feed coefficient decreased by 8.2%, indicating that water quality improvement can also enhance aquaculture efficiency (Liu et al., 2014). At larger scales, constructed wetlands used to treat tailwater from inland fish farms or shrimp farms can continuously remove 25%~70% of nitrogen species, more than 60% of phosphorus, and approximately 30%~40% of COD, often bringing total nitrogen, total phosphorus, and COD levels below national discharge standards (Li et al., 2024; Wang et al., 2024).

These multi-stage systems close material cycles through hydraulic retention, plant uptake, microbial transformation, and substrate adsorption/precipitation. Multi-stage surface-flow constructed wetlands composed of different aquatic plants, aeration, and biofiltration membranes can maintain total removal efficiencies above 60% across different seasons, with plant-associated biofilms and changing bacterial communities jointly supporting nitrogen, phosphorus, and organic matter removal. Cases of constructed wetlands based on settling ponds and native reeds have shown that, by optimizing hydraulic conditions, removal rates of organic matter, nutrients, and heavy metals from shrimp farm wastewater can reach as high as 80%~91% while shortening treatment time. Compared with systems that rely solely on internal pond regulation, this model has stronger stability and operability and can help promote freshwater pond aquaculture toward regional management, circular utilization, and green production.

5 Practical Pathways for Ecological Food Chain Closure in Freshwater Pond Aquaculture

5.1 Scientific design of pond ecological structure

The effective operation of ecological food chain closure first depends on the scientific and rational design of pond ecological structure. A freshwater pond is not a single aquaculture water body, but a composite ecosystem composed of multiple spatial units, including the water body, sediment, banks, inlet and drainage systems, plant purification zones, tailwater treatment zones, and ecological ditches. Therefore, the focus of pond ecological structure design lies not only in determining the size and shape of the aquaculture water surface, but also in systematically organizing the spatial pattern and hydraulic processes, so that water, nutrients, and pollutants can sequentially pass through multiple functional units such as production, sedimentation, absorption, decomposition, and purification before discharge or recycling. In practice, pond functional zones should be reasonably divided according to pond area, water depth, sediment type, water source conditions, main cultured species, and

production objectives, forming a spatial pattern in which the main culture zone, ecological buffer zone, plant purification zone, sedimentation zone, and tailwater collection and treatment zone are interconnected.

Ecological engineering practices in China indicate that eco-engineered pond recirculating systems generally integrate ecological ditches, ecological ponds, subsurface-flow constructed wetlands, and culture ponds to control water replenishment, regulate algal communities, and improve water quality (Liu et al., 2020). In these systems, constructed wetlands and ecological ditches can reduce harmful algal groups and increase the proportion of diatoms, thereby optimizing phytoplankton structure while removing nitrogen and phosphorus from tailwater (Liu et al., 2020). In the design of pond ecological structure, attention should also be paid to water-layer utilization, habitat diversity construction, and spatial planning at the regional scale. Different cultured organisms vary in their use of water space, and the upper, middle, and bottom water layers, as well as nearshore shallow-water areas, can all undertake different ecological functions. For example, the main cultured fish mainly provide economic output, aquatic plant zones are responsible for nutrient uptake and ecological buffering, sediment remediation zones undertake organic matter sedimentation and microbial decomposition, and ecological ditches and sedimentation zones provide preliminary tailwater purification. Through spatial zoning and functional allocation, niche conflicts among different organisms can be reduced, internal resource utilization efficiency in ponds can be improved, and system stability can be enhanced.

Pond cascade systems represent another important structural pathway. Through a series of interconnected ponds managed by batch discharge and refilling, they can achieve strong self-purification, reducing total phosphorus and total nitrogen in the terminal pond to approximately half of their initial concentrations, while supporting advanced fry production and multiple stocking-harvesting cycles (Kajgrová et al., 2023). At a broader scale, ecosystem-based spatial planning and functional zoning help arrange ponds rationally within watersheds, enabling them to meet ecological and social carrying capacity requirements while coordinating biosecurity, water resource management, and disease management among clustered farms. Remote-sensing assessments also show that pond siting, density, and the conversion of surrounding habitats affect sustainability at the landscape scale; therefore, scientifically determining the location and spatial configuration of pond areas is an important prerequisite for constructing ecological food chain closure.

5.2 Optimization of aquaculture species and trophic-level configuration

The key to constructing ecological food chain closure lies in optimizing aquaculture species combinations so that different organisms form complementary relationships in feeding habits, activity layers, and ecological functions. Traditional pond aquaculture often centers on a single main cultured species, which can easily lead to insufficient feed resource utilization, accumulation of residual feed and feces, and increased pressure on water quality regulation. Ecological closed-loop aquaculture should, on the basis of clearly identifying the main cultured species, reasonably combine functional organisms such as filter-feeding fish, omnivorous fish, benthic animals, aquatic plants, and beneficial microorganisms, so that formulated feed, plankton, organic detritus, and dissolved nutrients can be utilized stepwise by different trophic levels. This can improve comprehensive output per unit water body and reduce the impact of aquaculture wastes on water quality and sediment conditions. Studies on ecological engineering and fish polyculture have shown that, compared with single-species intensive ponds, polyculture systems based on species' ecological niches and feeding habits can significantly improve nitrogen utilization, energy conversion efficiency, and environmental performance. Cases of traditional multi-pond shrimp systems in China combined with bivalves and macroalgae have also shown higher equivalent shrimp output and increased input nitrogen utilization by more than 80%, indicating that carefully designed multi-trophic combinations have strong potential for nutrient recovery (Liu et al., 2020).

In specific configuration, the principles of "moderate polyculture, functional complementarity, and controllable density" should be followed. The stocking density of the main cultured species should match the carrying capacity of the pond and should not exceed the regulatory capacity of the ecosystem in pursuit of high yield. Companion species should be selected according to their ecological functions: filter-feeding fish can be used to regulate

plankton and suspended particles, benthic animals to utilize deposited organic detritus, aquatic plants to absorb nitrogen and phosphorus and improve habitats, and beneficial microorganisms to promote organic matter decomposition and sediment remediation. Meanwhile, feed input, aeration intensity, and biological configuration ratios should be dynamically adjusted according to seasonal changes, culture stages, and water quality conditions, so as to avoid system imbalance caused by excessive proliferation or insufficient function of a particular organism group. Recent freshwater IMTA studies using combinations of giant freshwater prawn, silver carp, bighead carp, and freshwater pearl mussels have shown that appropriate species combinations can suppress cyanobacterial blooms, increase beneficial sediment microorganisms, reduce phosphorus emissions in tailwater by approximately 60%, and improve nitrogen and phosphorus utilization rates by approximately 11%~13%. However, trade-offs such as high mussel mortality or inhibited growth of cyprinid fish also indicate that multi-trophic configuration still needs to be optimized according to density, species adaptability, and system carrying capacity (Tang et al., 2024).

In addition to empirical trials, species screening and compatibility pre-assessment tools can also support the design of ecological food chain closure. The AquaDesign assistant uses ecological niche hypervolumes and global abiotic environmental data to identify suitable environmental conditions for candidate species and determine whether different species combinations can coexist under shared abiotic environments in ponds, raceways, and recirculating systems (Butruille et al., 2022). Conceptual frameworks for polyculture design also emphasize species compatibility and complementarity and propose a stepwise procedure from candidate species screening, matching with culture environments, and management tool development to stakeholder collaboration (Thomas et al., 2020). In addition, analyses of "trophic level" in aquaculture caution against simply promoting nominally low-trophic species, because the effective trophic level is a property of the entire aquaculture system and is jointly influenced by feed sources, management practices, and system operational efficiency. Therefore, sustainability assessment should be based on actual feed use, management practices, and system performance. These insights collectively indicate that species configuration for ecological food chain closure should shift from "increasing species number" to "matching ecological functions" and "optimizing system efficiency."

5.3 Improvement of water quality monitoring and tailwater recycling systems

Water quality monitoring is an important support for ensuring the stable operation of ecological food chain closure. Freshwater pond ecosystems are characterized by obvious dynamic changes. Indicators such as water temperature, dissolved oxygen, pH, ammonia nitrogen, nitrite, total nitrogen, total phosphorus, transparency, turbidity, and conductivity are all affected by weather, feeding, stocking density, microbial activity, and algal changes. Without continuous monitoring and timely regulation, ecological closed-loop systems may lose balance due to hypoxia, excessive algal proliferation, sediment deterioration, or pollutant accumulation. Therefore, a management mechanism combining daily pond inspection, regular testing, and early warning of key indicators should be established to track water quality trends in a timely manner and adopt measures such as aeration, water exchange, feed control, sediment remediation, beneficial microbial supplementation, or adjustment of plant coverage based on monitoring results. In recent years, recirculating aquaculture systems (RAS) and pond-RAS hybrid systems have relied on mechanical and biological treatment units to remove particulate matter, nitrogen pollutants, phosphorus, carbon dioxide, antibiotics, and other hazardous substances, returning purified water to culture ponds or facilities and thereby greatly reducing overall water use and pollutant discharge (Liu et al., 2020).

Case studies have shown that tailwater purification ponds in ecological RAS can reduce antibiotic concentrations in aquaculture water by more than half, while recirculating systems can achieve more than 80% ammonia nitrogen removal and more than 70% phosphate removal, bringing multiple water quality parameters within recommended limits (Shi et al., 2022). Regarding tailwater utilization, pond drainage should be incorporated into the overall circulation system to avoid direct discharge of untreated aquaculture tailwater. Facilities such as sedimentation ponds, ecological ditches, constructed wetlands, ecological purification ponds, and plant purification belts can be built to achieve graded reduction of suspended solids, organic matter, nitrogen and phosphorus nutrients, antibiotic residues, and other potential pollutants in tailwater. Treated water can be reused for pond replenishment,

irrigation of surrounding farmland, or compliant discharge according to water quality conditions, thereby improving water resource utilization efficiency and reducing pressure on surrounding aquatic environments. Reviews of tailwater pollution control in large-scale aquaculture emphasize that constructed wetlands, ecological ditches, IMTA, and RAS models play complementary roles in wastewater treatment and internal water recycling on farms (Li et al., 2024).

Meanwhile, water quality monitoring and tailwater recycling systems require robust real-time monitoring support to operate efficiently and prevent system failures. Multi-sensor systems, Internet of Things (IoT) platforms, and artificial intelligence technologies can now continuously measure key parameters such as temperature, pH, dissolved oxygen, turbidity, conductivity, nutrients, and salinity, and provide early warning of critical events such as hypoxia, algal abnormalities, and sudden water quality changes. IoT-based RAS systems can also track water flow through biofilters, support assessment of biofilm formation, and enable remote automatic control of pumps and aeration equipment to maintain stable conditions under high stocking densities (Lindholm-Lehto, 2023; Tran-Minh, 2025). Therefore, integrating intelligent monitoring tools with ecological engineering units such as ecological ditches, constructed wetlands, purification ponds, and pond cascade systems can support adaptive management of water renewal, feed input, and treatment loading, promoting the transformation of ecological food chain closure from experience-based management to data-driven and intelligent management.

6 Major Problems in the Application of Ecological Food Chain Closure

6.1 System imbalance caused by unreasonable biological configuration

Ecological food chain closure emphasizes the coordinated configuration of different trophic levels and functional organisms, and its stable operation depends on rational species composition, stocking density, and trophic complementarity. However, in practical application, some aquaculture operators have insufficient understanding of pond ecosystem carrying capacity, biological niches, and interspecific relationships, and therefore tend to simply regard ecological closure as multi-species polyculture. If the proportions among the main cultured species, companion species, aquatic plants, and microorganisms lack a scientific basis, feeding competition, spatial competition, or functional overlap may occur among different organisms. For example, when filter-feeding fish are insufficiently configured, phytoplankton cannot be effectively regulated, making eutrophication more likely; when benthic animals are insufficient, deposited organic detritus cannot be utilized in a timely manner, increasing the risk of sediment deterioration; and when aquatic plants are excessively configured, water flow, nighttime dissolved oxygen levels, and the activity space of cultured animals may be affected. Relevant studies have also shown that inappropriate biological configuration may lead to interspecific competition, inefficient nutrient utilization, and environmental degradation.

In Mediterranean integrated multi-trophic aquaculture (IMTA) ponds, three fish species with different nominal trophic levels still mainly relied on commercial feed and competed for similar food sources, while macroalgae were barely utilized and only oysters effectively consumed excess phytoplankton. This indicates that, if feeding habits and natural productivity are not properly understood, the expected trophic complementarity may not be realized (Nahon et al., 2023). Studies on IMTA and biofloc technology have also pointed out that scientific species selection and appropriate population size are necessary conditions for achieving optimal biological and chemical processes; otherwise, ecosystem health and sustainability will be affected (Khanjani et al., 2022). In addition, unreasonable biological configuration may weaken the stability of ecological food chain closure. A closed-loop system does not mean that the more organisms of all types it contains, the better; instead, a dynamic balance among main production, resource utilization, and ecological regulation must be formed. If one trophic level is too strong or too weak, material cycling and energy transfer efficiency will both be affected.

Excessively high density of the main cultured fish increases feed input and metabolic waste discharge, making microbial decomposition and plant uptake capacity difficult to match. Insufficient aquatic plant coverage makes it difficult to perform nitrogen and phosphorus uptake and habitat stabilization functions. Improper supplementation of beneficial microorganisms may also fail to form dominant communities due to unsuitable water temperature,

dissolved oxygen, or sediment conditions. Model analyses and field assessments indicate that misjudging pond carrying capacity and species functions may lead to system instability. An ecosystem model of crab-shrimp-clam ponds showed that system benefits peak near a specific stocking density; if stocking density continues to increase beyond this threshold, environmental standards may be exceeded and system performance may decline (Dong et al., 2023). Large-scale IMTA practices in Sanggou Bay also show that although complex species combinations have good ecological and production potential, they are difficult to manage because interspecific interactions are not yet fully understood, making unbalanced configurations likely. Therefore, species in ecological closed-loop systems should have ecological complementarity, local adaptability, and economic feasibility; otherwise, organic and inorganic loads will continue to accumulate, thereby weakening the realization of both environmental and economic objectives (Biswas et al., 2020; Knowler et al., 2020).

6.2 Effects of environmental changes on closed-loop operation efficiency

The operational efficiency of ecological food chain closure in freshwater ponds is significantly affected by environmental conditions. Factors such as water temperature, light, rainfall, dissolved oxygen, pH, water source quality, and sediment condition can all alter biological activities and material transformation processes within the system. Closed and semi-closed aquaculture systems are particularly sensitive to environmental changes because their self-purification processes, microbial transformations, and trophic interactions all depend on temperature, pH, and other external drivers. When water temperature is high, feeding activity of cultured animals increases and microbial decomposition accelerates, but the risk of declining dissolved oxygen also rises. When water temperature is low, the growth of aquatic plants and microbial activity weaken, and nutrient uptake and organic matter decomposition efficiency decline. Heavy rainfall, continuous cloudy and rainy weather, or hot and humid conditions may also cause water stratification, pH fluctuation, abnormal algal proliferation, or hypoxic floating, thereby affecting the normal operation of ecological closed-loop systems. Long-term dynamic simulations of ecosystem-based pond aquaculture indicate that climate change can simultaneously alter physical, chemical, and biological processes and affect carp growth and pond ecosystem functions over long time scales; therefore, environmental changes must be incorporated into pond planning and operational management (Varga et al., 2020). Comprehensive studies on the effects of temperature increase in aquaculture also indicate that anthropogenic warming can directly affect the growth, survival, reproduction, immunity, and microbiomes of cultured species, while indirectly increasing disease risk, parasite loads, and contaminant toxicity, thereby threatening the productivity of closed systems with limited buffering capacity.

Sediment conditions, water source quality, and changes in microbial networks are also important factors affecting closed-loop efficiency. Long-term high-density aquaculture ponds often experience sediment organic matter accumulation, anaerobic conditions in bottom layers, and release of harmful substances. Even if aquatic plants and beneficial microorganisms are introduced, poor sediment conditions may make it difficult to rapidly restore ecological functions. If the external water source contains high levels of suspended solids, nutrients, or pollutants, it will also increase the burden of pond ecological regulation. Environmental fluctuations can also reshape microbial networks and food-web structures, thereby affecting the stability of ecological closure. Long-term lake data show that seasonal changes in temperature and pH can alter microbial network complexity and keystone taxa; the loss or weakening of these keystone taxa can destabilize communities and reduce ecological stability. Studies on coastal fish communities have also found that an approximately 20% increase in temperature can lead to declining community diversity, a shift of interaction networks toward instability, and the emergence of critical slowing down, manifested as the dominance of a few competitive species and the reduction of cooperative species. In ecosystem-based ponds and IMTA systems, microorganisms and lower trophic organisms are important drivers of nutrient cycling. Therefore, community shifts caused by environmental changes may reduce nutrient removal efficiency, alter system carrying capacity, and increase the risks of regime shifts, eutrophication, and production losses when management measures fail to adapt. Thus, ecological food chain closure is not a fixed model that can operate stably independent of environmental conditions, but a dynamic system jointly influenced by seasonal changes, climate fluctuations, and pond baseline conditions (Varga et al., 2020).

6.3 Need for improvement in technical management and economic evaluation systems

Ecological food chain closure involves multiple links, including aquaculture species configuration, water quality monitoring, feed management, microbial regulation, aquatic plant management, tailwater treatment, disease prevention and control, and ecological benefit assessment. Therefore, it places higher requirements on the comprehensive management capacity of aquaculture operators. Compared with traditional pond aquaculture, ecological closed-loop aquaculture relies more heavily on process management and dynamic regulation and cannot be conducted through extensive experience-based operation alone. For example, feeding amounts need to be adjusted in a timely manner according to weather, water temperature, feeding behavior, and water quality indicators; aquatic plant coverage should be maintained at an appropriate level to avoid excessive growth or decomposition; and microbial agents should be applied based on dissolved oxygen, sediment condition, and organic load. If management is inadequate, ecological closed-loop models may not only fail to achieve expected results but may also increase production risks. Although ecological food chain closure and IMTA are considered to have the potential to improve sustainability and profitability, related studies indicate that current technical management systems remain insufficient. Reviews of IMTA economics show that there is still a lack of bioeconomic models and tools for comparing different systems and fully internalizing environmental externalities, and that economic incentive mechanisms and eco-certification frameworks require further improvement (Knowler et al., 2020). A comprehensive assessment of freshwater aquaculture in China found that its average ecological efficiency was only 0.702, indicating considerable room for improvement and clear regional differences. This suggests that current management has not yet fully coordinated production efficiency with environmental performance (Wang et al., 2024).

Meanwhile, the current economic evaluation system for ecological food chain closure aquaculture remains insufficient. Some practices place greater emphasis on water quality improvement, tailwater emission reduction, and ecological demonstration effects, while lacking systematic accounting of economic factors such as facility investment, labor costs, management costs, culture cycles, comprehensive output, product premiums, and long-term maintenance expenses. Economic evaluations of eco-friendly technologies and closed-loop systems remain fragmented. Systematic reviews of cost-benefit analysis in aquaculture show that, although related studies are increasing, environmental impacts and social costs are still insufficiently considered, and most studies have not yet incorporated ecological benefits into formal cost-benefit frameworks. Life cycle and economic analyses of aquaponics and aquaponics-IMTA systems also indicate that infrastructure, energy, and labor costs account for a relatively high proportion; for certain species or production scales, the benefit-cost ratio may be marginal or even negative, and profitability depends on the precise optimization of technical parameters, species selection, production scale, and market matching. Case studies of eco-friendly management show that such technologies have the potential to increase yield and reduce water and electricity use, but they also emphasize the need to establish standardized performance indicators, such as Aquaculture Performance Indicators, to simultaneously reflect environmental, economic, and social outcomes. For aquaculture operators, whether ecological models can be continuously promoted depends not only on whether their ecological benefits are evident, but also on whether their economic returns are stable. Therefore, in the future, a comprehensive evaluation system that integrates ecological, production, economic, and social benefits should be established to provide clearer decision-making support for pond aquaculture across different regions, species, and scales.

7 Countermeasures for Optimizing Ecological Food Chain Closure Aquaculture

7.1 Establishing a site-specific model selection mechanism

Optimizing ecological food chain closure aquaculture first requires establishing a site-specific model selection mechanism based on the natural conditions, aquaculture foundation, technical capacity, and industrial needs of different regions. Freshwater pond aquaculture is affected by multiple factors, including climate, water sources, sediment conditions, pond size, main cultured species, market positioning, and regional environmental carrying capacity. Therefore, the same ecological closed-loop model should not be simply applied across different regions. For example, in areas with good water sources, large pond areas, and a foundation for multi-species coordinated aquaculture, priority may be given to multi-trophic coordinated models such as fish-shrimp-shellfish-algae

systems. In areas with high stocking densities and strong pressure from tailwater discharge, pond-ecological ditch-constructed wetland recycling purification systems are more suitable. In ponds with suitable conditions for aquatic plant cultivation or aquaponic development, fish-aquatic plant-microorganism ecological regulation models can be prioritized. Relevant studies have shown that spatial and habitat suitability models integrating hydrodynamics, water quality, and geomorphological characteristics can identify areas suitable for specific aquaculture types and reveal the feasibility and potential risks of models such as pond aquaculture, integrated multi-trophic aquaculture (IMTA), and shellfish culture in different regions (Falconer et al., 2016). Species distribution models and multi-criteria evaluation methods, such as Mahalanobis models, Maxent models, and multi-indicator suitability assessments, can also help planners screen models based on the characteristics of successful farms and optimal environmental thresholds, thereby reducing the negative impacts of inappropriate siting and spatial conflicts on sustainability.

In the process of model selection, ecological suitability, production feasibility, and economic rationality should be integrated. Aquaculture operators need to fully assess pond carrying capacity, baseline water quality, sediment conditions, tailwater discharge requirements, and the characteristics of the main cultured species before determining suitable biological configurations and facility construction plans. For small-scale farmers, low-input and easy-to-manage measures such as plant purification, microbial regulation, and moderate polyculture can be prioritized. For large-scale aquaculture bases, regional closed-loop systems can be constructed through ecological ditches, sedimentation ponds, constructed wetlands, recirculating aquaculture facilities, and digital monitoring platforms. At the farm and pond scale, ecosystem models and bioeconomic models can be used to simulate the effects of different stocking densities, species combinations, feed inputs, and tailwater treatment measures on aquaculture profits, nitrogen and phosphorus removal, and system stability, thereby providing quantitative support for site-specific integrated pond configurations (Biswas et al., 2020; Dong et al., 2023). Studies have also shown that integrated multi-trophic systems and recirculating systems generally outperform monoculture systems in terms of combined economic, ecological, and social benefits, providing an evidence base for local model selection and technical route formulation. Therefore, the selection of ecological food chain closure models should shift from experience-based judgment to an integrated decision-making process of "environmental suitability assessment-carrying capacity estimation-ecological-economic simulation-technical route matching."

7.2 Strengthening standardized technical systems and digital management

To achieve stable promotion of ecological food chain closure aquaculture, it is necessary to strengthen the development of standardized technical systems. At present, some ecological aquaculture practices still rely mainly on experience-based operation and lack unified standards in species matching ratios, stocking density, feeding management, aquatic plant coverage, microbial application frequency, tailwater treatment processes, and operational performance evaluation, resulting in unstable application outcomes. Therefore, operational, replicable, and evaluable technical standards should be established around key links such as pond renovation, biological configuration, feed management, water quality regulation, sediment remediation, tailwater purification, disease prevention and control, and product quality traceability. Standardized management can lower the implementation threshold of ecological closed-loop models and improve technical consistency and management comparability among different aquaculture operators. Relevant reviews have pointed out that standardized technical systems and digital tools are essential for the reliable operation of closed food-chain ponds under variable conditions. In the context of Industry 4.0, sensor networks, automated equipment, and decision-support tools can replace traditional manual and subjective monitoring, enabling real-time and evidence-based management of water quality, feeding, and system operation (Bernal-Higuera et al., 2023).

At the same time, the deep integration of digital technologies with ecological food chain closure aquaculture should be promoted. Freshwater pond ecosystems are characterized by dynamic changes, and relying solely on manual pond inspection and experience-based judgment makes it difficult to detect problems such as declining dissolved oxygen, rising ammonia nitrogen, abnormal algal communities, and sediment deterioration in a timely manner. By deploying online monitoring equipment for water temperature, pH, dissolved oxygen, ammonia

nitrogen, nitrite, turbidity, conductivity, and other parameters, and combining these tools with intelligent aeration, remote warning, and data analysis platforms, continuous monitoring and precise regulation of pond environments can be achieved. IoT-based freshwater recirculating aquaculture systems (RAS) and pond systems can rely on physical, network, and logical architectures, using edge or fog computing devices, secure communication, databases, and intelligent analytics to monitor key variables such as dissolved oxygen and pH, and to drive actuators such as aerators, water pumps, and feeders for closed-loop control (Hala et al., 2024).

Advanced control methods can further improve management precision and system robustness. For example, fuzzy logic systems that integrate multiple sensors with cloud platforms can autonomously maintain appropriate dissolved oxygen, salinity, and pH levels and have demonstrated high accuracy and stability in real pond trials. Intelligent analytical methods, such as M5 model trees, random forests, and technologies for fish tracking, counting, and behavior analysis, can also improve the prediction of key water quality parameters, feeding regulation, and health warning, providing a foundation for unified monitoring standards and evaluation protocols (Bernal-Higuita et al., 2023). Therefore, the standardization of ecological food chain closure should be advanced together with digital platforms, incorporating best practices, monitoring indicators, and data models into technical guidelines for freshwater aquaculture to improve the stability and replicability of model promotion.

7.3 Constructing a coordinated evaluation system for ecological and economic benefits

The promotion of ecological food chain closure aquaculture should not emphasize ecological value alone; instead, a comprehensive evaluation system that integrates ecological, production, economic, and social benefits should be established. In terms of ecological benefits, priority should be given to evaluating water quality improvement, nitrogen and phosphorus reduction in tailwater, sediment remediation effects, reduced drug use, changes in greenhouse gas emissions, and resource utilization of aquaculture wastes. In terms of production benefits, attention should be paid to survival rate, growth rate, yield per unit area, feed utilization efficiency, disease incidence, and multispecies comprehensive output. In terms of economic benefits, facility investment, labor costs, energy costs, management costs, product prices, overall returns, and long-term maintenance expenses should be comprehensively calculated. In terms of social benefits, employment creation, food safety, green product supply, and the sustainable development of aquaculture communities should also be considered.

Ecological-economic evaluation studies have shown that, when Δ DPSIR, emergy analysis, and cost-benefit analysis are used to compare monoculture with IMTA, IMTA can simultaneously reduce nitrogen emissions, greenhouse gas emissions, and external resource harvesting, while moderately increasing farm profits. When public environmental benefits are monetized, total benefits are much higher than the increase in private profits (Knowler et al., 2020; Nobre et al., 2010). Comprehensive indicators such as emergy-based sustainability indices, environmental sustainability indices, and relative coefficients can also quantify trade-offs among resource use, environmental load, and economic returns, and identify better models among alternatives. In practical promotion, attention should also be paid to the value transformation of ecological products and the standardization of evaluation methods. Ecological food chain closure aquaculture can improve the culture environment, reduce dependence on pharmaceuticals, lower tailwater pollution, and enhance aquatic product quality. These advantages need to be transformed into economic returns through brand building, quality certification, green product labels, eco-labeling, and marketing.

For large-scale aquaculture bases, production records, water quality monitoring records, tailwater treatment records, and product traceability systems can be established to enhance product credibility and market recognition. For local specialty aquatic products, ecological aquaculture models can be combined with regional branding, recreational fisheries, and agricultural tourism to expand industrial value-added space. Broader reviews and meta-analyses have pointed out that current life cycle assessment of IMTA systems still faces methodological challenges, including the selection of functional units, data gaps, and inconsistent impact categories. Therefore, it is necessary to establish aquaculture-specific databases and unified life cycle assessment protocols to improve the comparability of different studies and aquaculture models (Hala et al., 2024). A global systematic review of

shrimp farming models also shows that pond IMTA and recirculating systems perform better in terms of comprehensive, economic, ecological, and social benefits, but their results are sensitive to indicators such as survival rate, pH, and dissolved oxygen. This indicates that evaluation systems must incorporate both production process indicators and environmental process indicators. Therefore, evaluation of ecological food chain closure should integrate bioeconomic models, life cycle assessment, energy analysis, cost-benefit analysis, and social indicators to provide a decision-making framework for measuring model performance, internalizing environmental externalities, designing policy incentives, and promoting the sustainable transformation of freshwater pond aquaculture (Nobre et al., 2010; Biswas et al., 2020; Knowler et al., 2020; Dong et al., 2023).

8 Conclusion and Prospects

Ecological food chain closure is based on the principles of material cycling and energy flow in ecosystems. Through the rational configuration of producers, consumers, and decomposers, feed, residual feed, feces, organic detritus, and dissolved nutrients generated during freshwater pond aquaculture can be transformed and reused across multiple levels within the system. Compared with traditional pond aquaculture models characterized by high input and high discharge, this model places greater emphasis on functional complementarity among organisms and the regulation of ecological processes. It can, to a certain extent, reduce the accumulation of aquaculture wastes, improve water quality and sediment conditions, and enhance resource utilization efficiency. Especially in the context of increasing requirements for green development and tailwater emission reduction in aquaculture, ecological food chain closure provides an important pathway for freshwater pond aquaculture to shift from a production-oriented model to an ecologically efficient model. Its value is reflected not only in yield improvement, but also in comprehensive benefits such as enhanced ecological stability, reduced disease risk, and lower environmental load. Ecological food chain closure aquaculture is not simply multi-species polyculture or the application of a single purification technology. Rather, it is a systematic engineering approach involving pond ecological structure design, aquaculture species configuration, water quality regulation, sediment remediation, tailwater recycling, and production management optimization. Therefore, in practical promotion, technical support, refined management, and market transformation need to be integrated. On the one hand, suitable ecological closed-loop models should be selected according to water source conditions, climatic characteristics, pond foundations, and main cultured species in different regions, and standardized operating procedures should be established to improve the stability and replicability of model application. On the other hand, training for aquaculture operators and the development of digital management should be strengthened. Online monitoring, intelligent aeration, precise feeding, and tailwater treatment facilities can be used to improve system operation efficiency. Meanwhile, attention should also be paid to transforming the market value of ecological aquaculture products. Green certification, brand building, and quality traceability can enhance consumer trust, allowing ecological benefits to be converted into sustained economic returns and thereby increasing the willingness of aquaculture operators to participate in ecological closed-loop construction.

In the future, freshwater pond aquaculture will further develop toward ecologicalization, intelligentization, standardization, and circularization. Ecologicalization emphasizes the construction of more stable multi-trophic structures in pond systems to enhance water self-purification and ecological regulation capacity. Intelligentization emphasizes the use of IoT sensors, data analysis platforms, and intelligent equipment to achieve precise management of water quality, feeding, aeration, and tailwater treatment processes. Standardization requires the formation of technical specifications and evaluation systems applicable to aquaculture scenarios involving different regions, species, and scales. Circularization emphasizes the reuse of aquaculture tailwater, nutrients, and biological resources, promoting the connection of pond aquaculture with crop production, wetland purification, and regional ecological governance. Overall, ecological food chain closure is not only an important technical pathway for the green transformation of freshwater pond aquaculture, but will also become an important support for modern aquaculture to achieve efficient resource utilization, environmentally friendly production, and sustainable industrial development.

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Conflict of Interest Disclosure

The author affirms that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

References

- Bai D., Li X., Liu Z., Wan L., Song C., Zhou Y., and Cao X., 2023, Nitrogen and phosphorus turnover and coupling in ponds with different aquaculture species, *Aquaculture*, 563: 738997.
<https://doi.org/10.1016/j.aquaculture.2022.738997>
- Bernal-Higueta F., Acosta-Coll M., Ballester-Merelo F., and De-La-Hoz-Franco E., 2023, Implementation of information and communication technologies to increase sustainable productivity in freshwater finfish aquaculture-A review. *Journal of Cleaner Production*, 408: 137124.
<https://doi.org/10.1016/j.jclepro.2023.137124>
- Biswas G., Kumar P., Ghoshal T., Kailasam M., De D., Bera A., Mandal B., Sukumaran K., and Vijayan K., 2020, Integrated multi-trophic aquaculture (IMTA) outperforms conventional polyculture with respect to environmental remediation, productivity and economic return in brackishwater ponds. *Aquaculture*, 516: 734626.
<https://doi.org/10.1016/j.aquaculture.2019.734626>
- Boyd C., D'Abramo L., Glencross B., Huyben D. C., Juarez L. M., Lockwood G. S., McNevin A. A., Tacon A., Teletchea F., Tomasso J., Tucker C., and Valenti W., 2020, Achieving sustainable aquaculture: Historical and current perspectives and future needs and challenges, *Journal of the World Aquaculture Society*, 51(3): 578-633.
<https://doi.org/10.1111/jwas.12714>
- Butruille G., Thomas M., Pasquet A., Amoussou N., Toomey L., Rosenstein A., Chauchard S., and Lecocq T., 2022, AquaDesign: A tool to assist aquaculture production design based on abiotic requirements of animal species, *PLoS ONE*, 17(8): e0272508.
<https://doi.org/10.1371/journal.pone.0272508>
- Campanati C., Willer D., Schubert J., and Aldridge D., 2022, Sustainable intensification of aquaculture through nutrient recycling and circular economies: More fish, less waste, blue growth, *Reviews in Fisheries Science & Aquaculture*, 30(2): 143-169.
<https://doi.org/10.1080/23308249.2021.1897520>
- Chang Z., Neori A., He Y., Li J., Qiao L., Preston S., Liu P., and Li J., 2020, Development and current state of seawater shrimp farming, with an emphasis on integrated multi-trophic pond aquaculture farms, in China-A review, *Reviews in Aquaculture*, 12(4): 2544-2558.
<https://doi.org/10.1111/raq.12457>
- Dong S., Cao L., Liu W.J., Huang M., Sun Y.X., Zhang Y.Y., Yu S.E., Zhou Y., Li L., and Dong Y., 2025, System-specific aquaculture annual growth rates can mitigate the dilemma of production, pollution and carbon dioxide emissions in China, *Nature Food*, 6(4): 365-374.
<https://doi.org/10.1038/s43016-025-01122-1>
- Dong S., Liu W.J., Zhu B., Zhang D., and Wang F., 2023, Practice assessment of integrated marine pond aquaculture for increasing benefits and reducing environmental pollution using an ecosystem modeling approach, *Science of the Total Environment*, 141, 109154.
<https://doi.org/10.1016/j.scitotenv.2023.168408>
- Dong S., Shan H., Yu L., Liu X., Ren Z., and Wang F., 2022, An ecosystem approach for integrated pond aquaculture practice: Application of food web models and ecosystem indices, *Ecological Indicators*, 141: 109154.
<https://doi.org/10.1016/j.ecolind.2022.109154>
- Duan Y., Liu L., Tang R., Xie S., Yang M., Zhang Z., and Li D., 2024, Assessing changes in China's pond water quality from 1989 to 2020: Implications for green development in aquaculture, *Reviews in Aquaculture*, 17(1): e12997.
<https://doi.org/10.1111/raq.12997>
- Falconer L., Telfer T., and Ross L., 2016, Investigation of a novel approach for aquaculture site selection, *Journal of Environmental Management*, 181: 791-804.
<https://doi.org/10.1016/j.jenvman.2016.07.018>
- Goda A. M., Aboseif A., Taha M. K. S., Mohammady E. Y., Aboushabana N. M., Nazmi H. M., Zaher M., Aly H. A., El-Okaby M. A. S., Otazua N. I., and Ashour M., 2024, Optimizing nutrient utilization, hydraulic loading rate, and feed conversion ratios through freshwater IMTA-aquaponic and hydroponic systems as an environmentally sustainable aquaculture concept, *Scientific Reports*, 14(1): 144878.
<https://doi.org/10.1038/s41598-024-70935-0>
- Hala A. F., Chougule K., Cunha M. E., Caria M., Oliveira I., Bradley T., Forbes J., and Galileu S.L., 2024, Life cycle assessment of integrated multi-trophic aquaculture: A review on methodology and challenges for its sustainability evaluation, *Aquaculture*, 590: 741035.
<https://doi.org/10.1016/j.aquaculture.2024.741035>
- Henares M. N., Medeiros M. V., and Camargo A. F., 2020, Overview of strategies that contribute to the environmental sustainability of pond aquaculture: Rearing systems, residue treatment, and environmental assessment tools, *Reviews in Aquaculture*, 12(1): 453-470.
<https://doi.org/10.1111/raq.12327>

- Kajgróv L., Pecha O., Roy K., Dvořák J., Let M., Potužák J., Vrba J., and Bláha M., 2023, Pond cascades as a tool for ecological aquaculture allowing natural zooplankton succession, nutrient retention, and multiple stocking-harvesting cycles, *Aquacultural Engineering*, 104: 102374.
<https://doi.org/10.1016/j.aquaeng.2023.102374>
- Khanjani M., Zahedi S., and Mohammadi A., 2022, Integrated multitrophic aquaculture (IMTA) as an environmentally friendly system for sustainable aquaculture: Functionality, species, and application of biofloc technology (BFT), *Environmental Science and Pollution Research*, 29(45): 67513-67531.
<https://doi.org/10.1007/s11356-022-22371-8>
- Knowler D., Chopin T., Martínez-Españeira R., Neori A., Nobre A., Noce A., and Reid G., 2020, The economics of Integrated Multi-Trophic Aquaculture: Where are we now and where do we need to go? *Reviews in Aquaculture*, 12(3): 2039-2062.
<https://doi.org/10.1111/raq.12399>
- Li K., Jiang R., Qiu J., Liu J., Shao L., Zhang J., Liu Q., Jiang Z., Wang H., He W., and He P., 2024, How to control pollution from tailwater in large-scale aquaculture in China: A review, *Aquaculture*, 590: 741085.
<https://doi.org/10.1016/j.aquaculture.2024.741085>
- Lindholm-Lehto P., 2023, Water quality monitoring in recirculating aquaculture systems, *Aquaculture, Fish and Fisheries*, 3(2): 113-131.
<https://doi.org/10.1002/aff2.102>
- Liu X., Shao Z., Cheng G.-F., Lu S., Gu Z., Zhu H., Shen H., Wang J., and Chen X., 2020, Ecological engineering in pond aquaculture: A review from the whole-process perspective in China, *Reviews in Aquaculture*, 13(2): 1060-1076.
<https://doi.org/10.1111/raq.12512>
- Liu X., Xu H., Wang X., Wu Z.-F., and Bao X.-H., 2014, An ecological engineering pond aquaculture recirculating system for effluent purification and water quality control, *Clean-Soil, Air, Water*, 42(3): 221-228.
<https://doi.org/10.1002/clen.201200567>
- Lothmann R., and Sewilam H., 2022, Potential of innovative marine aquaculture techniques to close nutrient cycles. *Reviews in Aquaculture*, 14: 2324-2345.
<https://doi.org/10.1111/raq.12781>
- Moriarty D., 1997, The role of microorganisms in aquaculture ponds. *Aquaculture*, 151: 333-349.
[https://doi.org/10.1016/S0044-8486\(96\)01487-1](https://doi.org/10.1016/S0044-8486(96)01487-1)
- Nahon S., De Brito G. V., Quental-Ferreira H., Aubin J., Jaeger C., Menniti C., Kerhervé P., Larroquet L., and Cunha M.E., 2023, Food web in Mediterranean coastal integrated multi-trophic aquaculture ponds: Learnings from fatty acids and stable isotope tracers. *Aquaculture*, 569: 739292.
<https://doi.org/10.1016/j.aquaculture.2023.739292>
- Nenciu F., Voica I., Cocârță D., Vlăduț V., Matache M., and Arsenoaia V., 2022, "Zero-waste" food production system supporting the synergic interaction between aquaculture and horticulture. *Sustainability*, 14: 13396.
<https://doi.org/10.3390/su142013396>
- Nie Z., Zheng Z., Zhu H.-J., Sun Y., Gao J., Gao J., Xu P., and Xu G., 2023, Effects of submerged macrophytes (*Elodea nuttallii*) on water quality and microbial communities of largemouth bass (*Micropterus salmoides*) ponds, *Frontiers in Microbiology*, 13: 1050699.
<https://doi.org/10.3389/fmicb.2022.1050699>
- Nobre A., Robertson-Andersson D., Neori A., and Sankar K., 2010, Ecological-economic assessment of aquaculture options: Comparison between abalone monoculture and integrated multi-trophic aquaculture of abalone and seaweeds. *Aquaculture*, 306: 116-126.
<https://doi.org/10.1016/j.aquaculture.2010.06.002>
- Shi B., Cheng X., Chen H., Xie J., Zhou Z., Jiang S., Peng X., Zhang Y., Zhu D., and Lu Z., 2022, Occurrence, source tracking and removal of antibiotics in recirculating aquaculture systems (RAS) in southern China, *Journal of Environmental Management*, 324: 116311.
<https://doi.org/10.1016/j.jenvman.2022.116311>
- Takeuchi T., 2017, Closed ecological recirculating aquaculture systems. In: *Aquaculture Perspective of Multi-Use Sites in the Open Ocean*, 279-298. Springer, Cham.
https://doi.org/10.1007/978-4-431-56585-7_13
- Tang Y., Ju C., Mei R., Zhao L., Liu J., Yang Y., Guo X., Su C., Cheng Y., and Liu Q., 2024, Exploring the optimal integrated multi-trophic aquaculture (IMTA) patterns benefiting culture animals and natural water environment, *Aquaculture*, 589: 741011.
<https://doi.org/10.1016/j.aquaculture.2024.741011>
- Thomas M., Pasquet A., Aubin J., Nahon S., and Lecocq T., 2021, When more is more: Taking advantage of species diversity to move towards sustainable aquaculture, *Biological Reviews*, 96(2): 767-784.
<https://doi.org/10.1111/brv.12677>
- Tran-Minh N., 2025, An IoT-based water quality monitoring system for recirculating aquaculture systems. *Tạp chí Khoa học Đại học Công Thương*, 25(6): 366.
https://doi.org/10.62985/j.huit_ojs.vol25.no6.366
- Varga M., Berzi-Nagy L., Csukás B., and Gyalog G., 2020, Long-term dynamic simulation of environmental impacts on ecosystem-based pond aquaculture, *Environmental Modelling & Software*, 134: 104755.
<https://doi.org/10.1016/j.envsoft.2020.104755>
- Wang Z., Chen X., Shi L., Zhang X., and Hu Y., 2024, Productivity versus environmental sustainability: A broadscale assessment of freshwater aquaculture's technical efficiency and ecological efficiency in China's inland provinces. *Journal of the World Aquaculture Society*, 55(3): 1044-1064.
<https://doi.org/10.1111/jwas.13057>

- Zhang K., Jiang J., Li Z.F., Yu E. M., Gong W., Xia Y., Tian J.J., Li H., Xie W.P., Xie J., and Wang G., 2024, Evaluating the effects and mechanisms of the eco-substrate in aquaculture environment restoration from an ecosystem perspective via the Ecopath model, Sustainability, 16(7): 2955.
<https://doi.org/10.3390/su16072955>
- Zhong S., Li A., and Wu J., 2022, Eco-efficiency of freshwater aquaculture in China: An assessment considering the undesirable output of pollutant emissions, Environment, Development and Sustainability, 25(4): 3555-3576.
<https://doi.org/10.1007/s10668-022-02189-7>



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Research Report

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In-Vitro Probiotic Characterization and Molecular Identification of Autochthonous *Bacillus* spp. from *Oreochromis niloticus*

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Abstract The escalating global aquaculture production necessitates sustainable alternatives to traditional antibiotics due to concerns regarding antimicrobial resistance and environmental contamination. This study investigated the probiotic potential and molecular identity of autochthonous bacterial isolates from the gastrointestinal tract of *Oreochromis niloticus* (Nile tilapia). Microbial populations from the upper and lower intestinal segments were enumerated and characterized using morphological, biochemical, and molecular methods. Results revealed significant spatial variation in bacterial distribution along the gastrointestinal tract, with total bacterial abundance significantly higher ($p < 0.05$) in the lower intestine than in the upper intestine. Among the bacterial groups identified, *Bacillus* spp. showed strong probiotic potential due to their high abundance, spore-forming capacity, and tolerance to gastrointestinal stress conditions. The isolates were Gram-positive, non-hemolytic, and demonstrated strong survival under acidic conditions (pH 2.5~7) and bile salt concentrations up to 1%. Antimicrobial activity assays revealed significant inhibition of pathogenic bacteria including *Escherichia coli*, *Salmonella typhi*, and *Staphylococcus aureus*, with inhibition zones reaching 34 mm. Molecular characterization using 16S rRNA gene sequencing confirmed the isolates as *Bacillus clausii*. Phylogenetic analysis showed high genetic similarity with previously reported *Bacillus clausii* strains in the NCBI database. These findings suggest that autochthonous *Bacillus clausii* isolated from *O. niloticus* possess desirable probiotic characteristics and may serve as promising candidates for functional probiotic applications in sustainable aquaculture.

Keywords Probiotics; *Bacillus clausii*; Nile tilapia; Gut microbiota; Antimicrobial activity; Aquaculture biotechnology

1 Introduction

Global aquaculture production has expanded rapidly over the past few decades, providing a significant proportion of the world's animal protein supply (FAO, 2014). Among cultured species, *Oreochromis niloticus* (Nile tilapia) is one of the most economically important fish species globally, being the most widely cultured fish species in Africa and the second most cultured species in Nigeria (Cavalcante et al., 2020). Its importance is due to its fast growth, adaptability to diverse environmental conditions, and high consumer demand.

However, intensification of aquaculture practices has led to increased disease outbreaks caused by bacterial pathogens such as *Aeromonas*, *Vibrio*, and *Edwardsiella* species. Traditionally, antibiotics have been used to control such infections, but their widespread application has resulted in antimicrobial resistance, environmental contamination, and food safety concerns (Cabello, 2006; Defoirdt et al., 2011).

Consequently, probiotics have gained increasing attention as environmentally friendly alternatives to antibiotics. Probiotics are defined as live microorganisms that confer health benefits to the host when administered in adequate amounts (FAO/WHO, 2002). In aquaculture, probiotics have been shown to improve nutrient utilization, enhance immune responses, and suppress pathogenic bacteria (Nayak, 2010; Ringø et al., 2018).

Host-associated or autochthonous probiotics are particularly promising because they originate from the host microbiome and are therefore better adapted to colonize the gastrointestinal environment. The gut microbiota of fish plays essential roles in digestion, nutrient metabolism, immune regulation, and pathogen exclusion (Gatesoupe, 1999; Ringø and Olsen, 2014).

Among potential probiotic microorganisms, *Bacillus* species are widely studied due to their ability to produce antimicrobial compounds and extracellular enzymes, as well as their capacity to form spores that enhance survival under harsh environmental conditions (Cutting, 2011; Zorriehzahra et al., 2016).

Despite the increasing interest in probiotic development for aquaculture research in rainbow Trout (Ruholla et al., 2021), Catfish (Akanmu et al., 2016; Setufe et al., 2021), Nile Tilapia (Mohammadi et al., 2022), information on autochthonous probiotic bacteria associated with *O. niloticus* remains limited in many aquaculture systems (Cavalcante et al., 2020).

Therefore, the present study aimed to characterize the gut microbial population of *Oreochromis niloticus*, evaluate the in vitro probiotic properties of bacterial isolates, and identify promising probiotic strains using molecular techniques.

2 Materials and Methods

2.1 Sample collection and gut dissection

Healthy Nile tilapia were obtained and dissected under sterile conditions. The gastrointestinal tract was separated into upper and lower intestinal segments to investigate the spatial distribution of gut microbiota.

2.2 Bacterial isolation and enumeration

Gut contents were homogenized, serially diluted, and plated on nutrient agar plates. Colony forming units (CFU g⁻¹) were calculated after incubation. Isolation and enumeration of gut microbiota were performed following standard microbiological procedures described by Cappuccino and Welsh (2017) and Madigan et al. (2018).

2.3 Morphological and biochemical characterization

The isolates were characterized based on the following morphological and biochemical tests:

- 1 Gram staining was performed according to the classical method described by Beveridge (2001).
- 2 Endospore staining was conducted using the Schaeffer-Fulton staining technique (Schaeffer and Fulton, 1933).
- 3 Catalase activity was evaluated according to MacFaddin (2000).
- 4 Hemolytic activity was determined on blood agar following Verschuere et al. (2000).
- 5 These tests were used to evaluate the safety and probiotic potential of the bacterial isolates.

2.4 Acid tolerance test

Bacterial isolates were exposed to pH levels of 2.5, 3.0, 3.5, 4.0, and 7.0. Growth and survival were recorded. Acid tolerance assays were performed following methods described by Hyronimus et al. (2000) and Charteris *et al.* (1998).

2.5 Bile salt tolerance test

Bile tolerance was assessed using media containing different bile salt concentrations (0.2%, 0.4%, 0.6%, and 1.0%). Bile tolerance was evaluated according to the procedure described by Gilliland *et al.* (1984).

2.6 Antimicrobial activity assay

Antagonistic activity was evaluated against the following pathogenic bacteria:

- 1 *Escherichia coli*
- 2 *Salmonella typhi*
- 3 *Staphylococcus aureus*

The diameter of inhibition zones was measured in millimeters.

Antimicrobial activity was determined using the agar well diffusion method described by Tagg and McGiven (1971).

2.7 Molecular identification

Genomic DNA was extracted and amplified using universal 16S rRNA primers. PCR products were sequenced and analyzed using BLAST analysis. Genomic DNA was extracted using a modified CTAB protocol according to Saraniya and Jeevaratnam (2012).

2.8 Statistical analysis

All experiments were conducted in triplicate and data were expressed as mean $\pm$ standard deviation (SD). Statistical analyses were performed using one-way analysis of variance (ANOVA) to determine significant differences among treatments.

When significant differences were detected ($p < 0.05$), means were separated using Duncan's Multiple Range Test (DMRT). Superscript letters were used to denote statistical differences between treatments.

For microbial enumeration data, bacterial counts were $\log_{10}$ transformed prior to analysis to ensure normal distribution and homogeneity of variance.

The statistical model applied was

$$Y_{ij} = \mu + T_i + \epsilon_{ij}$$

Where:

- 1 Y_{ij} = observed value
- 2 μ = overall mean
- 3 T_i = treatment effect
- 4 ϵ_{ij} = experimental error

Significance was declared at $p < 0.05$.

All analyses were conducted using SPSS version 25.0 (IBM Corp., USA).

3 Results

3.1 Distribution of gut microbiota

The distribution of bacterial populations along the intestinal tract of *Oreochromis niloticus* is presented (Figure 1). The results indicate that the lower gut harboured consistently higher bacterial loads compared to the upper gut across all bacterial groups, including total heterotrophic bacteria, cocci, and *Bacillus* spp. This pattern may be associated with the more stable physicochemical conditions and nutrient availability in the distal intestine, which favor microbial proliferation and colonization.

Table 1 Relative abundance of bacterial groups in the gastrointestinal tract

Bacterial Group	Representative Genera	Upper Gut (CFU g ⁻¹)	Lower Gut (CFU g ⁻¹)	Probiotic Potential
<i>Helicobacter</i> / <i>Fusobacterium</i>	Opportunistic commensals	1.75×10^{1a}	6.65×10^{6b}	No
Cocci bacteria	Staphylococcus, Streptococcus	1.14×10^{6a}	3.05×10^{6b}	Variable
<i>Bacillus</i> spp.	Spore-forming rods	1.14×10^{6a}	3.05×10^{6b}	Yes

Different superscripts indicate significant differences ($p < 0.05$).

3.2 Morphological and biochemical characterization

3.2.1 Gram staining

Gram staining result presented shows both *Bacillus* spp. and Cocci bacteria to be gram positive while *Helicobacter* spp and *Fusobacterium* spp to be gram negative (Table 2).

3.2.2 Spore test

Table 3 shows that only *Bacillus* spp. is capable of sporulation, while the other isolates; Cocci bacteria, *Helicobacter* spp. and *Fusobacterium* spp. are non-spore forming bacteria.

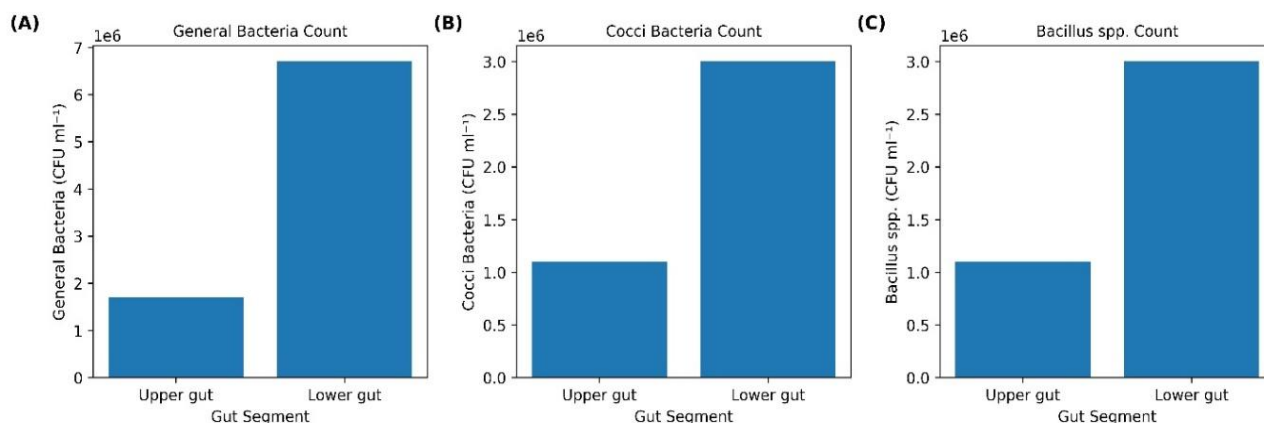


Figure 1 Comparative bacterial load in the intestinal tract of *Oreochromis niloticus*

Image caption: (A) Total heterotrophic bacteria, (B) cocci bacteria, and (C) *Bacillus* spp. counts in the upper and lower gut segments. Bacterial abundance is expressed as colony-forming units per millilitre (CFU mL⁻¹). The lower gut consistently exhibited higher bacterial populations than the upper gut.

Table 2 Gram staining results

Isolate	Morphology	Gram Reaction
<i>Bacillus</i> spp.	Rod-shaped cells	Gram positive
<i>Cocci</i> bacteria	Spherical cells	Gram positive
<i>Helicobacter</i> spp.	Spiral rods	Gram negative
<i>Fusobacterium</i> spp.	Spindle rods	Gram negative

Table 3 Spore staining

Isolate	Spore Formation
<i>Bacillus</i> spp.	Positive
<i>Cocci</i> bacteria	Negative
<i>Helicobacter</i> spp.	Negative
<i>Fusobacterium</i> spp.	Negative

3.2.3 Catalase and hemolysis test

The result of production of catalase and hemolytic test by the Isolates is presented in Table 4. Gas production from the isolates comes out positive for both *Cocci* and *Bacillus* spp., while both isolates were negative to hemolysis which is a good sign of potential probiotics

Table 4 Catalase and Hemolysis Test

Isolate	Catalase	Hemolysis
<i>Bacillus</i> spp.	Positive	Negative
<i>Cocci</i> bacteria	Positive	Negative

The absence of hemolytic activity suggests the isolates are non-pathogenic and safe for probiotic application.

3.3 Acid and bile salt tolerance

The results of acid tolerance and bile salt tolerance are presented in Tables 5 and 6, respectively. The results shows that selected isolates survived bile salt at all concentration, however, the tolerance of *Bacillus* spp. is greater than Cocci bacteria at all level of bile salt concentration. Also, isolates survived pH tolerance at all concentration, meanwhile, the tolerance level of *Bacillus* spp. at all pH concentration is higher in value than the value recorded for Cocci bacteria.

Table 5 Acid tolerance of isolates

Isolate	pH 2.5	pH 3.0	pH 3.5	pH 4	pH 7
<i>Bacillus</i> spp.	1.8±0.2	2.2±0.4	2.6±0.2	2.8±0.3	3.4±0.2
Cocci bacteria	0.4±0.1	0.7±0.2	0.9±0.1	1.1±0.2	1.8±0.3

Statistical analysis showed significantly higher acid tolerance ($p < 0.05$) for *Bacillus* isolates across all pH levels.

Table 6 Bile tolerance

Isolate	0.2%	0.4%	0.6%	1.0%
<i>Bacillus</i> spp.	2.8±0.3	2.2±0.1	1.9±0.2	1.6±0.1
Cocci bacteria	2.7±0.1	2.1±0.3	1.6±0.1	1.3±0.2

The superior tolerance exhibited by *Bacillus* spp. indicates their adaptation to intestinal bile conditions.

3.4 Antimicrobial activity

As presented in table 7 and figure 2, the inhibitory activity of the tested probiotic isolates against *Escherichia coli* isolates showed clear differences between *Cocci* spp. and *Bacillus* spp., as demonstrated by their respective zones of inhibition. *Bacillus* spp exhibited the highest antagonistic effect with a mean inhibition zone of 34 mm, while *Cocci* spp. produced a comparatively smaller zone of 26 mm. The significantly larger inhibition zone produced by *Bacillus* spp. indicates a stronger antimicrobial potency against the target pathogen.

Table 7 Zone of inhibition against pathogens

Pathogen	<i>Cocci</i> spp. (mm)	<i>Bacillus</i> spp. (mm)
<i>Escherichia coli</i>	26	34
<i>Salmonella typhi</i>	29	29
<i>Staphylococcus aureus</i>	39	39

Bacillus isolates demonstrated strong antagonistic activity, suggesting production of antimicrobial compounds.

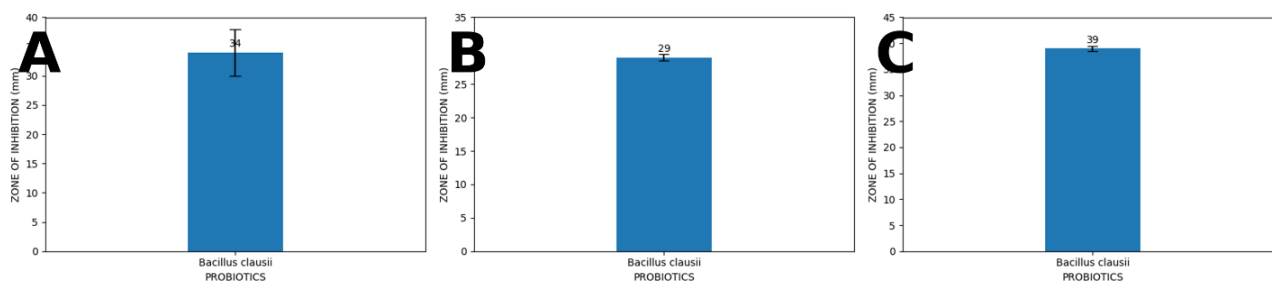


Figure 2 Diameter of inhibition zone of *Bacillus clausii* tested against A=*Escherichia coli* B=*Salmonella typhi* C=*Staphylococcus aureus* isolates.

Mean radii with different superscripts indicate significant ($\alpha_{0.05}$) difference according to DMRT.

3.5 Molecular identification

PCR amplification of the 16S rRNA gene produced single bands of approximately 1500 bp, confirming successful amplification. Sequencing analysis identified the isolate as *Bacillus clausii*. Phylogenetic analysis revealed a close evolutionary relationship between the identified strain and previously reported *Bacillus clausii* strains retrieved from the NCBI GenBank database (Figure 3). The identified strain showed close clustering with *Bacillus clausii* PRA25, *Bacillus clausii* AK31, and *Bacillus clausii* XJU2. Bootstrap values ranged from 86% to 98%, indicating strong support for the phylogenetic clustering.

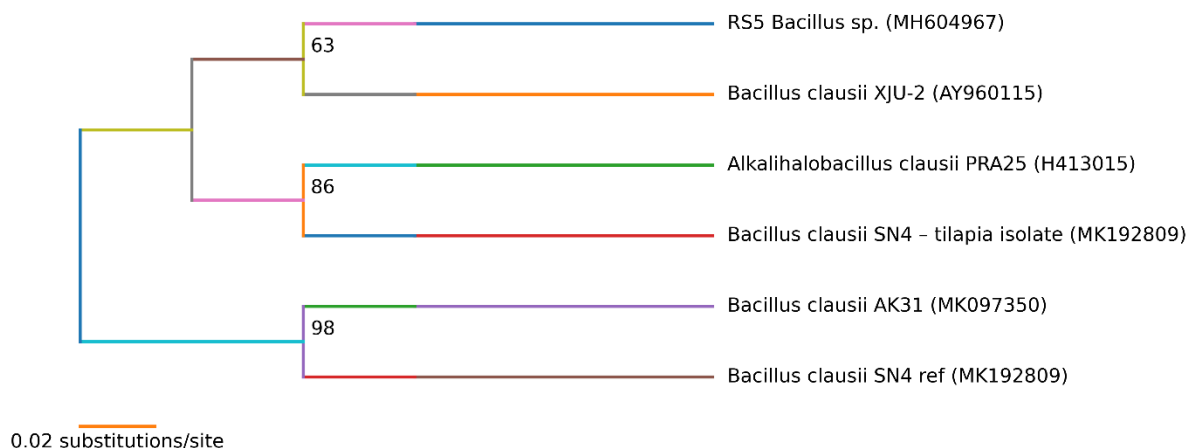


Figure 3 Phylogenetic tree based on 16S rRNA gene sequences

Image caption: The phylogenetic tree shows the relationship between the isolated strain *Bacillus clausii* SN4 from *Oreochromis niloticus* and closely related *Bacillus* species retrieved from GenBank. The tree was constructed using the Neighbor-Joining method, and bootstrap values (%) based on 1000 replicates are indicated at branch nodes. The scale bar represents 0.02 nucleotide substitutions per site.

4 Discussion

4.1 Gastrointestinal microbial distribution in *Oreochromis niloticus*

In the present study, microbial abundance was significantly higher in the lower intestine compared with the upper intestine (Table 1 and Figure 1). This pattern is consistent with the typical spatial organization of microbial communities in the gastrointestinal tract of teleost fish. The distal intestine generally provides more favorable conditions for microbial colonization due to lower oxygen concentrations, slower digesta transit, and greater availability of fermentable substrates. These environmental conditions promote the growth of facultative and anaerobic microorganisms that participate in fermentation processes and nutrient metabolism. Similar spatial distribution patterns have been reported in several fish species, including tilapia, salmonids, and carp, where the distal gut harbors higher microbial densities and greater microbial diversity (Nayak, 2010; Ringø et al., 2016). The higher bacterial counts observed in the lower intestine in this study therefore suggest that this region represents a major site for microbial metabolic activity and host–microbe interactions.

4.2 Probiotic potential of *Bacillus* species

Among the bacterial isolates obtained from the gastrointestinal tract of *O. niloticus*, members of the genus *Bacillus* demonstrated promising probiotic characteristics. *Bacillus* species are widely recognized as beneficial probiotic candidates in aquaculture due to several advantageous physiological traits, including their ability to form endospores, produce antimicrobial metabolites, and secrete extracellular digestive enzymes.

The spore-forming ability observed in the isolates as presented in Table 3 is particularly significant for probiotic applications. Endospores exhibit remarkable resistance to environmental stressors such as heat, desiccation, and acidic conditions. This resilience improves the stability of probiotic formulations during feed processing and storage while ensuring survival during passage through the gastrointestinal tract (Cutting, 2011; Hong et al., 2005). As a result, *Bacillus*-based probiotics are commonly incorporated into aquaculture feeds to improve digestive efficiency and disease resistance.

4.3 Safety assessment of the probiotic candidates

The safety evaluation showed that the isolates exhibited no hemolytic activity on blood agar, as shown in Table 4, indicating that they are non-hemolytic and therefore unlikely to be pathogenic. Hemolysis is commonly used as an indicator of bacterial virulence, and the absence of hemolytic activity is considered an important safety criterion

for probiotic candidates (Verschuere et al., 2000). The non-hemolytic behavior observed in this study therefore supports the suitability of these isolates for further probiotic development. Catalase activity was positive in *Bacillus* spp., indicating the ability of the isolates to produce catalase enzymes that degrade hydrogen peroxide into water and oxygen.

4.4 Acid and bile tolerance as probiotic selection criteria

Tolerance to acidic and bile environments represents a fundamental requirement for probiotic microorganisms, as they must survive the harsh conditions of the stomach and intestine before exerting beneficial effects within the host.

In the present study, the bacterial isolates demonstrated considerable survival across a wide pH range (2.5~7.0) table 5, with *Bacillus* isolates showing significantly higher tolerance compared to cocci bacteria. The ability to withstand acidic environments suggests that these isolates possess physiological adaptations that maintain cellular stability under gastric stress. Similar acid tolerance characteristics have been reported for *Bacillus clausii* strains used in commercial probiotic preparations, further supporting the robustness of this species under gastrointestinal conditions (Hong et al., 2005).

The isolates in this study exhibited tolerance to bile concentrations of up to 1% table 6, with *Bacillus* isolates showing superior tolerance compared to cocci bacteria. Such tolerance indicates that the isolates are capable of surviving within the intestinal environment where bile salts are present during digestion. Previous studies have suggested that bile tolerance in probiotic bacteria may be associated with mechanisms such as bile salt hydrolase activity and membrane modifications that protect cells from bile-induced stress (Gilliland et al., 1984). Therefore, the acid and bile tolerance exhibited by the isolates strongly supports their probiotic potential.

4.5 Antimicrobial activity against fish pathogens

The antimicrobial activity of *Bacillus* species is well documented and is often attributed to the production of bioactive compounds such as bacteriocins, lipopeptides (including surfactin, iturin, and fengycin), and organic acids (Zhou et al., 2010; Ringø et al., 2018). These antimicrobial substances inhibit pathogenic microorganisms through mechanisms such as membrane disruption, competitive exclusion, and nutrient competition. The strong inhibitory activity of *Bacillus* species against *Escherichia coli*, *Salmonella typhi* and *Staphylococcus aureus* as observed in this study is presented in table 7 and figure 2 therefore suggests that the isolates may contribute to maintaining microbial balance within the gastrointestinal tract and preventing pathogen colonization in *O. niloticus*.

4.6 Molecular identification and phylogenetic analysis

Molecular characterization using 16S rRNA gene sequencing identified the isolates as *Bacillus clausii*. This species is widely recognized for its probiotic properties and has been extensively used in both human and veterinary probiotic formulations. *Bacillus clausii* is known for its remarkable resilience to environmental stress and its ability to modulate host immune responses.

Previous studies have demonstrated that *B. clausii* can enhance innate immune responses, stabilize intestinal microbiota, and improve resistance to bacterial infections in aquaculture species (Ringø et al., 2018). The identification of this species within the gastrointestinal tract of *O. niloticus* therefore suggests that it may play a natural role in maintaining gut microbial homeostasis.

Phylogenetic analysis further confirmed the taxonomic identity of the isolate by demonstrating close genetic relationships with previously reported *Bacillus clausii* strains in the NCBI database. The high bootstrap support values (86%~98%) observed in the phylogenetic tree indicate strong confidence in the clustering of the isolate within the *B. clausii* lineage.

4.7 Significance of autochthonous probiotics in aquaculture

The identification of autochthonous probiotic microorganisms is particularly advantageous for aquaculture

applications because host-associated bacteria are more likely to colonize and persist within the gastrointestinal tract. Unlike allochthonous probiotics introduced from external sources, autochthonous strains are naturally adapted to the host's intestinal environment, which enhances their colonization efficiency and functional stability (Gatesoupe, 1999; Ringø and Olsen, 2014). The isolation of *Bacillus clausii*-like strain of *O. niloticus* therefore represents a promising step toward developing host-specific probiotic formulations that could improve fish health and aquaculture productivity.

4.8 Limitations and future research directions

Despite the promising findings obtained in this study, several limitations should be acknowledged. First, the probiotic properties of the isolates were evaluated only through in vitro assays, which may not fully reflect their performance within the complex biological environment of the fish gastrointestinal tract. Second, the study focused primarily on basic probiotic characteristics such as acid tolerance, bile tolerance, and antimicrobial activity, while other important functional properties—including enzyme production, adhesion ability, and immunomodulatory effects—were not investigated.

Future research should therefore focus on in vivo validation of the probiotic potential of the identified *Bacillus clausii* strain. Controlled feeding trials should be conducted to evaluate its effects on growth performance, feed conversion efficiency, immune responses, and disease resistance in *O. niloticus*. Additionally, advanced molecular approaches such as metagenomics and transcriptomics could provide deeper insights into the interactions between the probiotic strain, native gut microbiota, and host immune pathways.

4.9 Implications for sustainable aquaculture

Overall, the findings of this study contribute to the growing body of knowledge on fish gut microbiota and probiotic development for aquaculture. The isolation and characterization of *Bacillus clausii* from the gastrointestinal tract of *Oreochromis niloticus* highlight the potential of host-associated microorganisms as sustainable alternatives to antibiotics in aquaculture production systems. By promoting microbial balance, enhancing digestive efficiency, and suppressing pathogenic bacteria, such probiotics may play an important role in improving fish health, increasing aquaculture productivity, and supporting environmentally sustainable aquaculture practices.

Authors Contribution

O.A. Onada was responsible for conceptualization, methodology development, investigation, data curation, laboratory experiments, formal analysis, and writing the original draft. E.K. Ajani and E.F. Osho contributed to supervision, project administration, methodology validation, result interpretation, and manuscript review and editing. All authors have read and approved the final version of the manuscript.

Conflict of Interest Disclosure

The authors affirm that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

References

- Akanmu A.O., Adeyemo O.K., and Fagbenro O.A., 2016, Probiotic potential of *Bacillus* species isolated from the gastrointestinal tract of African catfish (*Clarias gariepinus*), *Aquaculture Research*, 47(10): 3314-3325.
- Beveridge T.J., 2001, Use of the Gram stain in microbiology, *Biotechnique & Histochemistry*, 76(3): 111-118.
<https://doi.org/10.1080/bih.76.3.111.118>
- Cabello F.C., 2006, Heavy use of prophylactic antibiotics in aquaculture: A growing problem for human and animal health and for the environment, *Environmental Microbiology*, 8(7): 1137-1144.
<https://doi.org/10.1111/j.1462-2920.2006.01054.x>
- Cappuccino J.G., and Welsh C., 2017, *Microbiology: A laboratory manual*, 11th ed., Boston: Pearson.
- Cavalcante R.B., Vieira F.N., Mourão J.L.P., and Seiffert W.Q., 2020, Autochthonous probiotics in aquaculture: A review of selection criteria and applications, *Aquaculture Research*, 51(2): 557-572.
- Charteris W.P., Kelly P.M., Morelli L., and Collins J.K., 1998, Development and application of an in vitro methodology to determine the probiotic potential of bacterial strains, *Applied and Environmental Microbiology*, 64: 364-370.
- Cutting S.M., 2011, *Bacillus* probiotics, *Food Microbiology*, 28: 214-220.

- <https://doi.org/10.1016/j.fm.2010.03.007>
- Defoirdt T., Sorgeloos P., and Bossier P., 2011, Alternatives to antibiotics for the control of bacterial disease in aquaculture, Trends in Biotechnology, 29: 251-258.
- <https://doi.org/10.1016/j.mib.2011.03.004>
- FAO, 2014, The state of world fisheries and aquaculture, Rome: Food and Agriculture Organization of the United Nations.
- FAO/WHO, 2002, Guidelines for the evaluation of probiotics in food, London, Ontario: Food and Agriculture Organization of the United Nations and World Health Organization.
- Gatesoupe F.J., 1999, The use of probiotics in aquaculture, Aquaculture, 180: 147-165.
- [https://doi.org/10.1016/S0044-8486\(99\)00187-8](https://doi.org/10.1016/S0044-8486(99)00187-8)
- Gilliland S.E., Staley T.E., and Bush L.J., 1984, Importance of bile tolerance of *Lactobacillus acidophilus* used as a dietary adjunct, Applied and Environmental Microbiology, 47: 1-5.
- Hong H.A., Duc L.H., and Cutting S.M., 2005, The use of bacterial spore formers as probiotics, FEMS Microbiology Reviews, 29: 813-835.
- <https://doi.org/10.1016/j.femsre.2004.12.001>
- Hyronimus B., Le Marrec C., Urdaci M.C., and Fons M., 2000, Acid and bile tolerance of probiotic bacteria, International Journal of Food Microbiology, 61: 193-201.
- [https://doi.org/10.1016/S0168-1605\(00\)00366-4](https://doi.org/10.1016/S0168-1605(00)00366-4)
- MacFaddin J.F., 2000, Biochemical tests for identification of medical bacteria, 3rd ed., Philadelphia: Lippincott Williams & Wilkins.
- Madigan M.T., Bender K.S., Buckley D.H., Sattley W.M., and Stahl D.A., 2018, Brock biology of microorganisms, 15th ed., Boston: Pearson.
- Mohammadi G., Rafiee G., El Basuini M.F., Van Doan H., Ahmed H.A., Dawood M.A.O., and Abdel-Latif H.M.R., 2022, Growth performance, immune responses and disease resistance of Nile tilapia fed probiotics: A review, Aquaculture Reports, 22: 100954.
- Nayak S.K., 2010, Probiotics and immunity: A fish perspective, Fish & Shellfish Immunology, 29: 2-14.
- <https://doi.org/10.1016/j.fsi.2010.02.017>
- Ringø E., and Olsen R.E., 2014, The role of gut microbiota in fish health, Aquaculture Research, 45: 1653-1665.
- Ringø E., Hoseinifar S.H., Ghosh K., Doan H.V., Beck B.R., and Song S.K., 2018, Lactic acid bacteria in finfish-An update, Frontiers in Microbiology, 9: 1818.
- <https://doi.org/10.3389/fmicb.2018.01818>
- Ringø E., Zhou Z., Vecino J.L.G., Wadsworth S., Romero J., Kroghdahl Å., Olsen R.E., Dimitroglou A., Foey A., Davies S., Owen M., Lauzon H.L., Martinsen L.L., De Schryver P., Bossier P., Sperstad S., and Merrifield D.L., 2016, Effect of dietary components on the gut microbiota in aquatic animals, Aquaculture Nutrition, 22: 219-231.
- <https://doi.org/10.1111/anu.12346>
- Ruholla S., Hoseinifar S.H., Van Doan H., and Ringø E., 2021, Probiotic applications in rainbow trout aquaculture, Aquaculture, 536: 736476.
- Saraniya A., and Jeevaratnam K., 2012, Screening of probiotic properties of *Lactobacillus* species isolated from fermented foods, Journal of Microbiology, 50: 1-7.
- Schaeffer A.B., and Fulton M., 1933, A simplified method of staining endospores, Science, 77: 194.
- <https://doi.org/10.1126/science.77.1990.194>
- Setufe S.B., Lebelo S.L., and Thantsha M.S., 2021, Evaluation of probiotic properties of *Bacillus* spp. isolated from the gut of African catfish, Aquaculture International, 29: 1965-1981.
- Tagg J.R., and McGiven A.R., 1971, Assay system for bacteriocins, Applied Microbiology, 21: 943.
- <https://doi.org/10.1128/am.21.5.943-943.1971>
- Verschuere L., Rombaut G., Sorgeloos P., and Verstraete W., 2000, Probiotic bacteria as biological control agents in aquaculture, Microbiology and Molecular Biology Reviews, 64: 655-671.
- <https://doi.org/10.1128/MMBR.64.4.655-671.2000>
- Zhou X., Tian Z., Wang Y., and Li W., 2010, Effect of probiotics as water additives on tilapia growth and immune response, Aquaculture Research, 41: e1-e8.
- Zorriehzahra M.J., Delshad S.T., Adel M., Tiwari R., Karthik K., Dhama K., and Lazado C.C., 2016, Probiotics as beneficial microbes in aquaculture: an update on their multiple modes of action: a review, Veterinary quarterly, 36(4): 228-241.



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Research Article

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Impact of Dietary Immunostimulants on Morphometric and Haematological Responses of *Catla catla* (Hamilton, 1822) Challenged with *Aeromonas hydrophila*

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Abstract Aquaculture is a rapidly expanding sector that contributes significantly to global food production by cultivating aquatic organisms under controlled conditions. However, a major obstacle to aquaculture is the widespread use of antibiotics since the emergence of antibiotic-resistant bacteria. Thus, the immunostimulants are replacing chemotherapeutic therapies for aquaculture disease control. In such a scenario, morphometric and haematological evaluations are necessary to check the impact of immunostimulants on the health of both healthy and pathogen-infected fish. Because of its high nutritional value and other beneficial effects on human health, *Catla catla* has been a popular cultured freshwater fish in India, limited information is available regarding the effects of dietary immunostimulants on *Aeromonas hydrophila*-challenged *C. catla*. Therefore, the study examined the effects of immunostimulants on *C. catla* morphometric and haematological parameters under normal and *Aeromonas*-infected conditions. This study found that oral administration of immunostimulants such as vitamin C, vitamin E, chitin, chitosan, and levamisole significantly increased body weight, feed intake, feed conversion, and biomass. Morphometric investigations demonstrated that immunostimulant-supplemented feed considerably affected body length, width, and weight. Among all the tested feeds, vitamin C showed the strongest growth-promoting effect under *Aeromonas hydrophila* infection. The haematological analysis demonstrated that immunostimulants substantially increased haemoglobin content, RBC, WBC, neutrophil, lymphocyte, monocyte counts, and ESR as well as decreased eosinophil count and do not exhibit any effect on basophil count. Among all the immunostimulants Vitamin E has shown more effect in improving the haematological parameters. In conclusion, immunostimulants promote *C. catla* growth, immunity, and survival under *Aeromonas* infection. Thus, among the above selected immunostimulants, Vitamin C and Vitamin E are more potent and can be used in developing alternative dietary strategies for improving fish health management and reducing dependence on antibiotics in aquaculture.

Keywords *Aeromonas hydrophila*; *Catla catla*; Haematology; Immunostimulants; Morphometry

1 Introduction

Aquaculture is a rapidly expanding food production sector involving the cultivation of aquatic organisms under controlled (Ahmed and Thompson, 2019). The worldwide fish production in 2018 accounted for around 179 million tonnes, according to the Food and Agriculture Organization (FAO, 2020). Cultivated fish serve as a primary food supply for impoverished individuals, offering a cost-effective source of animal protein (Nolle et al., 2020). The global fishery stock is extensively depleted as a result of overfishing. Due to the growing human population, there is a need to enhance the productivity of aquaculture, which has become the main way of satisfying the growing demand for fish (Gephart et al., 2020).

Furthermore, the rise of antibiotic-resistant microbes poses a significant barrier to the widespread use of antibiotics. The administration of costly chemotherapeutic agents and antibiotics for managing diseases has faced major challenges due to their adverse effects, such as the accumulation of residues in tissues, the emergence of drug resistance, and immunosuppression. As a result, the market demand for antibiotic-treated food fish has decreased (Anderson and Jeney, 1992). Preventing disease is more preferable to intervening to suppress or stop the disease progression after it has commenced. Therefore, rather than using chemotherapeutic treatments, there is a growing focus on utilising immunostimulants as a means of controlling disease in aquaculture. The growing trend of

exploring various synergistic approaches for minimising fish infections has led to the accelerating use of immunostimulants as an appealing and potential substitute for chemotherapeutants in aquaculture practices.

An immunostimulant is a substance that enhances the innate or non-specific immune response through direct interaction with immune system cells, leading to their activation. Immunostimulants can be categorised into various agents according to their source including bacterial preparations, polysaccharides, extracts from animals or plants, nutritional components, and cytokines (Sakai, 1999). Immunostimulants enhance the immune system, decrease vulnerability to disease, and protect fish against stress and diseases. This minimizes adverse environmental effects and lessens reliance on chemicals or pharmaceuticals. New developments indicate that probiotics and immunostimulants, which are environmentally friendly methods, can greatly improve fish cultivation and health management (Sakai, 1999).

Several recent studies of peer researchers such as Rodriguez et al. (2003), Sahoo and Mukherjee (2001), and Smith et al. (2003) recommend incorporating the use of immunostimulants into the overall health management strategy. Immunostimulant elicit a strong and effective immune response against pathogenic organisms, including viruses, bacteria, fungi, and parasites, while exhibiting no risk of toxicity, carcinogenicity, or tissue residues (Bairwa et al., 2012). However, the effect of adding immunostimulants to fish is reliant on a number of factors such as the species, stage of development, kind of immunostimulant, and the dosage, which may vary depending on the animal's growth (Ringo et al., 2010).

In addition, studying an organism's response to a stressful event involves two types of responses: direct which affect biochemical and metabolic function, and indirect which affect the food chain, habitat accessibility, and behavioural changes (Adams, 2005). Finding and analysing biomarkers, especially in fish, has been recognised as a useful way to learn more about the state of stressed ecosystems and how they interact with aquatic life (Viarengo et al., 2007). Research studies indicate that immunostimulants can have beneficial impacts on the haematological parameters of fish. For instance, Hoseinifar et al. (2017) examined the impact of immunostimulants on the haematological parameters of rainbow trout, and they discovered that adding β -glucan, a widely used immunostimulant to fish's food resulted in higher levels of white blood cells and haemoglobin concentration.

In this context, fish morphometric and haematological measurements have become useful diagnostic tools and promising stress indicators. These measurements are crucial for evaluating the overall health of fish and the intensity of stress-induced reactions. Thus, a morphometric and haematological assessment is required to screen for the effects of immunostimulants on the health of fish that are both normal and infected with pathogen. In India, there is a scarcity of information on the effect of immunostimulants on the *Aeromonas*-infected *C. catla*. *C. catla* exemplifies a successful cultivated freshwater species for consumption, attributed to its notable nutritional and health benefits (Ismail, 2005). Therefore, this study aimed to evaluate the effects of different dietary immunostimulants on growth performance, morphometric characteristics, and haematological responses of *Catla catla* particularly under *Aeromonas hydrophila* challenge conditions, compared with previous studies on other cultured fish species.

2 Materials and Methods

2.1 Collection and acclimatisation of fish samples

Live healthy and young *C. catla* fish samples, with an initial body weight of 142 ± 10 g, were collected at a private farm (Longitude: $83^{\circ}10' 14.88''$ to $83^{\circ}11' 6.72''$ E; Latitude: $17^{\circ}40' 23.52''$ to $17^{\circ}45' 57.6''$ N) in K. Kotapadu village, Visakhapatnam, Andhra Pradesh, India, from December 2023 to January 2024. The live samples were carried to the lab in sterile zip pouches with pond water and maintained under regulated conditions. All fish were kept for two weeks to acclimatise to the laboratory environment. Fish were maintained in $50 \times 30 \times 40$ cm plastic containers with aerated, dechlorinated tap water.

2.2 Experimental design

A study was performed to assess the effects of five dietary immunostimulants, namely vitamin C, vitamin E, chitin, chitosan, and levamisole, on the growth performance, morphometric characteristics, and haematological responses of *C. catla*. The experiment was carried out with four distinct groups as follows:

Group 1 (Control): The first group of fish was fed GrowFin fish feed without any immunostimulants and is referred to as the control group.

Group 2 (Immunostimulant Fed): The second cohort of fish was subsequently partitioned into five experimental sub-groups, each receiving a distinct diet supplemented with a particular immunostimulant.

Group 3 (*A. hydrophila* infected): In the third group, the fish were fed a control diet and infected with a concentrated *A. hydrophila* culture at a dosage of 200 µL/L (10^6 CFU/mL).

Group 4 (*A. hydrophila* infected + Immunostimulant Fed/ Treated): In the fourth treatment group, the fish were further divided into five experimental sub-groups. Each group was infected with *A. hydrophila* like third group and fed independently with five selected immunostimulant mixed feeds.

In each experimental group ($n=10$), the fish were administered 3 gm of feed at 12-hour intervals. The water temperature and pH were calibrated at 25°C and 5.6, respectively. The photoperiod is established at 12 hours of light and 12 hours of darkness. Aerators were affixed to tubs to enhance aeration. Every day, half of the water in the tub was replaced with fresh, dechlorinated tap water, and the fish were maintained for one month in all the experimental groups. Following 30 days of exposure, fish were sacrificed with the objective of conducting growth performance, morphometric, and haematological assessments.

2.3 Measurements of growth performance

The initial measurement of length, weight, and other morphometric characteristics of all fish groups were done at the beginning of the experiment. Then the fish in all the treatment groups were allowed to grow for one month. Subsequently, the fish were sacrificed, and all growth parameters were recorded to determine their growth performance. The growth performance was assessed by measuring the total biomass gain (TBG), weight gain (WG), and feed conversion ratio (FCR) using the following formulas.

$$\text{Total biomass gain (Kg/m}^3\text{)} = \frac{\text{Final biomass}-\text{Initial biomass}}{\text{Water volume (m}^3\text{)}}$$

$$\text{Weight gain(g)} = \text{Final weight (g)} - \text{Initial weight(g)}$$

$$\text{Feed Conversion Ratio} = \frac{\text{Consumed feed (g)}}{\text{Weight gain (g)}}$$

2.4 Measurements of morphometric parameters

Morphometrics refers to the quantitative measurements of the dimension such as length, width, and height of the body or specific body portions of a fish. Morphometric measurements were obtained from fish in all the treatment groups at the beginning and end of the experiment. The morphometric characters were measured encompassing 14 specific traits including Body Length (BL), Body Width (BW), Body Depth (BD), Weight (W), Head Length (HL), Head Depth (HD), Eye Diameter (ED), Pupil Diameter (PD), Caudal Peduncle Length (CPL), Caudal Peduncle Depth (CPD), Caudal Fin Length (CFL), Dorsal Fin Length (DFL), Pectoral Fin Length (PcFL), Pelvic Fin Length (PIFN), and Anal Fin Length (Haryono, 2001).

2.5 Estimation of haematological parameters

2.5.1 Collection of blood

Following the end of the experiment, blood samples were obtained from the fish in all of the experimental groups. The blood sample was collected from the caudal vein, located just beyond the anal fin, by injecting a disposable

syringe into the muscle tissues at a right angle to the ventral surface of the fish until the blood was drawn into the syringe. Following that, the collected blood was transferred into a vial containing EDTA and placed in a freezer.

2.5.2 Preparation and estimation of haemoglobin

The haemoglobin fraction was isolated using the methodology outlined by Adisa et al. (2004) based on the principle of hypotonic lysis. To extract haemoglobin, the blood from all the fish in each treatment group were taken in a separate centrifuge tube and centrifuged at 3000 rpm for 10 minutes. After centrifugation, the RBC pellet was washed three times with a 0.14 M NaCl solution. Followed by washing, the RBC cell pellet was dissolved in phosphate buffer saline. Then one volume of RBC suspension was lysed by adding two volumes of 0.01M phosphate buffer (pH 7.4) and 0.5 volume of CCl₄. The haemolysate was further purified by centrifugation at 2300 rpm for 15 minutes at room temperature to remove any debris. The fraction with a high concentration of haemoglobin, specifically the upper layer was isolated and used to estimation of haemoglobin. The cyanmethemoglobin method, which is internationally recommended, was employed for determining the quantity of haemoglobin. 5 mL of cyanmethemoglobin reagent (Drabkin's solution) was transferred into sterilised test tubes. Subsequently, 20 µL of hemolysate from each of the experimental group fish were added separately to the tubes. Following that, the tubes were gently agitated and allowed to kept for 10 minutes at room temperature. After the incubation, the absorbance was measured in a spectrophotometer at 540 nm.

2.6 Determination of total RBC and WBC counts

The enumeration of RBC and WBC was performed using a Neubauer hemocytometer, following the methodology described by Martins et al. (2004).

$$\text{Total RBC count} = \frac{\text{Number of cells counted (N)} \times \text{Dilution factor}}{\text{Area} \times \text{Depth}}$$

$$\text{Total WBC count} = \frac{\text{Number of cells counted (N)} \times \text{Dilution factor}}{\text{Area} \times \text{Depth}}$$

2.7 Enumeration of differential leucocytes count

The differential blood count of the fish from each experimental group was conducted using the procedure outlined by Hudson and Hay (1991). To carry out this experiment, a minute quantity of blood was placed on a glass slide, and a thin smear was made by using oil and a spreader slide. Afterward, the blood smear was allowed to dry in the air, and then the slide was treated with Leishman's stain. The slide was incubated for 5 minutes and subsequently diluted with distilled water, followed by an additional incubation period of 10 minutes. After staining, the slides were rinsed with tap water and left to air dry. Finally, the slides were examined by placing them under the microscope.

2.8 Assessment of erythrocyte sedimentation rate (ESR)

The ESR of the fish from all the experimental groups was determined using the Westergren method. In order to perform ESR, the blood drawn from each sample was transferred into a Westergren-Katz tube up to the 200 mm mark immediately following blood collection. Subsequently, the tube was positioned vertically in a rack and left undisturbed for 1 hour at room temperature. During this period, measurements of the distance between the lowest point of the surface meniscus and the topmost layer of the red cell sediment were taken. The ESR is a measure of the distance that erythrocytes decrease in millimetres over the course of one hour.

2.9 Statistical analysis

Results were given as Mean ± Standard Deviation (SD) obtained from three independent experiments, and the data was assessed by one-way analysis of variance (ANOVA). The '*p*' values less than 0.05, 0.01 and 0.001 were considered as significant, highly significant and extremely significant difference between the groups.

3 Results

3.1 Growth performance

The growth performance of the fish in all of the treatment groups was assessed by measuring the total biomass gain (TBG), weight gain (WG), and feed conversion ratio (FCR).

3.1.1 Total biomass gain

The total biomass gain after 1 month of feeding with different immunostimulant-supplemented feeds such as vitamin C, vitamin E, chitin, chitosan, and levamisole under exposure to *Aeromonas hydrophila* was shown in Table 1 and Figure 1. From these results, it was observed that the fish fed with vitamin E mixed feed exhibited greater biomass gain, whereas fish fed with chitin mixed feed exhibited lowest biomass gain. The fish fed with control diet showed the biomass gain of 2.53 kg m⁻³, while the fish which are infected with *A. hydrophila* and fed with control diet exhibited the total biomass gain of 2.13 kg m⁻³.

Table 1 Growth performance of *Catla catla* with different immunostimulant-supplemented feeds under healthy and *Aeromonas hydrophila* infected conditions

S. No.	Treatment group	TBG (kgm ⁻³)	WG (g)	FCR
1	Fed with control feed	2.53	18.7	9.62
2	Aeromonas + Control feed	2.13	15.58	11.55
3	Fed with vitamin C mixed feed	3.33	24.8	7.25
4	Aeromonas + vitamin C mixed feed	2.93	21.9	8.82
5	Fed with vitamin E mixed feed	6.27	47.5	3.78
6	Aeromonas + vitamin E mixed feed	3.23	15.6	11.53
7	Fed with chitin mixed feed	2.8	20.5	8.78
8	Aeromonas + chitin mixed feed	2.66	20.2	8.91
9	Fed with chitosan mixed feed	3.73	28.1	6.41
10	Aeromonas + chitosan mixed feed	3.06	22.8	7.89
11	Fed with levamisole mixed feed	4.1	30.8	5.84
12	Aeromonas + levamisole mixed feed	3.46	26	6.92

Table notes: TBG – total biomass gain, WG – weight, FCR – feed conversion ratio

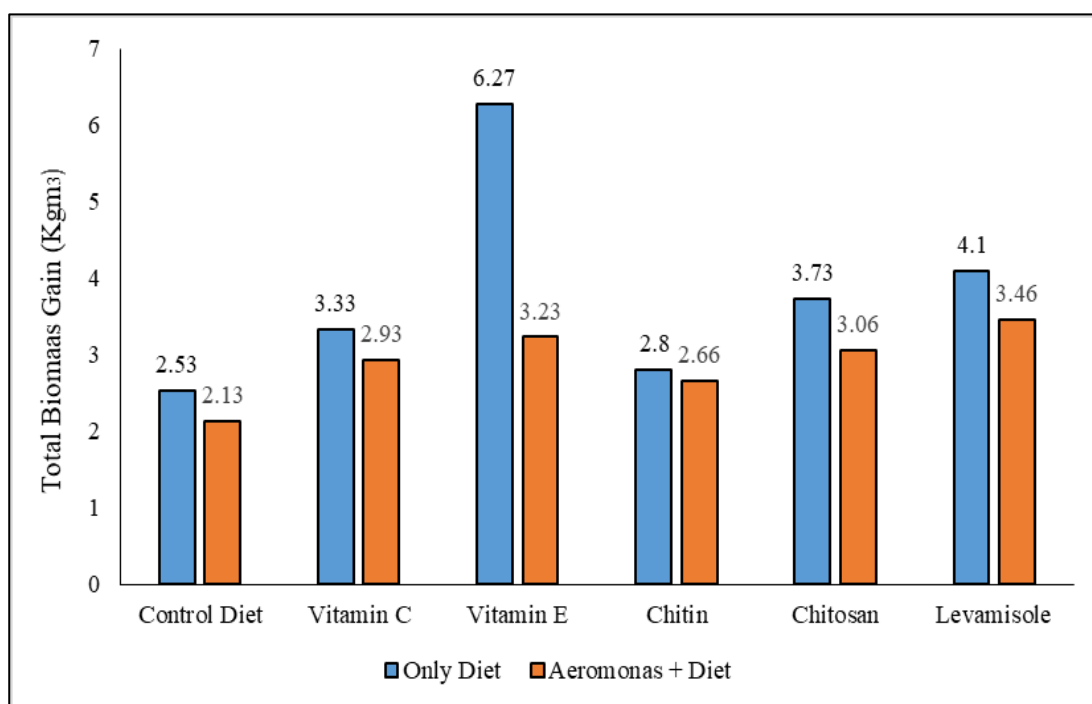


Figure 1 The total biomass gain with different immunostimulant-supplemented feeds under normal and *Aeromonas hydrophila* infected conditions

Furthermore, all the immunostimulant mixed feed significantly increases the total biomass gain when compared to the control groups. The total biomass gain in the fish fed with selected immunostimulant feeds such as vitamin C, vitamin E, chitin, chitosan, and levamisole were found to be 3.33, 6.27, 2.8, 3.73, and 4.1 kg m⁻³ respectively.

Whereas total biomass gain in the *A. hydrophila* infected fish fed with immunostimulant feeds such as vitamin C, vitamin E, chitin, chitosan, and levamisole were found to be 2.93, 3.23, 2.66, 3.06, and 3.46 kg m⁻³ respectively.

According to these results, vitamin E has a greater growth promoting properties than the other immunostimulant feeds, whereas under *Aeromonas* exposure, levamisole shows greater biomass gain. Furthermore, when compared to the *Aeromonas* infected fish with control diets, the fish fed with immunostimulant mixed feeds exhibited greater biomass gain.

3.1.2 Weight gain

The weight gain after 1 month of feeding with different immunostimulant-supplemented feeds such as vitamin C, vitamin E, chitin, chitosan, and levamisole under exposure to *A. hydrophila* was shown in Figure 2 and Table 1. From these results, it was observed that the fish fed with immunostimulant mixed feed exhibited greater weight gain than the fish fed with control feed.

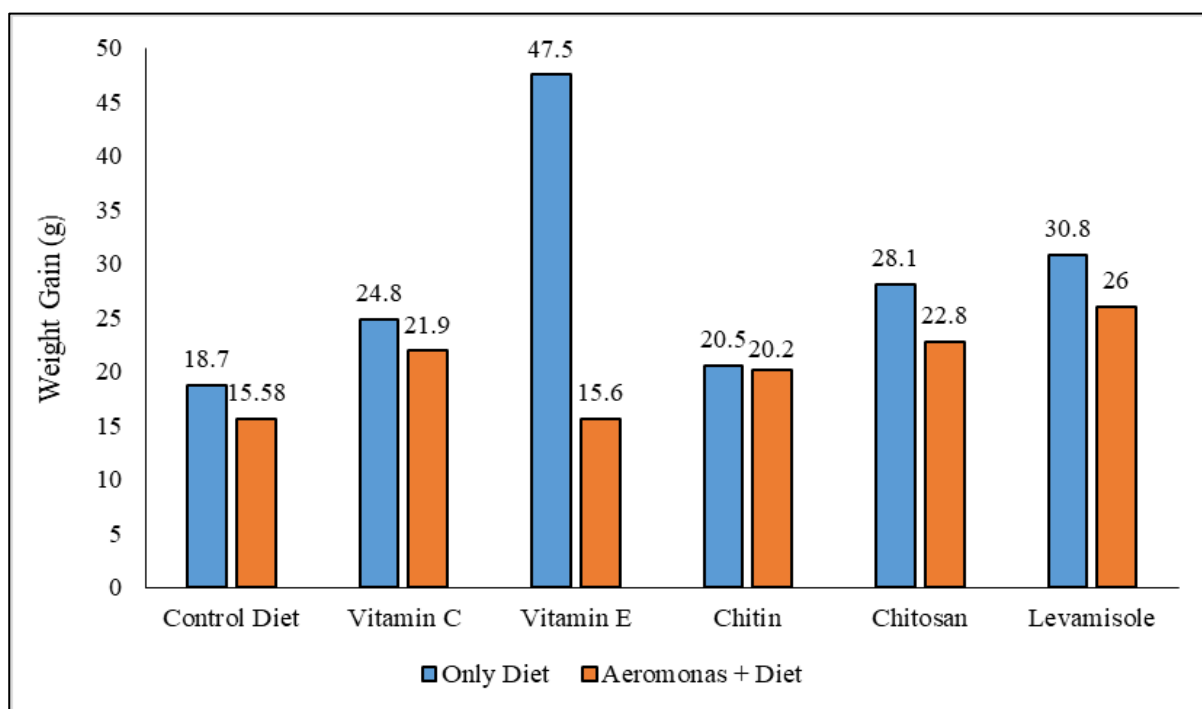


Figure 2 Weight gain with different immunostimulant-supplemented feeds under normal and *Aeromonas hydrophila* infected conditions

Furthermore, among all the tested immunostimulants, fish fed with vitamin E mixed feed exhibited greater weight gain. However, lowest weight gain was observed in *Aeromonas* infected fish fed with vitamin E. The fish fed with control diet shows the weight gain of 18.7 g, while the fish which are infected with and *A. hydrophila* and fed with control diet exhibited the weight gain of 15.58 g.

Furthermore, all the immunostimulant mixed feed significantly increased the weight gain when compared to the control groups. The weight gain in the fish fed with selected immunostimulant feeds such as vitamin C, vitamin E, chitin, chitosan, and levamisole were found to be 24.8, 47.5, 20.5, 28.1, and 30.8 g respectively.

Whereas, weight gain in the *A. hydrophila* infected fish fed with immunostimulant feeds such as vitamin C, vitamin E, chitin, chitosan, and levamisole were found to be 21.9, 15.6, 20.2, 22.8, and 26 g respectively. According to these results, vitamin E has a greater growth promoting properties than the other immunostimulant

feeds under normal conditions, whereas under *Aeromonas* exposure, levamisole shows greater weight gain. Furthermore, when compared to the *Aeromonas* infected fish with control diets, the fish fed with immunostimulant mixed feeds exhibited significantly increased weight gain.

Moreover, the fish fed with immunostimulant mixed feeds exhibited greater weight gain than the fish fed with control feed. The highest weight gain was observed in fish fed with vitamin E supplemented feed. After 30 days of treatment, the weight gain of fish fed with control feed as well as vitamin C, vitamin E, chitin, Chitosan, and levamisole supplemented feeds were found to be 18.7, 24.8, 47.5, 20.5, 28.1, and 30.8 g respectively. These results demonstrated that the vitamin C, vitamin E, and levamisole had the greater growth promoting properties.

3.1.3 Feed conversion ratio

Feed Conversion Ratio (FCR) is a metric that quantifies the amount of feed consumed by an animal relative to the weight gained by the animal within a specific time frame. Smaller FCR values suggest that a feed is turned into fish weight gain efficiently, but overfeeding or underfeeding increases the ratio (Bai et al., 2022).

The feed conversion ratio after 1 month of feeding with different immunostimulant-supplemented feeds such as vitamin C, vitamin E, chitin, chitosan, and levamisole under exposure to *A. hydrophila* was shown in Figure 3 and Table 1. From these results, it was observed that the fish fed with immunostimulant mixed feed exhibited lower FCR weight gain than the fish fed with control feed.

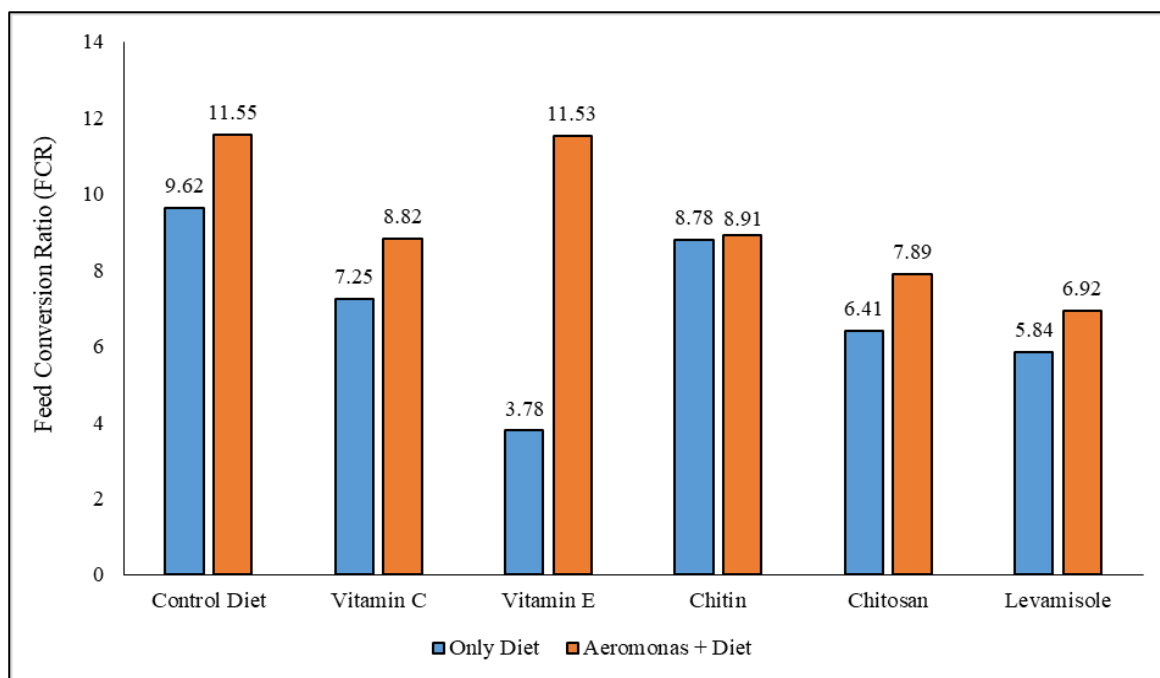


Figure 3 Feed Conversion Ratio (FCR) with different immunostimulant-supplemented feeds under normal and *Aeromonas hydrophila* infected conditions

Furthermore, among all the tested immunostimulants, fish fed with vitamin E mixed feed exhibited lower FCR, whereas highest FCR was observed in the fish grown under *Aeromonas* and control diets. The fish fed with control diet shows the FCR of 9.62, while the fish which are infected with *A. hydrophila* and fed with control diet exhibited the FCR of 11.55.

Furthermore, all the immunostimulant mixed feed significantly decreases the FCR when compared to the control groups. The FCR in the fish fed with selected immunostimulant feeds such as vitamin C, vitamin E, chitin, chitosan, and levamisole were found to be 7.25, 3.78, 8.78, 6.41, and 5.84 respectively. Whereas, FCR for the *A. hydrophila* infected fish fed with immunostimulant feeds such as vitamin C, vitamin E, chitin, chitosan, and levamisole were found to be 8.22, 11.53, 8.91, 7.89, and 6.92 respectively. According to these results, vitamin E

has a lower FCR properties than the other immunostimulant feeds under normal conditions, whereas under *Aeromonas* exposure, levamisole shows lower FCR values. Furthermore, when compared to the *Aeromonas* infected fish with control diets, the fish fed with immunostimulant mixed feeds exhibited significantly decreased FCR values.

3.2 Morphometric and growth-related traits

The following morphometric characterisation of the fish in all of the treatment groups was assessed by measuring Body Length (BL), Body Width (BW), Body Depth (BD), Weight (W), Head Length (HL), Head Depth (HD), Eye Diameter (ED), Pupil Diameter (PD), Caudal Peduncle Length (CPL), Caudal Peduncle Width (CPW), Caudal Fin Length (CFL), Dorsal Fin Length (DFL), Pectoral Fin Length (PcFL), Pelvic Fin Length (PIFN), and Anal Fin Length (AFL).

3.2.1 Morphometric characterisation of healthy fish fed with different immunostimulants

Table 2, depicts the initial and final measurements of morphometric parameters of healthy fish fed with control and immunostimulant mixed feeds. Table 3 presents the net gain of morphometric parameters of healthy fish fed with control and immunostimulant mixed feeds. From these results, it was found that the morphometric parameters including body length, body width, and weight were significantly varied with different immunostimulant feeds. While, the parameters including HL, HD, ED, PD, CPL, CPW, CFL, DFL, PcFL, PIFL, and AFL have not exhibited significant variation with the different immunostimulant feeds.

Table 2 Morphometric characteristics of healthy fish fed with control and immunostimulant mixed feed

S. No.	Parameter	Type of Diet											
		Control		Vitamin C		Vitamin E		Chitin		Chitosan		Levamisole	
		I	F	I	F	I	F	I	F	I	F	I	F
1	BL (cm)	15.9	17.3	18.7	21	21	23	16.4	18	9.8	11.7	13.3	15.1
2	BW (cm)	3.8	4.5	5	6	6.7	7.5	4	4.5	3	3.4	3	3.6
3	W (g)	154.5	173.2	148.7	171.5	161.3	208.8	155.7	171.2	130.2	158.3	145.8	176.6
4	HL (cm)	3.8	4.3	5	4.5	5.3	6	4	4.5	2.4	2.8	3.2	3.8
5	HD (cm)	2.9	3.4	4.1	4.5	4.5	5	3	3.5	1.4	1.7	1.7	2.8
6	ED (cm)	0.8	1	0.7	0.9	0.8	1	0.7	0.8	0.6	0.7	0.8	0.9
7	PD (cm)	0.4	0.5	0.35	0.5	0.4	0.5	0.3	0.4	0.3	0.4	0.3	0.4
8	CPL (cm)	1.6	2.3	2.6	3	2.7	3	2	2.5	1.1	1.4	1.7	2.1
9	CPW (cm)	1.7	1.9	1.8	2.3	2.1	2.5	1.5	1.9	0.8	1	1.1	1.5
10	CFL (cm)	3.7	4.1	4.3	4.8	4.4	5	3.9	4.4	2.1	2.7	3.1	3.6
11	DFL (cm)	3.3	3.8	3.9	4.3	3.5	4	3.5	4	2.4	2.9	2.7	3.1
12	PcFL (cm)	2.4	2.9	2.4	3	3	3.4	2.4	2.7	1.9	2.3	2.1	2.6
13	PIFL (cm)	2.3	2.7	2.9	3.5	2.9	3.3	2.6	3	1.6	2	1.9	2.4
14	AFL (cm)	2.7	3.1	2.7	3.2	2.9	3.5	2.3	2.7	1.2	1.6	1.7	2.2

Table 3 Net growth of morphometric parameters of healthy fish fed with control and immunostimulant mixed feed after 30 days of treatment

S. No.	Morphometric parameter	Type of Diet					
		Control	Vitamin C	Vitamin E	Chitin	Chitosan	Levamisole
1	BL (cm)	1.4	2.3	2	1.6	1.9	1.8
2	BW (cm)	0.7	1	0.8	0.5	0.4	0.6
3	W (g)	18.7	22.8	47.5	15.5	28.1	30.8
4	HL (cm)	0.5	0.5	0.7	0.5	0.4	0.6
5	HD (cm)	0.5	0.4	0.5	0.5	0.3	0.7
6	ED (cm)	0.2	0.2	0.2	0.1	0.1	0.1
7	PD (cm)	0.1	0.15	0.1	0.1	0.2	0.1
8	CPL (cm)	0.7	0.4	0.3	0.5	0.3	0.4
9	CPW (cm)	0.2	0.5	0.4	0.4	0.1	0.4
10	CFL (cm)	0.4	0.5	0.6	0.5	0.6	0.5
11	DFL (cm)	0.5	0.4	0.5	0.5	0.5	0.4
12	PcFL (cm)	0.5	0.6	0.4	0.3	0.5	0.5
13	PIFL (cm)	0.4	0.6	0.4	0.4	0.4	0.5
14	AFL (cm)	0.4	0.5	0.6	0.4	0.4	0.5

Furthermore, fish fed with immunostimulant-supplemented diets showed greater body length increments than the fish fed with control feed. Among, all the tested feeds, fish fed with vitamin C mixed feed exhibited greater body length gain. After 30 days of treatment, the length gain of fish fed with control feed as well as vitamin C, vitamin E, Chitin, Chitosan, and levamisole supplemented feeds were found to be 1.4, 2.3, 2, 1.6, 1.9, and 1.8 cm respectively.

However, the fish fed with vitamin C and vitamin E supplemented feed exhibited greater body width (BW) than the control. Whereas, the fish fed with chitin, chitosan, and levamisole supplemented feed shows lesser BW than the control. The highest BW gain observed in the fish fed with vitamin E supplemented feed. After 30 days of treatment, the BW gain of fish fed with control feed as well as vitamin C, vitamin E, chitin, chitosan, and levamisole supplemented feeds were found to be 0.7, 1, 0.8, 0.5, 0.4 and 0.6 cm respectively (Figure 4 to Figure 9).

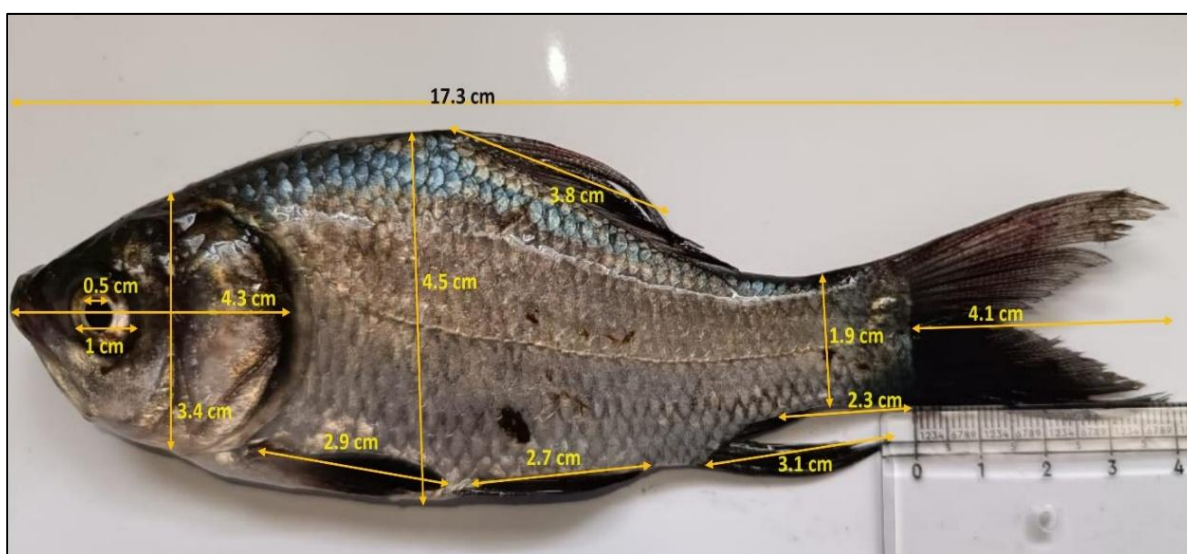


Figure 4 Morphometric measurements of *Catla catla* fed with normal feed after 30 days of treatment

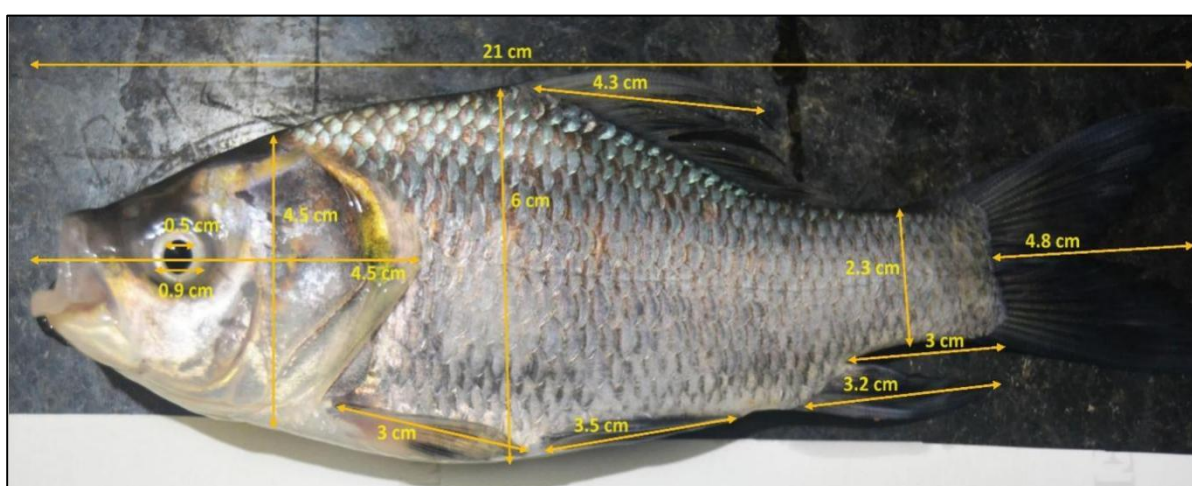


Figure 5 Morphometric measurements of *Catla catla* fed with vitamin C mixed feed after 30 days of treatment

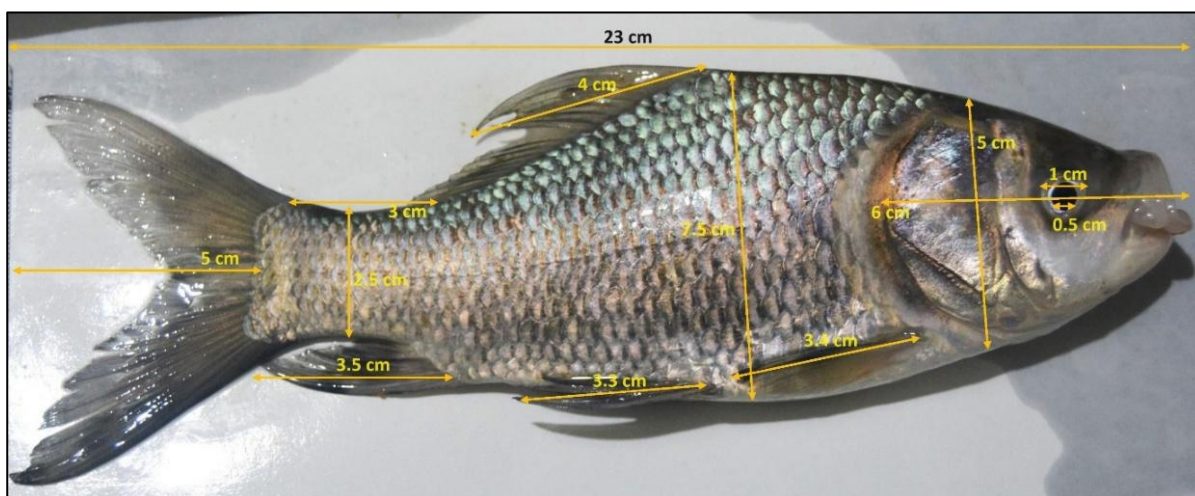


Figure 6 Morphometric measurements of *Catla catla* fed with vitamin E mixed feed after 30 days of treatment

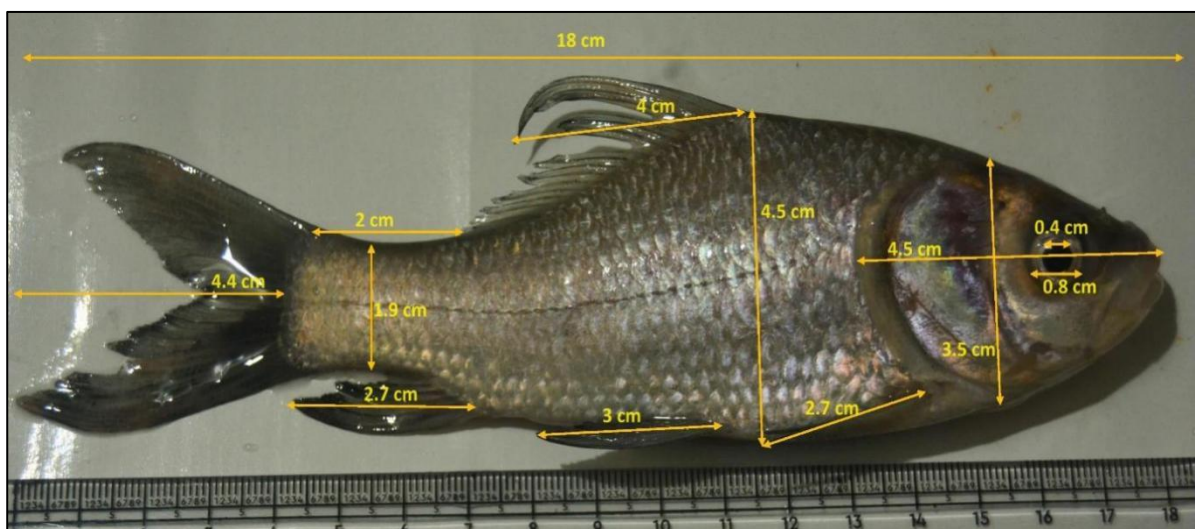


Figure 7 Morphometric measurements of *Catla catla* fed with Chitin mixed feed after 30 days of treatment

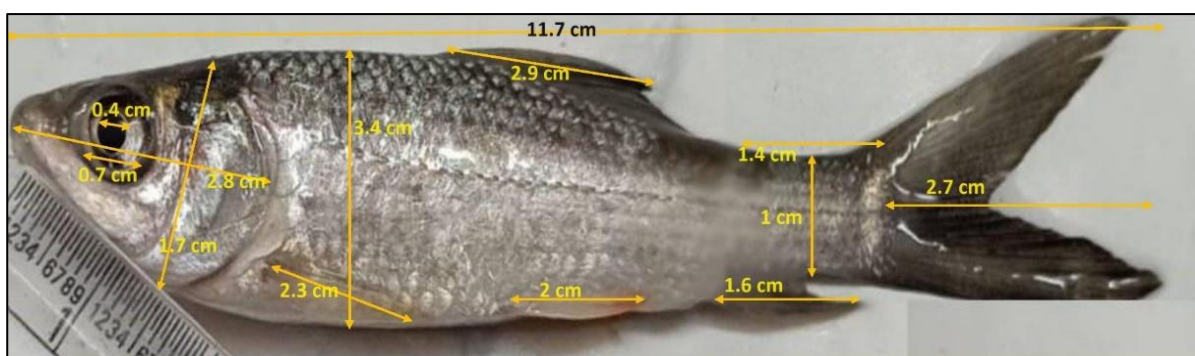


Figure 8 Morphometric measurements of *Catla catla* fed with Chitosan mixed feed after 30 days of treatment

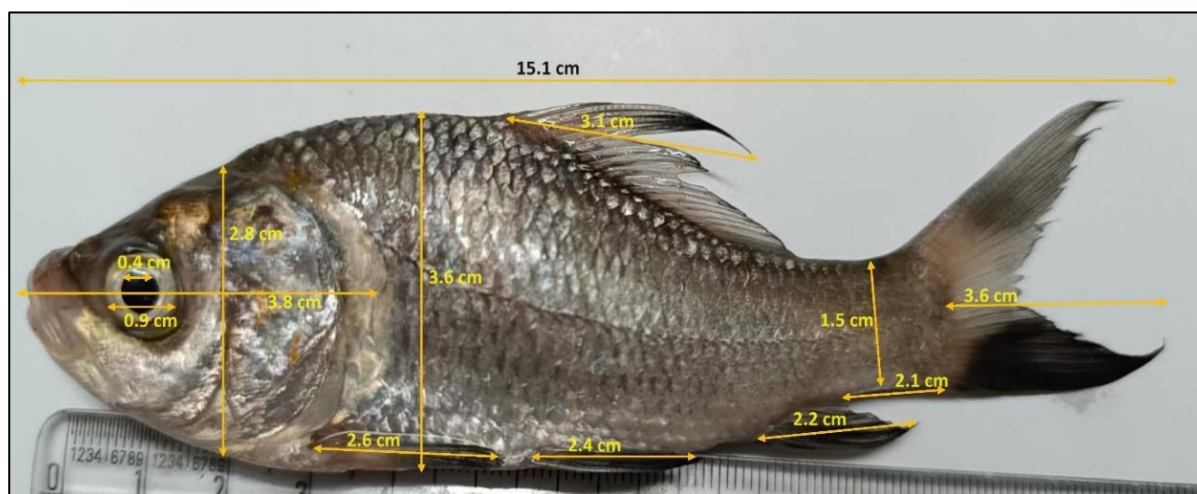


Figure 9 Morphometric measurements of *Catla catla* fed with Levamisole mixed feed after 30 days of treatment

3.2.2 Morphometric characterisation of *Aeromonas hydrophila* infected fish fed with different immunostimulants

The Table 4, depicts the initial and final measurements of morphometric parameters of *Aeromonas* infected fish fed with control and immunostimulant mixed feeds. The Table 5 illustrated the net gain of morphometric parameters of *Aeromonas* infected fish fed with control and immunostimulant mixed feeds. From these results, it was found that the morphometric parameters of infected fish including body length, body width, and weight were significantly varied with different immunostimulant feeds. While, there was no significant fluctuation observed in the metrics such as HL, HD, ED, PD, CPL, CPW, CFL, DFL, PcFL, PIFL, and AFL with the different immunostimulant diets.

Table 4 Morphometric characteristics of *Aeromonas hydrophila* infected fish fed with control and immunostimulant mixed feed

S. No.	Parameter	Type of Diet		Vitamin C		Vitamin E		Chitin		Chitosan		Levamisole	
		I	F	I	F	I	F	I	F	I	F	I	F
1	BL (cm)	16.2	17.5	20.2	22	20.3	22	16.3	17.8	10.4	11.9	13.5	15
2	BW (cm)	4.1	4.5	4.8	0.6	5.5	0.6	4.1	4.5	3.2	3.5	2.9	3.3
3	W (g)	157.8	173.4	137.3	153.2	186.3	162.3	148.3	168.7	141.3	164.1	164.5	138.5
4	HL (cm)	3.9	4.5	4.5	5	4.3	5	3.8	4.3	2.4	2.6	3.1	3.5
5	HD (cm)	3.8	4.3	3.6	4.1	3.6	4	3	3.4	1.4	1.6	2.1	2.5
6	ED (cm)	0.7	0.9	0.7	0.7	0.7	0.8	0.8	1	0.6	0.7	0.7	0.8
7	PD (cm)	0.4	0.5	0.3	0.35	0.3	0.4	0.4	0.5	0.3	0.4	0.3	0.4
8	CPL (cm)	1.7	2.2	2.1	2.6	2.1	2.5	1.7	2.3	1.6	2	1.2	1.5
9	CPW (cm)	1.9	2.1	1.8	2	1.7	2	1.5	1.9	1.1	1.4	1.1	1.4
10	CFL (cm)	3.8	4.1	4.3	4.9	4.7	5.3	3.5	4	2.1	2.5	3.1	3.5
11	DFL (cm)	3.5	3.9	3.9	4.5	4.1	4.5	4.9	5.4	1.7	2	2.7	3.2
12	PcFL (cm)	2.4	2.8	2.4	3.1	3.3	3.8	2.4	2.9	1.1	1.3	2	2.4
13	PIFL (cm)	2.5	2.9	2.9	3.4	2.9	3.5	2.3	2.7	1.7	2.2	1.8	2.3
14	AFL (cm)	2.5	2.7	2.7	2.8	3.1	3.6	1.6	2	1.5	1.8	1	1.2

Table 5 Net growth of morphometric parameters of *Aeromonas hydrophila* infected fish fed with control and immunostimulant mixed feed after 30 days of treatment

S. No.	Morphometric parameter	Type of Diet					
		Control	Vitamin C	Vitamin E	Chitin	Chitosan	Levamisole
1	BL (cm)	1.3	1.8	1.7	1.3	1.5	1.5
2	BW (cm)	0.4	1.2	0.5	0.4	0.3	0.4
3	W (g)	15.6	15.9	24	20.4	22.8	26
4	HL (cm)	0.6	0.5	0.7	0.5	0.2	0.4
5	HD (cm)	0.5	0.4	0.4	0.4	0.2	0.4
6	ED (cm)	0.2	0.1	0.1	0.2	0.1	0.1
7	PD (cm)	0.1	0.1	0.1	0.1	0.1	0.1
8	CPL (cm)	0.5	0.4	0.4	0.6	0.4	0.3
9	CPW (cm)	0.3	0.2	0.3	0.4	0.3	0.4
10	CFL (cm)	0.3	0.4	0.6	0.5	0.4	0.4
11	DFL (cm)	0.4	0.6	0.4	0.5	0.3	0.5
12	PcFL (cm)	0.4	0.4	0.5	0.5	0.2	0.4
13	PIFL (cm)	0.4	0.4	0.6	0.4	0.5	0.5
14	AFL (cm)	0.2	0.8	0.5	0.4	0.3	0.2

Furthermore, infected fish fed with all five selected immunostimulant mixed feed exhibited lower lengths than the normal fish fed with immunostimulant diets. Among, all the tested feeds, *Aeromonas* infected fish fed with vitamin C mixed feed exhibited greater body length gain. After 30 days of treatment, the length gain of *Aeromonas* infected fish fed with control feed as well as vitamin C, vitamin E, chitin, Chitosan, and levamisole supplemented feeds were found to be 1.3, 1.8, 1.7, 1.3, 1.5, and 1.5 cm respectively.

However, the infected fish fed with vitamin C and vitamin E supplemented feed exhibited greater body width (BW) than the control. Whereas, the fish fed with chitosan supplemented feed shows lesser BW than the control. The BW gain of infected fish with the chitosan, and levamisole mixed feed are equal to the BW gain of fish fed with control feed. The highest BW gain observed in the infected fish fed with vitamin C supplemented feed. After 30 days of treatment, the BW gain of infected fish fed with control feed as well as vitamin C, vitamin E, chitin, Chitosan, and levamisole supplemented feeds were found to be 0.4, 1.2, 0.5, 0.4, 0.3, and 0.4 cm respectively (Figure 10 to Figure 15).

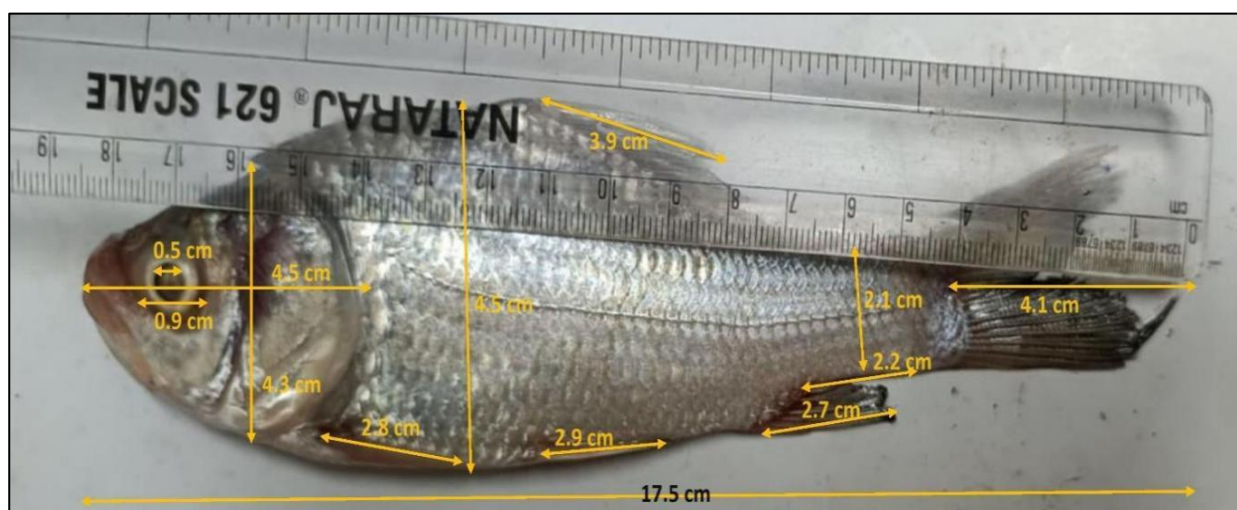


Figure 10 Morphometric measurements of *Aeromonas hydrophila* infected *Catla catla* fed with control feed after 30 days of treatment

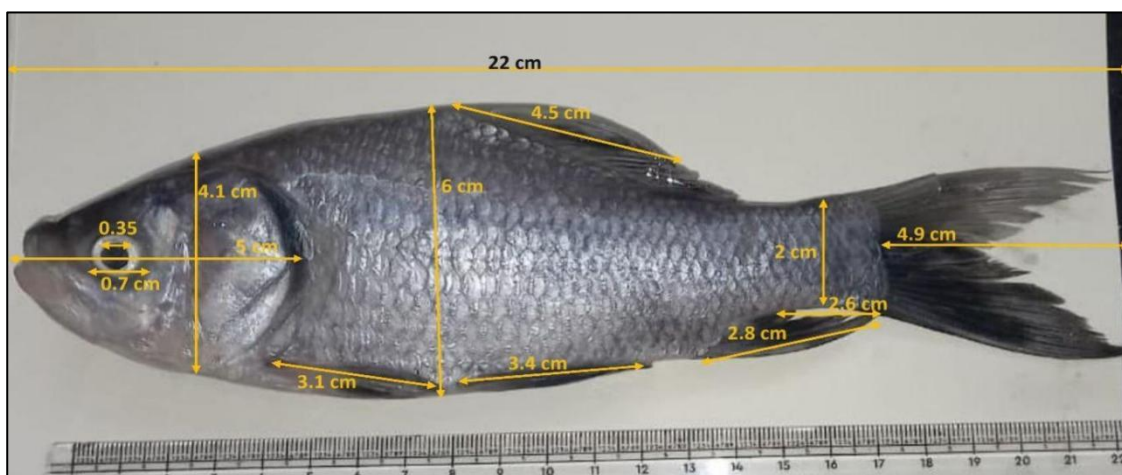


Figure 11 Morphometric measurements of *Aeromonas hydrophila* infected *Catla catla* fed with vitamin C mixed feed after 30 days of treatment

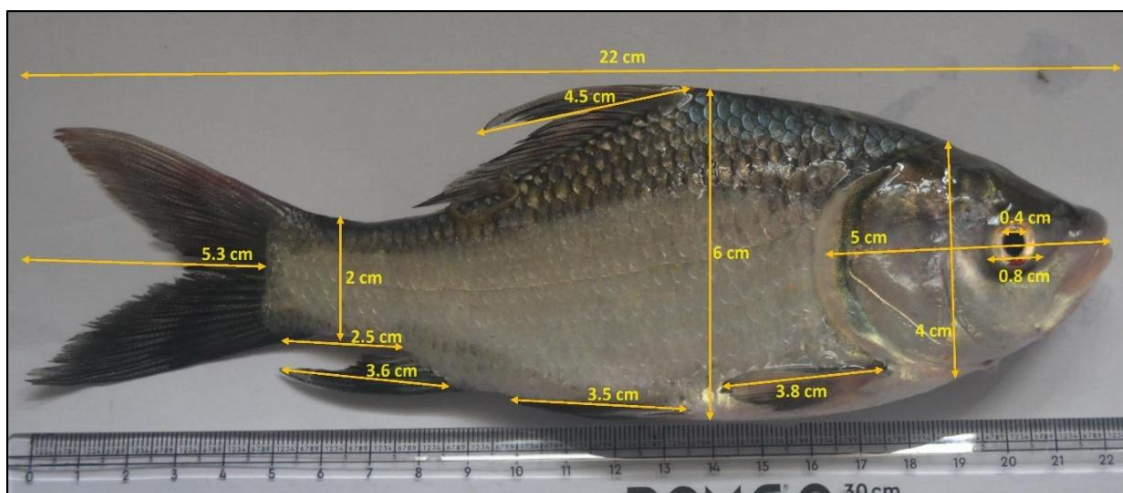


Figure 12 Morphometric measurements of *Aeromonas hydrophila* infected *Catla catla* fed with vitamin E mixed feed after 30 days of treatment

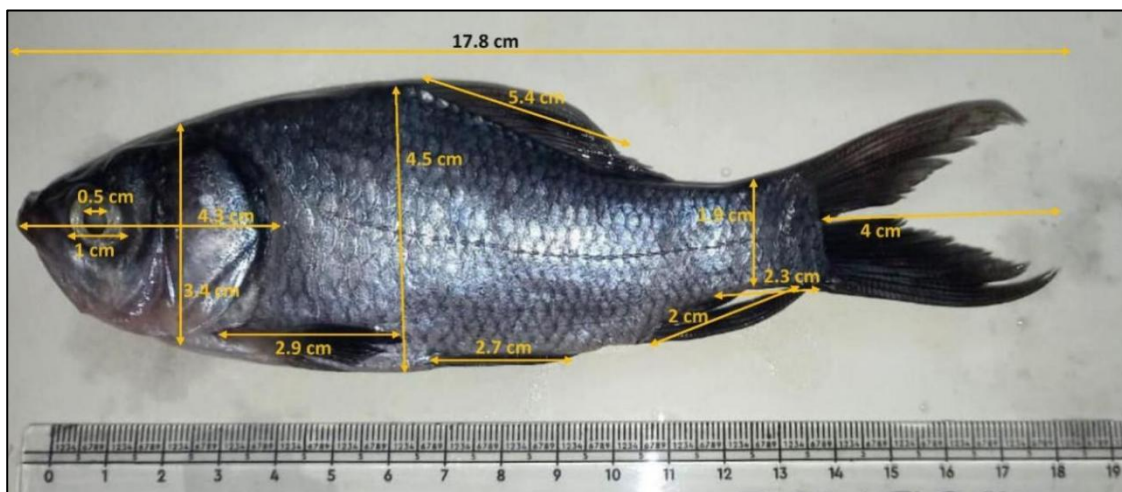


Figure 13 Morphometric measurements of *Aeromonas hydrophila* infected *Catla catla* fed with chitin mixed feed after 30 days of treatment

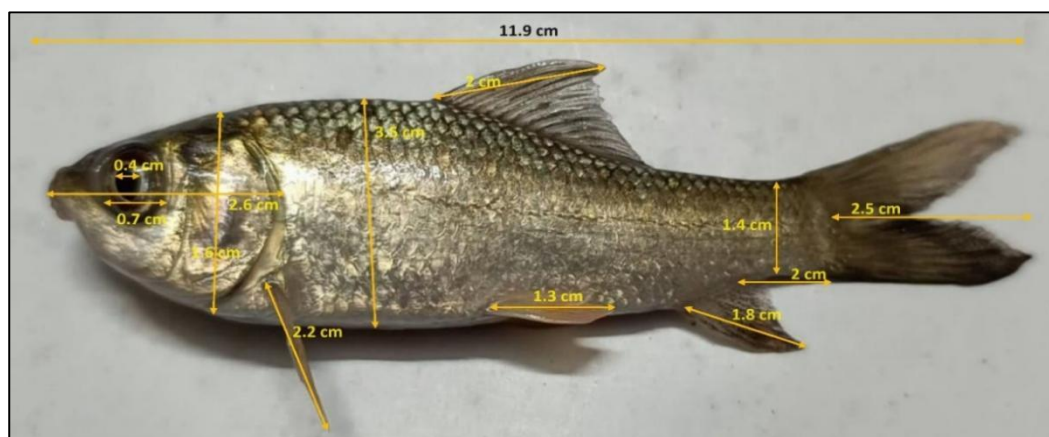


Figure 14 Morphometric measurements of *Aeromonas hydrophila* infected *Catla catla* fed with chitosan mixed feed after 30 days of treatment

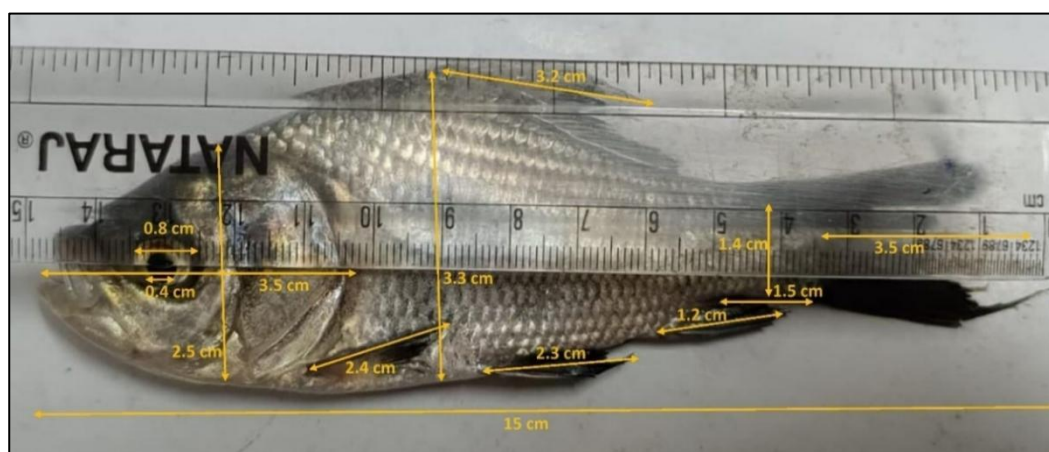


Figure 15 Morphometric measurements of *Aeromonas hydrophila* infected *Catla catla* fed with levamisole mixed feed after 30 days of treatment

From the Table 6 to Table 20 represents the comparative morphometric parameters of fish fed with control and the five immunostimulants mixed diets under healthy and *Aeromonas* infected conditions. One way ANOVA analysis and Tukey's HSD analysis of morphometric parameters of fish fed with control and the five immunostimulants mixed diets under healthy and *Aeromonas* infected conditions.

Table 6 Comparative morphometric parameters of fish fed with control and vitamin C mixed diets under normal and *Aeromonas hydrophila* infection

S. No.	Morphometric parameter	Healthy fish (T1)	Control feed		Vitamin C feed	
			<i>Aeromonas</i> infected fish (T2)	Healthy fish (T3)	<i>Aeromonas</i> infected fish (T4)	
1	BL (cm)	1.4	1.3	2.3	1.8	
2	BW (cm)	0.7	0.4	01	1.2	
3	W (g)	18.7	15.6	22.8	15.9	
4	HL (cm)	0.5	0.6	0.5	0.5	
5	HD (cm)	0.5	0.5	0.4	0.4	
6	ED (cm)	0.2	0.2	0.2	0.1	
7	PD (cm)	0.1	0.1	0.15	0.1	
8	CPL (cm)	0.7	0.5	0.4	0.4	
9	CPW (cm)	0.2	0.3	0.5	0.2	
10	CFL (cm)	0.4	0.3	0.5	0.4	
11	DFL (cm)	0.5	0.4	0.4	0.6	
12	PcFL (cm)	0.5	0.4	0.6	0.4	
13	PIFL (cm)	0.4	0.4	0.6	0.4	
14	AFL (cm)	0.4	0.2	0.5	0.8	

Table 7 One-way ANOVA analysis of morphometric parameters of fish fed with control and vitamin C mixed diets under normal and *Aeromonas hydrophila* infection

Control feed/ Vitamin C mixed feed	SS	DF	MS	F – value
Between groups	3.7066	03	1.2355	0.05331
Within groups	1205.1238	52	23.1755	
Total	1208.8303	55		

SS - Sum of Squares,

DF - Degrees of Freedom,

MS - Mean Squares,

F-value -- variation between mean values

Table 8 Tukey's HSD analysis of morphometric parameters of fish fed with control and vitamin C mixed diets under healthy and *Aeromonas hydrophila* infection

S. No.	Pairwise comparisons	Tukey's HSD	Q – value	p – value
1	T1 : T2	0.29	0.22	0.99860 ^{NS}
2	T1 : T3	0.40	0.31	0.99610 ^{NS}
3	T1 : T4	0.14	0.11	0.99982 ^{NS}
4	T2 : T3	0.69	0.54	0.98128 ^{NS}
5	T2 : T4	0.14	0.11	0.99982 ^{NS}
6	T3 : T4	0.55	0.42	0.99047 ^{NS}

T1 – Healthy fish fed with control diet,

T2 - *Aeromonas* infected fish fed with control diet,

T3 – Healthy fish fed with Vitamin C mixed diet,

T4 – *Aeromonas* infected fish fed with Vitamin C mixed diet,

Q-value - studentized range statistic value,

p < 0.05 - Significant

p-value* = Significant, p-value** = highly significant, p-value*** = extremely significant,

p-value^{NS} = Not Significant

Table 9 Comparative morphometric parameters of fish fed with control and vitamin E mixed diets under normal and *Aeromonas hydrophila* infection

S. No.	Morphometric parameter	Control feed		Vitamin E feed	
		Healthy fish (T1)	<i>Aeromonas</i> infected fish (T2)	Healthy fish (T3)	<i>Aeromonas</i> infected fish (T4)
1	BL (cm)	1.4	1.3	2	1.7
2	BW (cm)	0.7	0.4	0.8	0.5
3	W (g)	18.7	15.6	47.5	24
4	HL (cm)	0.5	0.6	0.7	0.7
5	HD (cm)	0.5	0.5	0.5	0.4
6	ED (cm)	0.2	0.2	0.2	0.1
7	PD (cm)	0.1	0.1	0.1	0.1
8	CPL (cm)	0.7	0.5	0.3	0.4
9	CPW (cm)	0.2	0.3	0.4	0.3
10	CFL (cm)	0.4	0.3	0.6	0.6
11	DFL (cm)	0.5	0.4	0.5	0.4
12	PcFL (cm)	0.5	0.4	0.4	0.5
13	PIFL (cm)	0.4	0.4	0.4	0.6
14	AFL (cm)	0.4	0.2	0.6	0.5

Table 10 One-way ANOVA analysis of morphometric parameters of fish fed with control and vitamin E mixed diets under normal and *Aeromonas hydrophila* infection

Normal feed/ Vitamin E mixed feed	SS	DF	MS	F – value
Between groups (Treatment)	49.2079	03	16.4026	0.27653
Within groups	3084.3857	52	59.3151	
Total	3133.5936	55		

SS - Sum of Squares,

DF - Degrees of Freedom,

MS - Mean Squares,

F-value -- variation between mean values

Table 11 Tukey's HSD analysis of morphometric parameters of fish fed with control and Vitamin E mixed diets under normal and *Aeromonas hydrophila* infection

S. No.	Pairwise comparison	Tukey's HSD	Q – value	p – value
1	T1 : T2	0.29	0.14	0.99966 ^{NS}
2	T1 : T3	2.13	1.03	0.88411 ^{NS}
3	T1 : T4	0.40	0.19	0.99906 ^{NS}
4	T2 : T3	2.41	1.17	0.84027 ^{NS}
5	T2 : T4	0.69	0.33	0.99534 ^{NS}
6	T3 : T4	1.73	0.84	0.93350 ^{NS}

T1 – Healthy fish fed with control diet

T2 - *Aeromonas* infected fish fed with control diet

T3 – Healthy fish fed with Vitamin E mixed diet

T4 – *Aeromonas* infected fish fed with Vitamin E mixed diet

Q-value - studentized range statistic value,

p < 0.05 - Significant

p-value* = Significant, p-value** = highly significant, p-value*** = extremely significant,

p-value^{NS} = Not Significant

Table 12 Comparative morphometric parameters of fish fed with control and Chitin mixed diets under normal and *Aeromonas hydrophila* infection

S. No.	Morphometric parameter	Control feed		Chitin feed	
		Healthy fish (T1)	<i>Aeromonas</i> infected fish (T2)	Healthy fish (T3)	<i>Aeromonas</i> infected fish (T4)
1	BL (cm)	1.4	1.3	1.6	1.3
2	BW (cm)	0.7	0.4	0.5	0.4
3	W (g)	18.7	15.6	15.5	20.4
4	HL (cm)	0.5	0.6	0.5	0.5
5	HD (cm)	0.5	0.5	0.5	0.4
6	ED (cm)	0.2	0.2	0.1	0.2
7	PD (cm)	0.1	0.1	0.1	0.1
8	CPL (cm)	0.7	0.5	0.5	0.6
9	CPW (cm)	0.2	0.3	0.4	0.4
10	CFL (cm)	0.4	0.3	0.5	0.5
11	DFL (cm)	0.5	0.4	0.5	0.5
12	PcFL (cm)	0.5	0.4	0.3	0.5
13	PIFL (cm)	0.4	0.4	0.4	0.4
14	AFL (cm)	0.4	0.2	0.4	0.4

Table 13 One-way ANOVA analysis of morphometric parameters of fish fed with control and Chitin mixed diets under normal and *Aeromonas hydrophila* infection

Normal Feed/ Chitin Feed	SS	DF	MS	F – value
Between groups (Treatment)	1.4657	3	0.4886	0.02301
Within groups	1104.0314	52	21.2314	
Total	1105.4971	55		

SS - Sum of Squares,

DF - Degrees of Freedom,

MS - Mean Squares,

F-value -- variation between mean values

Table 14 Tukey's HSD analysis of morphometric parameters of fish fed with control and Chitin mixed diets under normal and *Aeromonas hydrophila* infection

S. No.	Pairwise comparisons	Tukey's HSD	Q – value	p – value
1	T1 : T2	0.29	0.23	0.99841 ^{NS}
2	T1 : T3	0.24	0.20	0.99902 ^{NS}
3	T1 : T4	0.10	0.08	0.99993 ^{NS}
4	T2 : T3	0.04	0.03	0.99999 ^{NS}
5	T2 : T4	0.39	0.31	0.99612 ^{NS}
6	T3 : T4	0.34	0.28	0.99726 ^{NS}

T1 – Healthy fish fed with control diet,

T2 - *Aeromonas* infected fish fed with control diet,

T3 – Healthy fish fed with Chitin mixed diet,

T4 – *Aeromonas* infected fish fed with Chitin mixed diet,

Q-value - studentized range statistic value,

p < 0.05 - Significant

p-value* = Significant, p-value** = highly significant, p-value*** = extremely significant,

p-value^{NS} = Not Significant

Table 15 Comparative morphometric parameters of fish fed with control and Chitosan mixed diets under normal and *Aeromonas hydrophila* infection

S. No.	Morphometric parameter	Control feed		Chitosan feed	
		Healthy fish (T1)	<i>Aeromonas</i> infected fish (T2)	Healthy fish (T3)	<i>Aeromonas</i> infected fish (T4)
1	BL (cm)	1.4	1.3	1.9	1.5
2	BW (cm)	0.7	0.4	0.4	0.3
3	W (g)	18.7	15.6	28.1	22.8
4	HL (cm)	0.5	0.6	0.4	0.2
5	HD (cm)	0.5	0.5	0.3	0.2
6	ED (cm)	0.2	0.2	0.1	0.1
7	PD (cm)	0.1	0.1	0.2	0.1
8	CPL (cm)	0.7	0.5	0.3	0.4
9	CPW (cm)	0.2	0.3	0.1	0.3
10	CFL (cm)	0.4	0.3	0.6	0.4
11	DFL (cm)	0.5	0.4	0.5	0.3
12	PcFL (cm)	0.5	0.4	0.5	0.2
13	PIFL (cm)	0.4	0.4	0.4	0.5
14	AFL (cm)	0.4	0.2	0.4	0.3

Table 16 One-way ANOVA analysis of morphometric parameters of fish fed with control and Chitosan mixed diets under normal and *Aeromonas hydrophila* infection

Source	SS	DF	MS	F – value
Between groups (Treatment)	6.3621	3	2.1207	0.06473
Within groups	1703.72	52	32.7638	
Total	1710.0821	55		

SS - Sum of Squares,

DF - Degrees of Freedom,

MS - Mean Squares,

F-value -- variation between mean values

Table 17 Tukey's HSD analysis of morphometric parameters of fish fed with control and Chitosan mixed diets under normal and *Aeromonas hydrophila* infection

S. No.	Pairwise comparison	Tukey's HSD	Q – value	p – value
1	T1 : T2	0.29	0.19	0.99917 ^{NS}
2	T1 : T3	0.64	0.42	0.99077 ^{NS}
3	T1 : T4	0.17	0.11	0.99982 ^{NS}
4	T2 : T3	0.93	0.61	0.97320 ^{NS}
5	T2 : T4	0.46	0.30	0.99602 ^{NS}
6	T3 : T4	0.47	0.31	0.99630 ^{NS}

T1 – Healthy fish fed with control diet

T2 - *Aeromonas* infected fish fed with control diet

T3 – Healthy fish fed with Chitosan mixed diet

T4 – *Aeromonas* infected fish fed with Chitosan mixed diet

Q-value - studentized range statistic value

$p < 0.05$ - Significant

p -value* = Significant, p -value** = highly significant, p -value*** = extremely significant,

p -value^{NS} = Not Significant

Table 18 Comparative morphometric parameters of fish fed with control and Levamisole mixed diets under normal and *Aeromonas hydrophila* infection

S. No.	Morphometric parameter	Control feed		Chitosan feed	
		Healthy fish (T1)	<i>Aeromonas</i> infected fish (T2)	Healthy fish (T3)	<i>Aeromonas</i> infected (T4)
1	BL (cm)	1.4	1.3	1.8	1.5
2	BW (cm)	0.7	0.4	0.6	0.4
3	W (g)	18.7	15.6	30.8	26
4	HL (cm)	0.5	0.6	0.6	0.4
5	HD (cm)	0.5	0.5	0.7	0.4
6	ED (cm)	0.2	0.2	0.1	0.1
7	PD (cm)	0.1	0.1	0.1	0.1
8	CPL (cm)	0.7	0.5	0.4	0.3
9	CPW (cm)	0.2	0.3	0.4	0.4
10	CFL (cm)	0.4	0.3	0.5	0.4
11	DFL (cm)	0.5	0.4	0.4	0.5
12	PcFL (cm)	0.5	0.4	0.5	0.4
13	PIFL (cm)	0.4	0.4	0.5	0.5
14	AFL (cm)	0.4	0.2	0.5	0.2

Table 19 One-way ANOVA analysis of morphometric parameters of fish fed with control and Levamisole mixed diets under normal and *Aeromonas hydrophila* infection

Control diet/ Levamisole mixed diet	SS	DF	MS	F – value
Between groups	11.5177	3	3.8392	0.10062
Within groups	1984.0807	52	38.1554	
Total	1995.5984	55		

SS - Sum of Squares,

DF - Degrees of Freedom,

MS - Mean Squares,

F-value -- variation between mean values

Table 20 Tukey's HSD analysis of morphometric parameters of fish fed with control and Levamisole mixed diets under normal and *Aeromonas* infection

S. No.	Pairwise comparison	Tukey's HSD	Q – value	p – value
1	T1 : T2	0.29	0.17	0.99934 ^{NS}
2	T1 : T3	0.91	0.55	0.97986 ^{NS}
3	T1 : T4	0.46	0.28	0.99731 ^{NS}
4	T2 : T3	1.19	0.72	0.95613 ^{NS}
5	T2 : T4	0.74	0.45	0.98873 ^{NS}
6	T3 : T4	0.45	0.27	0.99743 ^{NS}

T1 – Healthy fish fed with control diet

T2 - *Aeromonas* infected fish fed with control diet

T3 – Healthy fish fed with Levamisole mixed diet

T4 – *Aeromonas* infected fish fed with Levamisole mixed diet

Q-value - studentized range statistic value

$p < 0.05$ - Significant

p -value* = Significant, p -value** = highly significant, p -value*** = extremely significant,

p -value^{NS} = Not Significant

Though there is no significant change between the groups, finally the vitamin C and vitamin E have shown the improvement in growth performance and morphometric characteristics of the experimental fish.

3.3 Haemoglobin

The Table 21, shows the mean haemoglobin values of healthy and *Aeromonas*-infected *Catla catla* fish that were treated with different immunostimulant mixed feeds such as vitamin C, vitamin E, chitin, chitosan, and levamisole as well as the control feed. Based on these data, it was shown that the average haemoglobin values in all test groups varied significantly. The average haemoglobin values in healthy fish treated with different immunostimulant-supplemented feeds ranged from 6.86 ± 0.37 to 14.61 ± 0.35 gm/dL. In *Aeromonas* infected fish, haemoglobin levels with different immunostimulant mixed feeds typically vary from 5.99 ± 0.66 to 13.04 ± 0.19 gm/dL.

Table 21 Haemoglobin content in the fish fed with different immunostimulant mixed feeds under normal and *Aeromonas hydrophila* infected conditions

S. No.	Experimental feed	Mean Haemoglobin (g/dL)		F – value	p – value
		Healthy fish	<i>Aeromonas</i> infected fish		
1	Control feed	6.86 ± 0.37	5.99 ± 0.66	3.92	0.118 ^{NS}
2	Vitamin C mixed feed	13.61 ± 0.35	12.81 ± 0.17	11.03	0.023*
3	Vitamin E mixed feed	14.61 ± 0.35	13.04 ± 0.19	48.28	0.022*
4	Chitin mixed feed	9.61 ± 0.21	7.84 ± 0.59	13.21	0.022*
5	Chitosan mixed feed	9.79 ± 0.49	8.83 ± 0.33	7.79	0.045*
6	Levamisole mixed feed	12.65 ± 0.22	11.77 ± 0.26	20.01	0.011*

p -value* = Significant, p -value** = highly significant, p -value*** = extremely significant, p -value^{NS} = Not Significant

The mean haemoglobin levels in healthy fish fed with immunostimulant mixed feed such as control, vitamin C, vitamin E, chitin, chitosan, and levamisole were found to be 6.86 ± 0.37 , 13.61 ± 0.4 , 14.61 ± 0.35 , 9.16 ± 0.21 , 9.79 ± 0.49 , and 12.65 ± 0.22 gm/dL respectively. As well as, the mean haemoglobin levels in *Aeromonas* infected fish fed with immunostimulant mixed feed such as control, vitamin C, vitamin E, chitin, chitosan, and levamisole were found to be 5.99 ± 0.66 , 12.81 ± 0.17 , 13.04 ± 0.19 , 7.84 ± 0.59 , 8.83 ± 0.33 , and 11.77 ± 0.26 g/dL respectively.

The quantity of haemoglobin significantly varies between healthy and *Aeromonas* infected fish from all the treatment groups. The p -values for the haemoglobin content of the healthy and *Aeromonas* infected fish in the treatment groups of control, vitamin C, vitamin E, chitin, chitosan, and levamisole were measured as 0.118, 0.023, 0.002, 0.022, 0.045, and 0.011 respectively. These results demonstrated that the tested immunostimulants significantly increased haemoglobin content in both healthy and *Aeromonas* infected fish when compared to the control.

3.4 Total RBC count

The mean total RBC count in the fish fed with different immunostimulant mixed feeds under normal and *Aeromonas* infected conditions is shown in Table 22. These results demonstrated significant variation in the mean values of the total RBC count across all test groups. The average total RBC count in healthy fish treated with different immunostimulant-supplemented feeds ranged from 1.24 ± 0.08 to $2.85 \pm 2.09 \times 10^6/\text{mm}^3$. In *Aeromonas* infected fish, total RBC count with different immunostimulant mixed feeds typically varies from 1.16 ± 0.05 to $2.25 \pm 0.08 \times 10^6/\text{mm}^3$.

Table 22 Total RBC count in the fish fed with different immunostimulant mixed feeds under normal and *Aeromonas hydrophila* infected conditions

S. No.	Experimental feed	Mean Total RBC count ($\times 10^6/\text{mm}^3$)		F – value	p – value
		Healthy fish	<i>Aeromonas</i> infected fish		
1	Control feed	1.24 ± 0.08	1.16 ± 0.05	2.41	0.196 ^{NS}
2	Vitamin C mixed feed	2.59 ± 0.05	2.05 ± 0.07	121.13	0.0004***
3	Vitamin E mixed feed	2.85 ± 0.09	2.25 ± 0.08	73.64	0.001***
4	Chitin mixed feed	1.67 ± 0.06	1.26 ± 0.05	99.53	0.0006***
5	Chitosan mixed feed	1.77 ± 0.06	1.33 ± 0.04	109.49	0.0005***
6	Levamisole mixed feed	2.21 ± 0.03	1.78 ± 0.05	214.51	0.0001***

p-value* = Significant, p-value** = highly significant, p-value*** = extremely significant, p-value^{NS} = Not Significant

The mean total RBC count in healthy fish fed with immunostimulant mixed feed such as control, vitamin C, vitamin E, chitin, chitosan, and levamisole was found to be 1.24 ± 0.08 , 2.59 ± 0.05 , 2.85 ± 0.09 , 1.67 ± 0.06 , 1.77 ± 0.06 , and $2.21 \pm 0.03 \times 10^6/\text{mm}^3$ respectively. As well as, the mean total RBC count in *Aeromonas* infected fish fed with immunostimulant mixed feed such as control, vitamin C, vitamin E, chitin, chitosan, and levamisole was found to be 1.16 ± 0.05 , 2.05 ± 0.07 , 2.25 ± 0.08 , 1.26 ± 0.05 , 1.33 ± 0.04 , and $1.78 \pm 0.05 \times 10^6/\text{mm}^3$ respectively.

The total RBC count significantly varies between healthy and *Aeromonas* infected fish from all the treatment groups. The p - values for the total RBC count between healthy and *Aeromonas* infected fish in the treatment groups of control, vitamin C, vitamin E, chitin, chitosan, and levamisole were measured as 0.196, 0.0004, 0.001, 0.0006, 0.0005, and 0.0001 respectively. These results demonstrated that the tested immunostimulants significantly increased the total RBC count in both healthy and *Aeromonas* infected fish when compared to the control control-fed group.

3.5 Total WBC count

The mean total WBC count in the fish fed with different immunostimulant mixed feeds under normal and *Aeromonas* infected conditions is shown in Table 23. From these results, it was observed that the mean WBC count in all test groups varied significantly. The average total WBC count in healthy fish treated with different immunostimulant-supplemented feeds ranged from 4077 ± 0.93 to 7535 ± 1.07 cells/ mm^3 . In *Aeromonas* infected fish, the total WBC count with different immunostimulant mixed feeds typically ranges from 4342 ± 0.87 to 7757 ± 1.06 cells/ mm^3 .

Table 23 Total WBC count in the fish fed with different immunostimulant mixed feeds under normal and *Aeromonas hydrophila* infected conditions

S. No.	Experimental feed	Mean Total WBC count (cells/ mm^3)		F- value	p – value
		Healthy fish	<i>Aeromonas</i> infected fish		
1	Control feed	4077 ± 0.93	4342 ± 0.87	12.94	0.0228*
2	Vitamin C mixed feed	6855 ± 0.86	7130 ± 0.43	17.43	0.134 ^{NS}
3	Vitamin E mixed feed	7535 ± 1.07	7757 ± 1.06	6.5	0.063 ^{NS}
4	Chitin mixed feed	6613 ± 0.74	6849 ± 1.12	9.1	0.034*
5	Chitosan mixed feed	6322 ± 0.84	6435 ± 0.92	2.41	0.195 ^{NS}
6	Levamisole mixed feed	7248 ± 0.82	7373 ± 0.95	2.97	0.156 ^{NS}

p-value* = Significant, p-value** = highly significant, p-value*** = extremely significant, p-value^{NS} = Not Significant

The mean total WBC count in healthy fish fed with immunostimulant mixed feed such as control, vitamin C, vitamin E, chitin, chitosan, and levamisole was found to be 4077 ± 0.93 , 6855 ± 0.861 , 7535 ± 1.07 , 6613 ± 0.74 , 6322 ± 0.84 , and 7248 ± 0.82 cells/mm³ respectively. As well as, the mean total WBC count in *Aeromonas* infected fish fed with immunostimulant mixed feed such as control, vitamin C, vitamin E, chitin, chitosan, and levamisole were found to be 4342 ± 0.87 , 7130 ± 0.43 , 7757 ± 1.06 , 6849 ± 1.12 , 6435 ± 0.92 , and $7373 \pm 0.95 \times 10^3/\text{mm}^3$ respectively.

The total WBC count significantly varies between healthy and *Aeromonas* infected fish from all the treatment groups. The *p* - values for the total WBC count content between healthy and *Aeromonas* infected fish in the treatment groups of control, vitamin C, vitamin E, chitin, chitosan, and levamisole were measured as 0.0228, 0.134, 0.063, 0.034, 0.195, and 0.156 respectively. These results demonstrated that the chitin mixed feed significantly increased the total WBC count content in both healthy and *Aeromonas* infected fish when compared to the control control-fed group.

3.6 Differential leucocyte count

Differential blood counts of the fish fed with different immunostimulant mixed feeds under normal and *Aeromonas* infected conditions were performed by counting the blood cells including eosinophils, basophils, neutrophils, lymphocytes, and monocytes.

3.6.1 Eosinophils

The Table 24 shows the mean eosinophil count of healthy and *Aeromonas* infected *Catla catla* fish that were treated with different immunostimulant mixed feeds. According to these results, it was observed that the average eosinophil count in all test groups varied significantly. The average eosinophil count in healthy fish treated with different immunostimulant-supplemented feeds ranged from 357 ± 53.07 to 475 ± 45.76 cells/mm³. In *Aeromonas* infected fish, eosinophil count with different immunostimulant mixed feeds typically ranges from 369 ± 50.21 to 543.66 ± 52.51 cells/mm³.

Table 24 Eosinophil count in the fish fed with different immunostimulant mixed feeds under normal and *Aeromonas hydrophila* infected conditions

S. No.	Experimental feed	Eosinophils (cells/mm ³)		F- value	p- value
		Healthy fish	<i>Aeromonas</i> infected fish		
1	Control feed	475 ± 45.76	543.66 ± 52.51	2.86	0.166 ^{NS}
2	Vitamin C mixed feed	412 ± 49.31	408 ± 44.65	0.01	0.925 ^{NS}
3	Vitamin E mixed feed	357 ± 53.07	369 ± 50.21	0.09	0.779 ^{NS}
4	Chitin mixed feed	417 ± 53.02	454 ± 52.003	0.77	0.421 ^{NS}
5	Chitosan mixed feed	430 ± 47.12	466 ± 46.36	0.91	0.394 ^{NS}
6	Levamisole mixed feed	358 ± 41.06	393 ± 67.002	0.61	0.47 ^{NS}

p-value* = Significant, *p*-value** = highly significant, *p*-value*** = extremely significant, *p*-value^{NS} = Not Significant

The mean eosinophil count in healthy fish fed with immunostimulant mixed feeds such as control, vitamin C, vitamin E, chitin, chitosan, and levamisole was found to be 475 ± 45.76 , 412 ± 49.31 , 357 ± 53.07 , 417 ± 53.02 , 430 ± 47.12 , and 358 ± 41.06 cells/mm³ respectively. As well as, the mean eosinophil count in *Aeromonas* infected fish fed with immunostimulant mixed feed such as control, vitamin C, vitamin E, chitin, chitosan, and levamisole was found to be 543.66 ± 52.51 , 408 ± 44.65 , 369 ± 50.21 , 454 ± 52.003 , 466 ± 46.36 , and 393 ± 67.002 cells/mm³ respectively. The number of eosinophils significantly varies between healthy and *Aeromonas* infected fish from all the treatment groups.

The *p* - values for the eosinophil count healthy and *Aeromonas* infected fish in the treatment groups of control, vitamin C, vitamin E, chitin, chitosan, and levamisole were measured as 0.166, 0.925, 0.779, 0.421, 0.394, and 0.47 respectively. These results demonstrated that the tested immunostimulants decreased eosinophil count but not significantly in both healthy and *Aeromonas* infected fish when compared to the control control-fed group.

3.6.2 Basophils

The basophil counts in the fish fed with different immunostimulant mixed feeds under normal and *Aeromonas* infected conditions are shown in Table 25. Based on these findings, it was determined that there was no statistically significant variation in the average basophil counts across the groups being tested. The average basophil counts in healthy fish treated with different immunostimulants mixed feed ranged from 38 ± 9 to 48 ± 9 cells/mm³. In *Aeromonas* infected fish, basophil counts with different immunostimulant mixed feeds typically ranged from 34 ± 6.5 to 42.6 ± 6.02 cells/mm³.

The mean basophil counts in healthy fish fed with immunostimulant mixed feed such as control, vitamin C, vitamin E, chitin, chitosan, and levamisole was found to be 38 ± 9 , 44.3 ± 8.50 , 48 ± 9 , 38 ± 9.53 , 43.3 ± 11.06 , and 46.33 ± 8.50 cells/mm³ respectively. As well as, the mean basophil counts in *Aeromonas* infected fish fed with immunostimulant mixed feed such as control, vitamin C, vitamin E, chitin, chitosan, and levamisole was found to be 34 ± 6.5 , 39 ± 8 , 42.66 ± 6.02 , 36.33 ± 8.50 , 39 ± 8.54 , and 42.66 ± 11.5 cells/mm³ respectively.

Table 25 Basophils count in the fish fed with different immunostimulant mixed feeds under normal and *Aeromonas hydrophila* infected conditions

S. No.	Experimental feed	Basophils (cells/mm ³)		F- value	p- value
		Healthy fish	<i>Aeromonas</i> Infected fish		
1	Control feed	38.0 ± 9	34.0 ± 6.5	0.33	0.596 ^{NS}
2	Vitamin C mixed feed	44.3 ± 8.50	39.0 ± 8	0.63	0.47 ^{NS}
3	Vitamin E mixed feed	48.0 ± 9	42.66 ± 6.02	0.73	0.441 ^{NS}
4	Chitin mixed feed	38.0 ± 9.53	36.33 ± 8.50	0.05	0.834 ^{NS}
5	Chitosan mixed feed	43.3 ± 11.06	39.0 ± 8.54	0.29	0.618 ^{NS}
6	Levamisole mixed feed	46.33 ± 8.50	42.66 ± 11.5	0.2	0.677 ^{NS}

p-value* = Significant, p-value** = highly significant, p-value*** = extremely significant, p-value^{NS} = Not Significant

The number of basophils insignificantly varies between healthy and *Aeromonas* infected fish from all the treatment groups. The p - values for the basophil counts between healthy and *Aeromonas* infected fish in the treatment groups of control, vitamin C, vitamin E, chitin, chitosan, and levamisole were measured as 0.596, 0.471, 0.441, 0.834, 0.618, and 0.677 respectively. These results demonstrated that the tested immunostimulants don't exhibit a significant impact on the basophil count in both healthy and *Aeromonas* infected fish when compared to the control control-fed group.

3.6.3 Neutrophils

The Table 26 shows the mean neutrophil counts of healthy and *Aeromonas* infected *Catla catla* fish that were fed with different immunostimulant-supplemented feeds. From these results, it was shown that the average neutrophils count in all test groups varied significantly. The mean neutrophil counts in healthy fish treated with different immunostimulants mixed feed ranged from 2650 ± 0.525 to 3340 ± 0.49 cells/mm³. Whereas, in *Aeromonas* infected fish, neutrophil counts with different immunostimulant mixed feeds ranged between 5060 ± 0.89 and 7570 ± 0.62 cells/mm³.

Table 26 Neutrophil counts in the fish fed with different immunostimulant mixed feeds under normal and *Aeromonas hydrophila* infected conditions.

S. No.	Experimental feed	Neutrophils (cells/mm ³)		F- value	p- value
		Healthy fish	<i>Aeromonas</i> infected fish		
1	Control feed	2650 ± 0.525	5060 ± 0.89	8.46	0.043*
2	Vitamin C mixed feed	2806 ± 0.62	6890 ± 0.77	51.11	0.0020**
3	Vitamin E mixed feed	3340 ± 0.49	7570 ± 0.62	110.1	0.0005***
4	Chitin mixed feed	3150 ± 0.625	5640 ± 0.525	28.04	0.0061**
5	Chitosan mixed feed	2860 ± 0.576	6206 ± 0.765	36.6	0.0037**
6	Levamisole mixed feed	2910 ± 0.672	7100 ± 1.030	37.79	0.0041**

p-value* = Significant, p-value** = highly significant, p-value*** = extremely significant, p-value^{NS} = Not Significant

The mean neutrophil counts in healthy fish fed with immunostimulant mixed feed such as control, vitamin C, vitamin E, chitin, chitosan, and levamisole were found to be 2650 ± 0.525 , 2806 ± 0.62 , 3340 ± 0.49 , 3150 ± 0.625 , 2860 ± 0.576 , and 2910 ± 0.672 cells/mm³ respectively. As well as, the mean neutrophil number in *Aeromonas* infected fish fed with immunostimulant mixed feed such as control, vitamin C, vitamin E, chitin, chitosan, and levamisole were found to be 5060 ± 0.89 , 6890 ± 0.77 , 7570 ± 0.62 , 5640 ± 0.525 , 6206 ± 0.765 , and 7100 ± 1.030 cells/mm³ respectively.

The number of neutrophils significantly varies between healthy and *Aeromonas* infected fish from all the treatment groups. The *p* - values of the neutrophil counts between healthy and *Aeromonas* infected fish in the treatment groups of control, vitamin C, vitamin E, chitin, chitosan, and levamisole were measured as 0.043, 0.0020, 0.00046, 0.0061, 0.0037, and 0.0041 respectively. These results demonstrated that the tested immunostimulants significantly increased the neutrophils content in both healthy and *Aeromonas* infected fish when compared to the control.

3.6.4 Lymphocytes

The Table 27, presents the mean lymphocyte counts of healthy and *Aeromonas*-infected *Catla catla* fish that were given various immunostimulant-mixed feeds, along with a control feed. Based on these findings, it was noted that the average lymphocyte counts in all the test groups, with the exception of the chitosan mixed diet group, showed significant variation. The mean lymphocyte counts in healthy fish that treated with different immunostimulant mixed feeds ranged from 2531 ± 0.79 to 3072 ± 0.99 cells/mm³. Whereas, in *Aeromonas*-infected fish, the lymphocyte counts varied between 2290 ± 1.095 and 2740 ± 1.270 cells/mm³ with the use of different immunostimulant mixed feeds.

Table 27 Lymphocyte counts in the fish fed with different immunostimulant mixed feeds under normal and *Aeromonas hydrophila* infected conditions

S. No.	Experimental feed	Lymphocytes (cells/mm ³)		F - value	<i>p</i> - value
		Healthy fish	<i>Aeromonas</i> infected fish		
1	Control feed	2531 ± 0.79	2229 ± 1.095	14.92	0.018*
2	Vitamin C mixed feed	2745 ± 0.87	2459 ± 1.046	13.3	0.021*
3	Vitamin E mixed feed	3072 ± 0.99	2740 ± 1.270	12.7	0.023*
4	Chitin mixed feed	2629 ± 0.92	2353 ± 1.084	11.28	0.028*
5	Chitosan mixed feed	2544 ± 1.17	2360 ± 1.06	4.03	0.115 ^{NS}
6	Levamisole mixed feed	2850 ± 0.89	2553 ± 1.22	11.6	0.027*

p-value* = Significant, *p*-value** = highly significant, *p*-value*** = extremely significant, *p*-value^{NS} = Not Significant

The average lymphocyte counts in healthy fish that were fed with different types of immunostimulant mixed feeds including control, vitamin C, vitamin E, chitin, chitosan, and levamisole, were measured to be 2531 ± 0.79 , 2531 ± 0.79 , 3072 ± 0.99 , 2629 ± 0.92 , 2544 ± 1.17 , and 2850 ± 0.89 cells/mm³ respectively. As well as, the mean counts of lymphocytes in *Aeromonas* infected fish that were fed with different types of immunostimulant mixed feeds including control, vitamin C, vitamin E, chitin, chitosan, and levamisole were reported to be 2229 ± 1.095 , 2459 ± 1.046 , 2740 ± 1.270 , 2353 ± 1.084 , 2360 ± 1.06 , and 2553 ± 1.22 cells/mm³ respectively.

The number of lymphocytes shows considerable variation between healthy fish and those infected with *Aeromonas* across all treatment groups. The *p* - values for the lymphocyte counts between healthy and *Aeromonas* infected fish in the treatment groups of control, vitamin C, vitamin E, chitin, chitosan, and levamisole were 0.018, 0.021, 0.023, 0.028, 0.115, and 0.027 respectively. These results indicated that the tested immunostimulants had a substantial effect on increasing significantly the number of lymphocytes in both healthy and *Aeromonas* infected fish when compared to the control control-fed group.

3.6.5 Monocytes

The average monocyte counts of healthy and *Aeromonas* infected *Catla catla* fish treated with various immunostimulant mixed diets are shown in Table 28. According to these data, it was demonstrated that the average monocyte count differed considerably across all test groups. The mean monocyte counts in healthy fish treated with various immunostimulant mixed feeds ranged from 178 ± 0.64 to 370 ± 0.51 cells/mm³. Whereas, in *Aeromonas*-infected fish, the monocyte counts varied between 462 ± 0.56 and 685 ± 0.77 cells/mm³ with the use of different immunostimulant mixed feeds.

Table 28 Monocyte counts in the fish fed with different immunostimulant mixed feeds under normal and *Aeromonas hydrophila* infected conditions

S. No.	Experimental feed	Monocytes (cells/mm ³)		F- value	p-value
		Healthy fish	<i>Aeromonas</i> infected fish		
1	Control feed	178 ± 0.64	462 ± 0.56	32.53	0.0046**
2	Vitamin C mixed feed	287 ± 0.62	654 ± 0.61	52.88	0.0019**
3	Vitamin E mixed feed	370 ± 0.51	685 ± 0.77	34.5	0.0041**
4	Chitin mixed feed	247 ± 0.4007	547 ± 0.625	48.75	0.0022**
5	Chitosan mixed feed	261 ± 0.45	556 ± 0.62	41.14	0.0030**
6	Levamisole mixed feed	274 ± 0.66	492 ± 0.72	14.85	0.018*

p-value* = Significant, p-value** = highly significant, p-value*** = extremely significant, p-value^{NS} = Not Significant

The mean monocyte counts in healthy fish fed with immunostimulant mixed feed such as control, vitamin C, vitamin E, chitin, chitosan, and levamisole were found to be 178 ± 0.64 , 287 ± 0.62 , 370 ± 0.51 , 247 ± 0.4007 , 261 ± 0.45 , and 274 ± 0.66 cells/mm³ respectively. As well as, the mean monocyte counts in *Aeromonas* infected fish fed with immunostimulant mixed feed such as control, vitamin C, vitamin E, chitin, chitosan, and levamisole were found to be 462 ± 0.56 , 654 ± 0.61 , 685 ± 0.77 , 547 ± 0.625 , 556 ± 0.62 , and 492 ± 0.72 cells/mm³ respectively.

The monocyte counts show significant variation between healthy fish and those infected with *Aeromonas* across all treatment groups. The *p* - values for the monocyte counts between healthy and *Aeromonas* infected fish were examined in the following treatment groups: control, vitamin C, vitamin E, chitin, chitosan, and levamisole. The *p* - values for these groups were 0.0046, 0.0019, 0.0041, 0.0022, 0.0030, and 0.018, respectively. These results indicate that the tested immunostimulants significantly enhance the monocyte count in both healthy and *Aeromonas* infected fish, as compared to the control feed.

3.7 Erythrocyte sedimentation rate (ESR)

The Table 29 shows the mean ESR values of healthy and *Aeromonas* infected *Catla catla* fish which were treated with different immunostimulant-supplemented feeds. From these results, it was observed that there was no statistically significant variation in the average ESR across the groups being tested. The mean ESR values in healthy fish treated with different immunostimulant mixed feeds ranged from 0.27 ± 0.13 to 0.39 ± 0.17 cm/hour. Whereas, in *Aeromonas* infected fish, the ESR with different immunostimulant mixed feeds vary between 0.33 ± 0.11 and 0.45 ± 0.14 cm/hour.

Table 29 ESR in the fish fed with different immunostimulant mixed feeds under normal and *Aeromonas hydrophila* infected conditions

S. No.	Experimental feed	ESR (cm/hour)		F- value	p -value
		Healthy Fish	<i>Aeromonas</i> infected fish		
1	Control feed	0.39 ± 0.17	0.45 ± 0.14	0.28	0.624 ^{NS}
2	Vitamin C mixed feed	0.33 ± 0.12	0.33 ± 0.11	0.0	1.00 ^{NS}
3	Vitamin E mixed feed	0.27 ± 0.13	0.34 ± 0.15	0.42	0.552 ^{NS}
4	Chitin mixed feed	0.37 ± 0.14	0.43 ± 0.15	0.23	0.656 ^{NS}
5	Chitosan mixed feed	0.35 ± 0.14	0.39 ± 0.17	0.13	0.736 ^{NS}
6	Levamisole mixed feed	0.33 ± 0.13	0.33 ± 1.13	0.0	1.00 ^{NS}

p-value* = Significant, p-value** = highly significant, p-value*** = extremely significant, p-value^{NS} = Not Significant

The mean ESR values in healthy fish fed with immunostimulant mixed feed such as control, vitamin C, vitamin E, chitin, chitosan, and levamisole were found to be 0.39 ± 0.17 , 0.33 ± 0.12 , 0.27 ± 0.13 , 0.37 ± 0.14 , 0.35 ± 0.14 , and 0.33 ± 0.13 cm/hour respectively. As well as, the mean ESR in *Aeromonas* infected fish fed with immunostimulant mixed feeds such as control, vitamin C, vitamin E, chitin, chitosan, and levamisole were found to be 0.45 ± 0.14 , 0.33 ± 0.11 , 0.34 ± 0.15 , 0.43 ± 0.15 , 0.39 ± 0.17 , and 0.33 ± 1.13 cm/hour respectively.

In this study, the ESR insignificantly varies between healthy and *Aeromonas* infected fish from all the treatment groups. The *p* - values for the ESR between healthy and *Aeromonas* infected fish in the treatment groups of control, vitamin C, vitamin E, chitin, chitosan, and levamisole were measured as 0.624, 1.00, 0.552, 0.656, 0.736, and 1.00 respectively. These results demonstrated that the tested immunostimulants insignificantly increased the ESR in both healthy and *Aeromonas* infected fish when compared to the control control-fed group.

4 Discussion

Aquaculture offers a sustainable source of cost-effective and nutritious protein for humans to consume, thus enhancing human health. Regrettably, the occurrence of epidemics of diseases remains a substantial hindrance to the attainment of profitable production through technological improvement. Researchers in the aquaculture field are currently studying a number of biologically safe and environmentally acceptable substances that have the potential to regulate the immune system, enhance growth performance, and prevent diseases in fish (Mansour et al., 2021).

In addition, they serve the purpose of safeguarding aquaculture organisms against many environmental factors such as water pollution, low temperatures, and excessive population density (Refaey et al., 2022). Immunostimulants positively affect growth rate efficiency and enhance the sustainability of aquaculture (Yossa et al., 2018). Immunostimulants have significant value in maintaining fish diseases and can be beneficial in fish farming. Studies have demonstrated the immunostimulatory properties of glucan, chitin, lactoferrin, and levamisole in fish and crustaceans. Vitamin B and C, growth hormone, and prolactin are also known to have immunostimulatory effects.

Immunostimulants enhance the activity of natural killer cells, complement, lysozyme, and antibody reactions in fish. The stimulation of these immune activities is linked to the enhanced defence against infectious pathogens (Sakai, 1999). Hence, the present research aims to assess the potential impacts of different immunostimulants on the growth performance and morphometric parameters of the *Catla catla*. The growth performance of the fish in all groups of treatment was evaluated by measuring the total biomass gain (TBG), weight gain (WG), and feed conversion ratio (FCR).

The findings of this study showed that the inclusion of immunostimulant mixed feed resulted in a significant increase in overall biomass gain compared to control. Based on these findings, vitamin E has superior growth-promoting characteristics compared to the other immunostimulant diets. The research conducted by Aathi et al. (2013), supports the argument that adding chitosan mixed fish feed can enhance the proximate value of crude protein in Indian large carp, *Labeo rohita*. The observed outcomes can be attributed to the augmentation of digestive enzymes, which aid in enhancing nutrition and facilitating the absorption of nutrients in the intestines. Consequently, this leads to a rise in fish growth (Abdel-Tawwab et al., 2020).

The present study revealed a significant rise in the weight gain of fish that were fed with immunostimulant diet compared to the control group after a period of 30 days. The FCR (Feed Conversion Ratio) of all the groups that received treatment was greater than that of the control group. These results were in accordance with the observations made by Abdelkhalek et al. (2008) and Awad et al. (2013) who found that nourishing sea bream with *Nigella sativa*, or synthetic immunostimulants, over a period of 30 to 60 days led to a substantial rise in weight gain and the rate of survival.

The findings of this study correlate with the research conducted by Rozi et al. (2018) who showed that including chitosan in the diet resulted in a significant rise of 12.19 ± 3.45 g in the relative weight growth of *Oreochromis niloticus*. In another study, Sarker et al. (2023) found that the average weight gain of fish at the end of the experiment was 6.88 ± 0.046 , 8.97 ± 0.042 , 5.94 ± 0.036 , and 4.65 ± 0.051 in the treatment groups T1, T2, T3, and the

control group respectively. Payung et al. (2017) reported that the addition of ginger to diets at concentrations ranging from 1.0 to 8.5 g/kg resulted in growth promoting benefits in catla, Nile tilapia, and rohu accordingly. Similarly, Talpur et al. (2013) observed a notable increase in rate of development when rainbow trout were fed with ginger containing feed.

Setijaningsih et al. (2021) examined the impact of various immunostimulants on the growth rate of *Oreochromis niloticus*, and they found that treatment B resulted in the greatest size increase, with an increase in weight of 17.75 ± 0.48 g, followed by treatment C with a weight gain of 15.91 ± 0.71 g, treatment D with a weight gain of 11.92 ± 0.21 g, and treatment A with a weight gain of 11.97 ± 0.42 g accordingly. Purbomartono et al. (2021) found that the weight gain of African catfish nourished with a diet containing 7.5 g/kg of ginger (T3) and diets containing 25, 50, and 75 g/kg of turmeric were significantly different from the weight gain of fish nourished with the control diet. The magnitude of weight gain is greater than the magnitude of length increase. The inherent biology of the fish is one of the sources of this condition. Positive allometry refers to a growth pattern in which the rate of weight increase is greater than the rate of length increase (Dewi et al., 2017).

The feed conversion ratio (FCR) represents the proportion of feed required to generate 1 kg of cultured fish flesh. A lower feed conversion ratio indicates a more efficient transformation of feed into meat, whereas a higher ratio suggests a less efficient conversion. The current findings indicate that there is substantial variation in the feed conversion ratio across various immunostimulant diets in *Catla catla* fish. The feed conversion ratio is commonly employed to assess the quality of the feed provided for fish development (Saputra et al., 2018). According to Defrizal and Khalil (2015) the FCR variability is affected by factors such as feed quality, feed quantity, species type, and fish size. Mudjiman (2011) stated that the FCR in fish typically ranges between 1.5 and 8. According to this statement, it can be concluded that the FCR of most treatments in the current study is favourable as it is within an acceptable range.

Furthermore, the present findings are consistent with the results reported by Khattabya et al. (2004) who showed that the Biogen® diet led to an enhancement of consumption of feed, FCR, protein efficiency ratio, and other metabolic parameters such as crude protein, solvent extract, ash, and moisture in fish. Novian et al. (2023) reported that following a treatment period of 42 days, the feed conversion ratio ranged from 1.6 to 2.3. The treatment with the highest FCR value is A, whereas the treatment with the lowest FCR value is C. The symbiotic supplementation of 2.5 g/kg and 5 g/kg resulted in a ratio of 2.3 ± 0.3 . Udo et al. (2018) stated that the inclusion of chitosan in the *Clarias gariepinus* meal at the ideal dosage can enhance the survival rate of fish. The study conducted by Sarker et al. (2023) observed that the average FCR of fish treatments T1, T2, T3, and the control were 1.35 ± 0.009 , 1.10 ± 0.029 , 1.56 ± 0.010 , and 1.56 ± 0.022 respectively. In another study, Hassan et al. (2018) found that the FCR was considerably improved in the treatment groups supplemented with turmeric, rosemary, and thyme compared to the control group. Purbomartono et al. (2021) found that the FCR of African catfish fed with ginger and turmeric diets was significantly different from the control group.

In addition, the FCR of the turmeric diet and the ginger diet at a dosage of 75 g/kg exhibited the most optimal results. The results of this study showed that these treatments influenced haematological responses associated with immune status in *Catla catla*, thereby effectively enhancing metrics related to growth promotion. The fish fed with vitamin E mixed feed had the most favourable outcomes, as evident by notable enhancements in the final body weight, weight gain percentage, and FCR, in comparison to the fish that were fed with a control diet.

In the present study, it was found that a 30-day dietary supplementation of immunostimulants substantially influences the body length, feed intake, feed conversion, and biomass in *Catla catla*. Vitamins are vital chemical substances that play a crucial role in sustaining life, as they are necessary in small quantities for regular growth, reproduction, and overall health (Gouda et al., 2020). L-ascorbic acid phosphate is a stable variant of vitamin C that can be used in feed as a supplement and it plays several important functions including immunostimulant and free radical scavenger.

Multiple research studies such as Abdel Rahman et al. (2020) and Liua et al. (2018) have found that adding vitamin C to fish feed can improve both developmental and immunological responses. Ibrahim et al. (2020) found that the final body weight, total weight gain, daily weight gain, and specific growth rate in Nile tilapia showed an exponential rise in the three experimental groups that received vitamin C supplementation when compared with the control group that did not receive vitamin C supplementation. However, the differences in feed intake, FCR, and PER across the experimental groups were not statistically significant.

Similarly, Xu et al. (2022) found that maintaining an appropriate quantity of vitamin C in the diet significantly enhanced the growth performance of Coho salmon post-smolts. But the amounts of dietary vitamin C didn't have any major effect on the ability to survive Coho salmon post-smolts. In addition, the specific growth rate of fish enhanced but the FCR dropped when they were fed with varying concentrations of vitamin C supplemented diets. The variables HIS, VSI, and CF did not have any *substantial impact when exposed to varying doses of vitamin C*. Padua et al. (2015) demonstrated that feeding *O. niloticus* with Aquate FishTM® supplemented diets had a greater survival rate; however, there was no difference in weight gain or specific growth rate. In contrast to the current findings, previous research conducted by Simic and Karel (1980) and Gao et al. (2012) found that the consumption of dietary vitamin C led to a reduction in feed intake and slower growth in fish. This was attributed to the alteration of the diet flavour and odour resulting from the deterioration of dietary lipids.

The vitamin C requirements of fish are influenced by several factors such as age, size, species, and conditions of rearing (Dawood and Koshio, 2016). Research on the vitamin C supplemented feed specifications for several fish species indicates that fish require 20~50 mg/kg of feed to meet their demands (NRC, 2011). In the current study, the addition of vitamin C to the diet of *Catla catla* resulted in a considerable enhancement in their growth performance compared to the control group. This may be caused by the vitamin C induced overexpression of growth hormone levels, improved intestinal health, and improved fish gut absorptive surface (Abdel Rahman et al., 2018). Faramarzi (2012) stated that the greater rate of development and weight gain seen in Nile tilapia when given vitamin C supplements can be attributed to the acceleration of protein synthesis.

Vitamin E refers to a group of eight naturally occurring lipophilic substances. The most biologically active form of vitamin E is α -tocopherol, which has an important role in normal metabolic functioning and biological processes (Combs and McClung, 2017). Fish are unable to produce all the biologically functional kinds of vitamin E on their own and therefore need external sources in their diet to obtain it (Peng and Gatlin, 2009). Inadequate diets that lack vitamin E can result in stunted growth, poor production of RBC, muscular weakness, darker skin, exudative diathesis, skin depigmentation, liver fat deterioration, and mortality (NRC, 2011).

Multiple research studies including Lu et al. (2016) and Bae et al. (2013) have documented that the application of dietary vitamin E supplementation improves the growth performance of fish. The present study demonstrates that the addition of vitamin E to the diet for a period of 30 days leads to a substantial enhancement in body growth and biomass. The results of this study agree with the findings of Taveekijakarn et al. (1996) who observed that a certain dosage of vitamin E stimulated the growth of aquatic animals. Similarly, He et al. (2017) found that catfish fed with a vitamin E supplemented diet significantly increased growth parameters such as mean weight gain, daily mean growth, and PER compared to those fed with the control diet. Furthermore, the greatest results were observed in fish receiving a diet supplemented with 100 mg/kg of vitamin E.

Saheli et al. (2021) reported that raising the dietary vitamin E concentration had a significant positive effect on the weight gain rate, specific growth rate, and protein efficacy ratio in Caspian trout. The greatest values were observed in Caspian trout fed with a diet supplemented with 78.8 mg/kg of vitamin E. In contrast, the FCR showed a linear and exponential reduction as the vitamin E concentration increased. The lowest FCR value was observed in fish that were fed a diet supplemented with 78.8 mg/kg of vitamin E. Rohani et al. (2023) found that adding vitamin E to the fish diet significantly increased their growth indices including weight gain and specific growth rate, when compared to fish fed with a control diet. In the same manner, feed utilisation markers such as FCR and PER were considerably increased without any noticeable differences in the control group.

James et al. (2008) reported that vitamin E has a considerable impact on the eating, growth, reproduction, and leukocyte count of *C. auratus*. Furthermore, they noted that fish fed a diet containing 300 mg of vitamin E per kg exhibited superior growth, gonad weight, gonadosomatic index, egg production, egg size, and hatching rate. Mehrad et al. (2012) found a notable disparity in body weight increase among zebrafish that were fed with a supplemental diet containing 500 and 1000 mg/kg of vitamin E, in comparison with the other groups. Additionally, the fish that were fed with a diet containing 1000 mg/kg of vitamin E exhibited the highest BWG. According to Wassef et al. (2001) the inclusion of vitamin E in the diet of aquatic organisms inhibits the oxidative degradation of unsaturated fatty acids in their diets and tissues. This assists in maintaining a typical metabolism and leads to increased survival rate, weight gain, and feed efficiency in these animals. Furthermore, He et al. (2017) found that vitamin E had a substantial impact on the development of the gut in fish. It was achieved by strengthening the structure of the intestinal wrinkles and increasing the thickness of the mucosal membrane. Since, the digestive and absorptive abilities of fish were enhanced, leading to improved growth. The findings of this study demonstrate that incorporating vitamin E into the diet is crucial for enhancing the survival rate and promoting the growth of *Catla catla*.

Chitin is the second most commonly found polysaccharide, following cellulose, and it makes up a significant portion of the exoskeleton structure of crustaceans (Boric et al., 2020). Chitin and its derived compounds possess several applications attributed to their biocompatibility (Barikani et al., 2014), biodegradability (Zhu et al., 2019), antibacterial and antioxidative characteristics (Ahmad et al., 2020). Danulat (1987) reported that the gastrointestinal system of *Gadus morhua* can digest up to 90% of chitin. Additionally, it has chitinase activity throughout its gastrointestinal system. In addition, Fines and Holt (2010) discovered that young *Rachycentron canadum* possess chitinolytic activity in their gastrointestinal system.

In this study, the addition of chitin to the diet over a period of 30 days leads to an increase in overall body weight. These outcomes are clearly evident as Kono et al. (1987) found that adding chitin to the diet of different kinds of fish such as yellowtail, sea bream, and Japanese eel, resulted in an increased growth rate and improved feed effectiveness. Shiau and Yu (1999) reported that the growth performance and digestibility of diet ingredients in hybrid tilapia fingerlings decrease as the amount of additional chitin in their diet increases. Elserafy et al. (2021) observed that the addition of chitin to the base diet of Nile tilapia at concentrations of 2 and 5% did not exhibit a significant improvement in growth performance when compared to the control group.

Chitosan is a biopolymer composed of glucosamine and N-acetylglucosamine that is obtained from the chitin found in the exoskeletons of crustaceans like crabs, lobsters, prawns, and krill, as well as the cell walls of fungi (El-Naggar et al., 2021). The addition of chitosan has a beneficial impact on the growth of fish, and additionally, on the structure and function of the intestines and the process of nutrient digestion and absorption. Abdel-Ghany and Salem (2019) and Maqsood et al. (2010) found that adding 2% chitosan to the diet of common carp can decrease fish mortality and improve their ability to grow.

Similarly, Salam et al. (2021) found that the chitosan supplemented feed at a dose of 1 g/kg increased body weight gain at days 15, 30, 45, and 60 after the treatment in juvenile *Barbonymus gonionotus*. Zaki et al. (2015) observed that the mean percentage of feed body weight and specific growth rate per day were significantly greater in the three groups of *Dicentrarchus labrax* that received a diet supplemented with 1 g chitosan per kg diet. The findings of Zhao et al. (2022) have substantiated that chitosan has the ability to inhibit the digestion of Dp-Ca (peptide-calcium chelate) in the stomach. This, in turn, leads to an increased amount of calcium being introduced into the intestine, thereby facilitating the absorption of calcium.

Levamisole, a synthetic compound belonging to the phenylimidazolthiazole class, is currently widely employed in human as well as animal medicine as an anthelmintic drug (Janssen, 1976). Levamisole was originally used in fish to augment the innate immune response (Baba et al., 1993) and used as an adjuvant in combination with a vaccine (Jeney and Anderson, 1993). Maqsood et al. (2009) found that adding 250 mg of levamisole per kg of feed

considerably improved the specific growth rate of *Cyprinus caprio* compared to fish that were fed a control diet. They also observed that the levamisole supplementation enhanced the FCR.

Siwicki and Korwin-Kossakowski (1988) demonstrated that levamisole enhances the growth of *Cyprinus caprio* larvae while having no impact on their survival or developmental pace. Mulero et al. (1988) found that *Sparus aurata* fish that received levamisole exhibited greater size and weight at the end of the trial when compared to the control group. Alvarez Pellitero et al. (2006) reported that *Scophthalmus maximus* treated with the 500 mg/kg supplemented levamisole diet exhibited a greater specific growth rate compared to the control group. Similarly, Niki et al. (1991) demonstrated that levamisole has a substantial impact on the specific growth rate of fish, resulting in a significant rise when compared with the control group. Bhatnagar and Lamba (2016) reported a statistically significant increase in the growth and feed efficiency of *C. mrigala* after 60 days of ingesting a diet with varying concentrations of levamisole. The results of this study indicate that adding levamisole to the diet of *Catla catla* significantly improves their innate immune response, boosts their resistance to disease, reduces mortality, and promotes fish growth.

Haematology is the branch of science that focuses on the examination of the fundamental elements including blood cells, and the identification of anomalies in the normal functioning of these cells. Pathology intricately connects with haematology to assess the physical condition of fish, determining whether they are in a state of health or disease. The composition of the blood will undergo alterations, particularly if it becomes contaminated. Alterations in the fish's physiology are indicated by variations in the blood composition, including measurements of haematocrit levels, haemoglobin levels, and blood cell counts.

Esepeid et al. (1987) assert that alterations in the blood composition of fish can serve as reliable markers for both infection and stress-related circumstances. Hence, blood measures play a vital role in assessing the nutritional adequacy, level of substances, and potential harm to the circulatory system in aquatic animals (Dash et al., 2015). Immunostimulants are critical in enhancing aquatic organisms' growth productivity by increasing their ability for feed digestion, utilisation, and haematological, as well as Immunological responses (Ringo et al., 2012).

Therefore, the objective of this study was to investigate the impact of several immunostimulants on the haematological parameters of *Catla catla* in both normal and *Aeromonas* infected conditions. The blood parameters were measured by assessing the concentration of haemoglobin, total RBC count, total WBC count, differential count, and erythrocyte sedimentation ratio. Ramesh et al. (2015) found that the haematological parameters were enhanced during the experiment due to the influence of feed additives.

Haemoglobin consists of polypeptide chains, referred to as globins, each of which contains a prosthetic group called heme. Globins are present in all organisms and tissues, displaying a wide range of quaternary structures and multiple functions in addition to transporting and storing oxygen, as demonstrated by cytoglobins and neuroglobins (Fago et al., 2004). Haemoglobin, found in red blood cells, enable the solubility of large amounts of gas and carry it to the tissues. Within the tissues, oxygen acts as the final recipient of electrons produced by oxidative catabolic reactions (Giardina et al., 2004).

Haemoglobin primarily serves the purpose of transporting oxygen from the gas-exchange organs to the peripheral tissues. Depending on the partial pressure of the gas, it must have the capacity to both firmly bind oxygen and release it when needed (Perutz, 1978). Riggs (1976) observed that haemoglobin appears to adapt to the diverse metabolic requirements of animals and the consistent fluctuations in their environment. According to Landini et al. (2002) haemoglobin play a crucial role in fish adaptation by serving as a connection between the organism and its surroundings. Fish, in comparison to terrestrial species, encounter a highly fluctuating environment with frequent changes in oxygen levels, both in terms of time and space.

Multiple studies have documented alterations in haemoglobin levels in fish in response to varying environmental conditions. For instance, Bastiawan et al. (1995) documented that stress in fish resulted in alterations in various blood parameters such as haemoglobin, hematocrit, RBC and WBC count. There is a noticeable rise in haemoglobin

and hematocrit levels in Nile tilapia, *O. niloticus*, when they are fed with *Bacillus amyloliquefaciens* supplemented feed (Reda and Selim, 2015). Similarly, Kumar et al. (2008) reported an increase in WBC count in *Labeo rohita* when fed with *Bacillus subtilis* supplemented diets.

In the present study, the mean haemoglobin concentration in healthy fish fed with various immunostimulant supplemented diets varied between 6.86 ± 0.37 and 14.61 ± 0.35 gm/dL. The haemoglobin concentrations in fish infected with *Aeromonas* and fed with different immunostimulant mixed feeds ranged from 5.99 ± 0.66 to 13.04 ± 0.19 gm/dL. These results demonstrated that the fish fed with immunostimulant mixed feed exhibited significantly greater haemoglobin content in both healthy and *Aeromonas* infected fish when compared to fish fed with control feed.

Furthermore, the vitamin E mixed feeds induce greater haemoglobin content in both normal and *Aeromonas* infected conditions than the other immunostimulants. The current results are consistent with the findings of Wu et al. (2013) who reported that the haemoglobin percentage was elevated ($p > 0.05$) in the C0.5 group on days 28 and 42 and in the C1.0 group on days 14, 28, and 42 when compared to the control with the treatment of *Coriolus versicolor* polysaccharides in all gynogenetic crucian carp.

The present study found that healthy fish treated with immunostimulant mixed feeds had an average total RBC count ranging from 1.24 ± 0.08 to $2.85 \pm 2.09 \times 10^6/\text{mm}^3$. *Aeromonas* infected fish had a total RBC count ranging from 1.16 ± 0.05 to $2.25 \pm 0.08 \times 10^6/\text{mm}^3$ when fed immunostimulant mixed diets. The current findings showed that fish fed with immunostimulant mixed feed had considerably higher RBC counts in both healthy and *Aeromonas* infected fish as compared to fish given control feed.

Furthermore, vitamin E mixed feed significantly enhances the RBC count in both normal and *Aeromonas* infected fish. Ahmad (1982) reported that the reference fish had a mean RBC count of 2.2×10^6 cells/ mm^3 of blood. The current findings are consistent with those of Zhou et al. (2012) who found a considerably greater RBC number in *Rachycentron canadum* fed with vitamin-C supplemented feed. Similarly, Burrells et al. (2001) found enhanced RBC number in Atlantic salmon fed with a nucleotide supplemented diet; however, the difference was not statistically significant. These findings suggest that the ingredients of the experimental feed might have an influence on haematocrit levels.

In accordance with the present study, Rairakhwada et al. (2007) found that channel catfish fed with β -1,3/1,6 glucan had a higher RBC count. Muahiddah and Diamahesa (2023) found that prechallenged *Cyprinus carpio* had a maximum RBC count of $2.63 \pm 0.034 \times 10^6$ cells/ mm^3 when treated with 25 g of *Euphorbia hirta* leaf extract per Kg. Following infection with the pathogen, a maximal RBC count of $1.93 \pm 0.012 \times 10^6$ cells/ mm^3 was observed on the 5th day with 25 g of *E. hirta* leaf extract per Kg and 50, 20, 10, and 5 g leaf extracts.

In contrast to the current findings, Kumar et al. (2006) discovered that supplanting with dietary starch fails to improve RBC count or haemoglobin concentration. Furthermore, *Aeromonas* infected fish had significantly fewer RBC than control fish. Pulungan et al. (2022) stated that hemolysin produced by the *A. hydrophila* bacterium can cause red blood cell lysis.

The total white blood cell count provides insight into the immunological condition of the fish, as WBC are the primary constituents of the immune system. Leukocytes, often known as blood cells, are responsible for targeting and destroying infections by engulfing them through a process called phagocytosis. The application of immunostimulants in the diet resulted in a rise in the total number of leukocytes, indicating an enhancement of cellular defence mechanisms.

According to Zainun (2007) leukocytes are a kind of blood cells that serve as non-specific defences by localising and eliminating infections. The reduction in leukocyte count following an infection is due to the migration of active leukocytes from blood vessels into the diseased tissue. The study conducted by Nuryati et al. (2010) demonstrates the ability of fish to identify and remember various types of infections that invade their bodies.

Kresno (2001) stated that an elevated leukocyte count suggests that the fish's immune system is reacting to the existence of foreign substances in the body. An elevation in leukocyte count indicates the effectiveness of the fish's immune system in generating a cellular and immunological response as a stimulus for an immune reaction. Suprayudi et al. (2006) suggested that fish enhance their ability to survive by augmenting the quantity of leukocytes, which serve as defensive cells. Sudiono (2014) reported that administering immunostimulants orally leads to a rise in the total WBC count. This reaction suggests that immunostimulants encourage fish to enhance their immune system by raising the quantity of leukocytes, which are involved in non-specific defensive mechanisms.

In the present study, the mean total WBC count in healthy fish treated with different immunostimulant-supplemented feeds ranged from 4077 ± 0.93 to 7535 ± 1.07 cells/mm³. In *Aeromonas* infected fish, the total WBC count with different immunostimulant mixed feeds ranges from 4342 ± 0.87 to 7757 ± 1.06 cells/mm³. These results demonstrated that the activities of leukocytes have decreased against bacterial infection significantly, when they were fed with different immunostimulant mixed feeds in compared to simple infection, which signifies the immunostimulatory activity of immunostimulants.

In addition, the vitamin E mixed feeds considerably enhance the white blood cell count in both healthy and *Aeromonas*-infected fish compared to other tested immunostimulants. The current results evident with the findings of Anderson and Jeney (1992) who observed that the injection of QAC resulted in an increase in the total WBC counts. Similarly, Harikrishnan et al. (2003) observed that elevated WBC counts in *C. carpio* following administration of *Azardicha indica* extracts. Lin et al. (2011) found that koi fish given β -1,3-glucan had noticeable increased peripheral leukocyte counts. Similarly, Sahoo and Mukherjee (2001) reported considerably higher leukocyte counts in *Labeo rohita* after administering β -1,3-glucan orally.

Furthermore, Sanchez-Martinez et al. (2017) found that a feed enriched with β -1,3/1,6-glucan led to a considerable increase in the number of leukocytes in *Ictalurus punctatus*. In their study, Selvaraj et al. (2005) found that the leukocyte counts in *Cyprinus carpio* remained unchanged when they were given yeast-derived β -glucan orally or by bath therapy. Indriani et al. (2020) reported that the total number of leukocytes in treatments A and B showed a greater rise compared to treatment C after receiving an immunostimulant till day 14. The post-day test challenge revealed a reduction in the overall number of leukocytes in all treatment groups of catfish when exposed to *Aeromonas hydrophila*.

The proportion of various cell types among the white blood cells is also a significant measure since it provides insight into the activation of the leukocytes (Fernandez et al., 2002). Leucocytes in vertebrates are classified based on their physical characteristics. This categorization allows for the identification of several types of cells, including eosinophils, basophils, lymphocytes, monocytes, and neutrophils (Ellis, 1977).

Granulocytes consist of three types of cells: eosinophils, basophils, and neutrophils. Granulocytes exhibit a response when foreign substances enter the body, although they do so without specifically recognising antigens. When an invasion occurs in tissues, these cells move and eliminate foreign particles either by phagocytosis or by destroying them through a cytotoxic reaction (Fernandez et al., 2002).

Lymphocytes possess a high metabolic capacity as a result of their many organelles in the cytoplasm, including the golgi apparatus, mitochondria, ribosomes, and endoplasmic reticulum. Lymphocytes are further categorised into B lymphocytes and T lymphocytes based on their maturation locations in the bone marrow and thymus, respectively. T cells are important for cell-mediated immunity and also aid B lymphocytes, which are accountable for producing antibodies against antigens (Ronald, 2001). Lymphocytes primarily serve to generate antibodies, establish immunological memory, and release regulatory substances known as lymphokines when subjected to the humoral and cell-specific immune systems (Campbell and Murru, 1990). Macrophages are crucial to the body's defence since they play a vital role in eliminating infections. Fernandez et al. (2002) established that macrophages, the primary phagocytic cells in fish, eliminate infections by two mechanisms: the release of harmful chemicals and phagocytosis.

In the present study, the differential blood counts of the fish fed with different immunostimulant mixed feeds under normal and *Aeromonas* infected conditions were performed by counting the blood cells including eosinophils, basophils, neutrophils, lymphocytes, and monocytes. The results of differential counts demonstrated that the average eosinophil, basophil, and neutrophil count in healthy fish treated with different immunostimulant-supplemented feeds ranged from 357 ± 53.07 to 475 ± 45.76 cells/mm³, 38 ± 9 to 48 ± 9 cells/mm³, and 2650 ± 0.525 to 3340 ± 0.49 cells/mm³ respectively.

Whereas, in *Aeromonas* infected fish, eosinophil, basophil, and neutrophil count with different immunostimulant mixed feeds varied between 369 ± 50.21 to 543.66 ± 52.51 cells/mm³, 34 ± 6.5 to 42.6 ± 6.02 cells/mm³, and 5060 ± 0.89 and 7570 ± 0.62 cells/mm³ respectively. The results of eosinophil counts indicated that the tested immunostimulants significantly increased eosinophil count in both healthy and *Aeromonas* infected fish when compared to the control. While, the results of basophil counts indicated that the tested immunostimulants don't exhibit a significant impact on the basophil count in both healthy and *Aeromonas* infected fish when compared to the control.

Furthermore, the results of neutrophil counts demonstrated that the tested immunostimulants significantly decrease the neutrophils content in both healthy and *Aeromonas* infected fish when compared to the control. The results of mean lymphocyte count in healthy fish that treated with different immunostimulant mixed feeds ranged from 2531 ± 0.79 to 3072 ± 0.99 cells/mm³. Whereas, in *Aeromonas*-infected fish, the lymphocyte counts varied between 2290 ± 1.095 and 2740 ± 1.270 cells/mm³ with the use of different immunostimulant mixed feeds. While, the mean monocyte counts in healthy fish treated with various immunostimulant mixed feeds ranged from 178 ± 0.64 to 370 ± 0.51 cells/mm³. Whereas, in *Aeromonas*-infected fish, the monocyte counts varied between 462 ± 0.56 and 685 ± 0.77 cells/mm³ with the use of different immunostimulant mixed feeds. These results of lymphocyte and monocyte counts indicated that the tested immunostimulants had a substantial effect on increasing the number of lymphocytes and decreasing number of neutrophils in both healthy and *Aeromonas* infected fish, as compared to the control group.

The current findings are consistent with the prior research conducted by Selvaraj et al. (2005) which demonstrated that intraperitoneal administration of β -glucan led to a considerable rise in the number of peripheral neutrophils and monocytes in a manner that appeared to be dependent on the dosage. Additionally, Chin and Woo (2005) found that Atlantic salmon had lymphocytes comprising 66.4~75.1% of their blood cells, granulocytes making up 1.6~3.6%, monocytes accounting for 0.19~1.1%, and thrombocytes constituting 20.1~31.9%. Hardie et al. (1991) observed that lymphocytes accounted for 68.0~80.3% of the total count, thrombocytes accounted for 16.8~23.3%, neutrophils accounted for 2.0~7.5%, and monocytes accounted for 1.0~2.8%. Hardie et al. (1991) reported that the percentage of lymphocytes ranged from 59.7% to 65.9%, thrombocytes ranged from 30.3% to 39.1%, neutrophils ranged from 2.1% to 2.6%, and monocytes ranged from 0.9% to 1.4%.

Contrary to the current study, Wojtaszek et al. (2002) found that administering cortisol resulted in a decrease in the number of circulating lymphocytes and eosinophils, but the number of neutrophils increased. Narnaware and Baker (1996) observed a decrease in lymphocytes and thrombocytes in the bloodstream, along with an increase in neutrophils and monocytes, in rainbow trout under stress. Ellsaesser and Clem (1987) discovered a decrease in lymphocyte count that was associated with an increase in neutrophils following the administration of physiological quantities of cortisol to channel catfish. While comparing the current data to earlier reports, it is evident that there is a relatively large number of lymphocytes, but the fraction of neutrophils is lower than the expected range. The change in the erythrocyte sedimentation rate has been attributed to components present in both the plasma and red blood cells (Al-Homrany, 2002). An elevated ESR level signifies a rise in the formation of erythrocyte rouleaux, which can be harmful to red blood cells as they pass through the microcirculation (Qian et al., 2002).

In the present study, the mean ESR values in healthy fish treated with different immunostimulant mixed feeds ranged from 0.27 ± 0.13 to 0.39 ± 0.17 cm/hour. Whereas, in *Aeromonas*-infected fish, the ESR with various immunostimulant mixed feeds ranges from 0.33 ± 0.111 to 0.45 ± 0.14 cm/hour. The results indicated that the

immunostimulants examined did not have a significant effect on the ESR in both healthy and *Aeromonas* infected fish, as compared to the control group. The present results align with the findings of Harkal et al. (2011) who observed a rise in ESR from 7.25 mm/h to 8.10 mm/hr over a period of 24 to 96 hours respectively, in presence of a 15-ppm concentration of aqueous garlic extract. Similarly, they noted an increase in ESR from 7.80 mm/h to 9.11 mm/h over the same time frame.

Moreover, it has been indicated that an elevation in ESR might be attributed to an augmentation in the level of fibrinogen, resulting in fibrinogenemia, which can be caused by exposure to garlic or by chemical compounds such as sulphur. Similarly, Wu et al. (2013) found that the erythrocyte sedimentation rate reduced in the groups receiving dosages of 0.5 and 1.0 g/kg. In the groups receiving dosages of 2.0 and 4.0 g/kg, the ESR reduced from days 0 to 14, but rise from days 28 to 56. Malla et al. (2009) and Kumar & Benerjee (1990) have documented an elevation in ESR in *Channa punctatus* and *Clarias batrachus* following a seven-day exposure to chlorpyrifos.

5 Conclusion

It is concluded that the infected fish fed with all the five selected immunostimulant mixed feed exhibited lower lengths than the normal fish fed with immunostimulant diets. Among all the tested feeds, *Aeromonas*- infected fish fed with vitamin C mixed feed exhibited greater body length gain. The tested immunostimulants significantly enhance the Hemoglobin percentage, as well as RBC, WBC, neutrophils, lymphocytes, and monocyte count; decrease the eosinophil count and not shown a significant impact on the basophils count and ESR in both healthy and *Aeromonas* infected fish when compared to the control feed administered group. Thus, among the above selected immunostimulants, Vitamin C and Vitamin E have shown better impact on the morphometry and haematology of *Cata catla* fish under *Aeromonas hydrophila* infected conditions and restored all the above parameters as in healthy fish.

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Conflict of Interest Disclosure

The authors declare that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

References

- Aathi K., Ramasubramanian V., Venkatachalam U., and Munirasu S., 2013, Effect of chitosan supplemented diet on survival, growth, hematological, biochemical and immunological responses of Indian major carp *Labeo rohita*, *International Research Journal of Pharmacy*, 4(5): 141-147.
<https://doi.org/10.7897/2230-8407.04529>
- Abdel Rahman A.N., Khalil A.A., Abdallah H.M., and ElHady M., 2018, The effects of the dietary supplementation of *Echinacea purpurea* extract and/or vitamin C on the intestinal histomorphology, phagocytic activity, and gene expression of the Nile tilapia. *Fish & Shellfish Immunology*, 82: 312-318.
<https://doi.org/10.1016/j.fsi.2018.08.024>
- Abdel-Ghany H.M., and Salem M.E.S., 2019, Effects of dietary chitosan supplementation on farmed fish; a review, *Reviews in Aquaculture*, 12(1): 438-452.
<https://doi.org/10.1111/raq.12326>
- Abdelkhalek N.K., Zaki V.H. and Yousef M., 2008, Effect of some immunostimulants on health status and disease resistance of Nile tilapia (*Oreochromis niloticus*), *Proceedings of 8th International Symposium on Tilapia in Aquaculture (ISTA VIII)*, pages1037-1088.
- Abdel-Tawwab M., Mounes H.A., Shady S.H. and Ahmed K.M., 2020, Effects of yucca, *Yucca schidigera*, extract and/or yeast, *Saccharomyces cerevisiae*, as water additives on growth, biochemical, and antioxidants/oxidant biomarkers of Nile tilapia, *Oreochromis niloticus*, *Aquaculture*, 533: 736122.
<https://doi.org/10.1016/j.aquaculture.2020.736122>
- Adams S.M., 2005, Assessing cause and effect of multiple stressors on marine systems. *Marine Pollution Bulletin*, 51: 649-657.
<https://doi.org/10.1016/j.marpolbul.2004.11.040>
- Ahmad M.R., 1982, Leucocytes of *Clarias batrachus* (L). With special reference to body weight, *Indian Journal of Zoology*, 23(2): 105-111.
- Ahmad S.I., Ahmad R., Khan M.S., Kant R., Shahid S., Gautam L., Hasan G.M. and Hassan M.I., 2020, Chitin and its derivatives: Structural properties and biomedical applications, *International Journal of Biological Macromolecules*, 164: 526-539.
<https://doi.org/10.1016/j.ijbiomac.2020.07.098>
- Ahmed N. and Thompson S., 2019, The blue dimensions of aquaculture: A global synthesis, *Science of the Total Environment*, 652: 851-861.
<https://doi.org/10.1016/j.scitotenv.2018.10.163>

- Al-Homrany M., 2002. The significance of extreme elevation of the erythrocyte sedimentation rate in hemodialysis patients, Saudi Journal of Kidney Diseases and Transplantation, 13(2): 141-145.
<https://pubmed.ncbi.nlm.nih.gov/17660652/>
- Alvarez Pellitero P., Sitja Bobadilla A., Bermudez R. and Quiroga M.I., 2006, Levamisole activates several innate immune factors in *Scophthalmus maximus* (L.) Teleostei, International Journal of Immunopathology and Pharmacology, 19(4): 727-738.
<https://doi.org/10.1177/039463200601900403>
- Anderson D.P. and Jeney G., 1992, Immunostimulants added to injected *Aeromonas salmonicida* bacterin enhance the defense mechanisms and protection in rainbow trout (*Oncorhynchus mykiss*). Veterinary Immunology and Immunopathology, 34(3-4): 379-389.
[https://doi.org/10.1016/0165-2427\(92\)90177-R](https://doi.org/10.1016/0165-2427(92)90177-R)
- Awad E., Austin D. and Lyndon A.R., 2013, Effect of black cumin seed oil (*Nigella sativa*) and nettle extract (Quercetin) on enhancement of immunity in rainbow trout, *Oncorhynchus mykiss* (Walbaum), Aquaculture, 388: 193-197.
<https://doi.org/10.1016/j.aquaculture.2013.01.008>
- Baba T., Watase Y. and Yoshinaga Y., 1993, Activation of mononuclear phagocytes function by levamisole immersion in carp, Nippon Suisan Gakkaishi, 59: 301-307.
<https://doi.org/10.2331/suisan.59.301>
- Bae J.Y., Park G.H., Yoo K.Y., Lee J.Y., Kim D.J. and Bai, S.C., 2013, Evaluation of optimum dietary vitamin E requirements using DL α -tocopheryl acetate in the juvenile eel, *Anguilla japonica*, Journal of Applied Ichthyology, 29(1): 213-217.
<https://doi.org/10.1111/jai.12001>
- Bai S.C., Hardy R.W. and Hamidoghli A., 2022, Diet analysis and evaluation In Fish nutrition, Academic Press, pages 709-743.
<https://doi.org/10.1016/B978-0-12-819587-1.00010-0>
- Bairwa M.K., Jakhar J.K., Satyanarayana Y. and Reddy A.D., 2012, Animal and plant originated immunostimulants used in aquaculture, Journal of Natural Product and Plant Resources, 2(3): 397-400.
- Barikani M., Oliaei E., Seddiqi H. and Honarkar H., 2014, Preparation and application of chitin and its derivatives: A review, Iranian Polymer Journal (English Edition), 23(4): 307-326.
<https://doi.org/10.1007/s13726-014-0225-z>
- Bastiawan D., Tauhid M., Alifuddin T.S. and Dermawati, 1995, Perubahan hematologic dan jaringan ikan lele dumbo *Clarias gariepinus* yang diinfeksi cendawan *Aphanomyces* spt, Jurnal Penelitian Perikanan Indonesia, 1(2): 106-115.
<https://doi.org/10.15578/jppi.1.2.1995.106-115>
- Bhatnagar A. and Lamba R., 2016, Immunostimulating and growth promoting activity of dietary levamisole on *Cirrhinus mrigala* fingerlings, Journal of Aquaculture & Marine Biology, 4(6): 1-9.
<https://doi.org/10.15406/jamb.2016.04.00102>
- Boric M., Vicente F.A., Jurkovic D.L., Novak U. and Likoza B., 2020, Chitin isolation from crustacean waste using a hybrid demineralization/DBD plasma process, Carbohydrate Polymers, 246: 1-8.
<https://doi.org/10.1016/j.carbpol.2020.116648>
- Burrells C., Williams P., Southgate P. and Wadsworth S., 2001, Dietary nucleotides: a novel supplement in fish feeds: 2. Effects on vaccination, salt water transfer, growth rates and physiology of Atlantic salmon (*Salmo salar* L.), Aquaculture, 199(1-2): 171-84.
[https://doi.org/10.1016/S0044-8486\(01\)00576-2](https://doi.org/10.1016/S0044-8486(01)00576-2)
- Campbell T. and Murru F., 1990, An introduction to fish hematology, The Compendium on Continuing Education for the Practicing Veterinarian, 12: 525-532.
<https://api.semanticscholar.org/CorpusID:70951850>
- Chin A. and Woo P.T., 2005, Innate cell-mediated immune response and peripheral leukocyte populations in Atlantic salmon, *Salmo salar* L., to a live *Cryptobia salmositica* vaccine, Parasitology Research, 95(5): 299-304.
<https://doi.org/10.1007/s00436-004-1270-x>
- Combs G.F. and McClung J.P., 2017, Chapter 8 vitamin E. In: Combs, G.F., McClung, J.P. (Eds.), The Vitamins, 5th edition, Academic Press, 207-242.
<https://doi.org/10.1016/B978-0-12-802965-7.00008-3>
- Danulat E., 1987, Digestibility of chitin in cod, *Gadus morhua*, in vivo, Helgoländer Meeresuntersuchungen (Helgoland Marine Research), 41(4): 425-436.
<https://doi.org/10.1007/BF02365402>
- Dash G., Raman R.P., Prasad K.P., Makesh M., Pradeep M. and Sen S., 2015, Evaluation of paraprobiotic applicability of *Lactobacillus plantarum* in improving the immune response and disease protection in giant freshwater prawn, *Macrobrachium rosenbergii* (de Man, 1879), Fish & Shellfish Immunology, 43: 167-174.
<https://doi.org/10.1016/j.fsi.2014.12.007>
- Dawood M.A. and Koshio S., 2016, Recent advances in the role of probiotics and prebiotics in carp aquaculture: a review, Aquaculture, 454: 243-251.
<https://doi.org/10.1016/j.aquaculture.2015.12.033>
- Defrizal D. and Khalil M., 2015, Pengaruh formulasi yang berbeda pada pakan pelet terhadap pertumbuhan ikan lele dumbo (*Clarias gariepinus*). Acta Aquatica, 2(2): 101-106.
<https://doi.org/10.29103/aa.v2i2.342>
- Dewi M., Efizon D. and Eddiwan E., 2017, Morphometric, meristic and growth pattern of *Osphrenemus gouramy* Lac. from the pinang luar oxbow lake, buluhcina village, kampar regency, riau, Jurnal Online Mahasiswa (JOM) Universitas Riau, 4(2): 1-11.
<https://api.semanticscholar.org/CorpusID:155237037>

- Ellis A.E., 1977, The leucocytes of fish: A review, *Journal of Fish Biology*, 11:453-491.
<https://doi.org/10.1111/j.1095-8649.1977.tb04140.x>
- Ellsaesser C.F. and Clem L.W., 1987, Cortisol-induced hematologic and immunologic changes in channel catfish (*Ictalurus punctatus*), *Comparative Biochemistry and Physiology Part A: Physiology*, 87(2): 405-408.
[https://doi.org/10.1016/0300-9629\(87\)90143-5](https://doi.org/10.1016/0300-9629(87)90143-5)
- El-Naggar M., Salaah S., El-Shabaka H., El-Rahman A., Khalil M.T. and Suloma A., 2021, Efficacy of dietary chitosan and chitosan nanoparticles supplementation on health status of Nile tilapia, *Oreochromis niloticus* (L.), *Aquaculture Reports*, 19(4): 100628.
<https://doi.org/10.1016/j.aqrep.2021.100628>
- Elsefay S.S., Abdel-Hameid H.N.A., Abdel-Salam A.H. and Dakrouni M.A., 2021, Effect of shrimp waste extracted chitin on growth and some biochemical parameters of the Nile tilapia, *Egyptian Journal of Aquatic Biology and Fisheries*, 25(1): 313-329.
<https://doi.org/10.21608/ejafb.2021.143244>
- Esepid S., Hjelmeland K. and Jorgensen T.O., 1987, *Developmental & Comparative Immunology*, Summer, 11(3): 529-537.
[https://doi.org/10.1016/0145-305X\(87\)90042-5](https://doi.org/10.1016/0145-305X(87)90042-5)
- Fago A., Hundahl C., Malte H. and Weber R.E., 2004, Functional properties of neuroglobin and cytoglobin. Insights into the ancestral physiological roles of globins, *IUBMB Life*, 56: 689-696.
<https://doi.org/10.1080/15216540500037299>
- FAO., 2020, The State of World Fisheries and Aquaculture 2020 (SOFIA): Sustainability in action, Food and Agriculture Organization of the United Nations.
- Faramarzi M., 2012, Effect of dietary vitamin C on growth and feeding parameters, carcass composition and survival rate of Common Carp (*Cyprinus carpio*), *Global Veterinaria*, 8(5): 507-510.
[https://www.idosi.org/gv/GV8\(5\)12/14.pdf](https://www.idosi.org/gv/GV8(5)12/14.pdf)
- Fernandez A.B., de Blas I. and Ruiz I., 2002, Immunological system in Teleost. Cells and organs. *Aquatic Magazine*, 16, Spanish.
- Fines B.C. and Holt G.J., 2010, Chitinase and apparent digestibility of chitin in the digestive tract of juvenile cobia, *Rachycentron canadum*, *Aquaculture*, 303: 34-39.
<https://doi.org/10.1016/j.aquaculture.2010.03.010>
- Gao J., Koshio S., Ishikawa M., Yokoyama S., Ren T.J., Komilus C.F. and Han Y.Z., 2012, Effects of dietary palm oil supplements with oxidized and non-oxidized fish oil on growth performances and fatty acid compositions of juvenile Japanese sea bass, *Lateolabrax japonicus*, *Aquaculture*, 324: 97-103.
<https://doi.org/10.1016/j.aquaculture.2011.10.031>
- Gephart J.A., Golden C.D., Asche F., Belton B., Brugere C., Froehlich H.E., Fry J.P., Halpern B.S., Hicks C.C., Jones R.C., Klinger D.H., Little D.C., McCauley D.J., Thilsted S.H., Troell M. and Allison E.H., 2020, Scenarios for global aquaculture and its role in human nutrition, *Reviews in Fisheries Science and Aquaculture*, 1-17.
<https://doi.org/10.1080/23308249.2020.1782342>
- Giardina B., Mosca D. and De Rosa M.C., 2004, The Bohr effect of haemoglobin in vertebrates: an example of molecular adaptation to different physiological requirements, *Acta Physiologica Scandinavica*, 182: 229-244.
<https://doi.org/10.1111/j.1365-201X.2004.01360.x>
- Gouda A., Amer S.A., Gabr S., Tolba S.A., 2020, Effect of dietary supplemental ascorbic acid and folic acid on the growth performance, redox status, and immune status of broiler chickens under heat stress. *Tropical ANIMAL Health and Production*, 52(6): 2987-2996.
<https://doi.org/10.1007/s11250-020-02316-4>
- Hardie L., Fletcher T. and Secombes C., 1990, The effect of vitamin E on the immune response of the Atlantic salmon (*Salmo salar* L.), *Aquaculture*, 87(1): 1-13.
[https://doi.org/10.1016/0044-8486\(90\)90206-3](https://doi.org/10.1016/0044-8486(90)90206-3)
- Hardie L., Fletcher T. and Secombes C., 1991, The effect of dietary vitamin C on the immune response of the Atlantic salmon (*Salmo salar* L.), *Aquaculture*, 95(3-4): 201-214.
[https://doi.org/10.1016/0044-8486\(91\)90087-N](https://doi.org/10.1016/0044-8486(91)90087-N)
- Harikrishnan R., Nisha R.M. and Balasundaram C., 2003, Hematological and biochemical parameters in common carp, *Cyprinus carpio*, following herbal treatment for *Aeromonas hydrophila* infection, *Aquaculture*, 221(1-4): 41-50.
[https://doi.org/10.1016/S0044-8486\(03\)00023-1](https://doi.org/10.1016/S0044-8486(03)00023-1)
- Harkal A.B., Jagtap A.R., Padewar S.K. and Mali R.P., 2011, Toxic effect of aqueous extract of garlic on erythrocyte sedimentation rate in fish, *Channa punctatus*, *The Asian Journal of Animal Science*, 6(2): 206-207.
https://researchjournal.co.in/upload/assignments/6_206-207.pdf
- Haryono, 2001, Variasi Morfologi dan Morfometri Ikan Dokun (*Puntius Lateristriga*) di Sumatera, *Jurnal Biota*, 6(3): 109-116.
- Hassan A., Yacout M.H.M., Khalel M.S., Hafsa S.A., Ibrahim M.A.R., Mocuta D., Rahoveanu A.T. and Lorena D., 2018, Effects of some herbal plant supplements on growth performance and the immune response in tilapia (*Oreochromis niloticus*). *Sciencio*, 1(1): 134-141.
<https://doi.org/10.2478/alife-2018-0020>
- He M., Wang K., Liang X., Fang J., Geng Y., Chen Z., Pu H., Hu Y., Li X. and Liu L. 2017, Effects of dietary vitamin E on growth performance as well as intestinal structure and function of channel catfish (*Ictalurus punctatus*, Rafinesque 1818), *Experimental and Therapeutic Medicine*, 14(6): 5703-5710.
<https://doi.org/10.3892/etm.2017.5295>

- Hoseinifar S.H., Dadar M. and Ringo E., 2017, Effect of β -glucan on growth performance, hematological parameters, and skin mucus immune responses in common carp (*Cyprinus carpio*) fingerlings, *Fish & Shellfish Immunology*, 67: 119-124.
<https://doi.org/10.1016/j.fsi.2017.06.023>
- Hudson I. and Hay C.F., 1991, Antibody interaction with antigen. In: *Practical immunology*, 3rd edition, Blackwell Scientific Publications, Oxford, pages 208-260.
- Ibrahim R.E., Ahmed S.A., Amer S.A., Al-Gabri N.A., Ahmed A.I., Abdel-Warith A.W.A., Younis E.S.M. and Metwally, A.E., 2020, Influence of vitamin C feed supplementation on the growth, antioxidant activity, immune status, tissue histomorphology, and disease resistance in Nile tilapia, *Oreochromis niloticus*, *Aquaculture Reports*, 18: 100545.
<https://doi.org/10.1016/j.aqrep.2020.100545>
- Indriani F., Puspitasari I., Setyastuti T.A. and Santika A., 2020, Haematological parameters of Catfish (*Clarias* sp.) fed by immunostimulant added with Cr+3-Yeast (*Saccaromices cerevisiae*) and Garlic, In *IOP Conference Series: Earth and Environmental Science*, IOP Publishing, 441(1): 012115.
<https://doi.org/10.1088/1755-1315/441/1/012115>
- Ismail H.M., 2005, The role of omega-3 fatty acids in cardiac protection: an overview, *Frontiers in Bioscience*, 10: 1079-1088.
<https://doi.org/10.2741/1601>
- James R., Vasudhevan I. and Sampath K., 2008, Effect of dietary vitamin E on growth, fecundity, and leukocyte count in goldfish (*Carassius auratus*), *The Israeli Journal of Aquaculture - Bamidgeh*, 60(2): 121-127.
<https://doi.org/10.46989/001c.20485>
- Janssen P.A., 1976, The levamisole story. *Progress in Drug Research*, 20: 347-383.
https://doi.org/10.1007/978-3-0348-7094-8_11
- Jeney G. and Anderson D.P., 1993, Enhanced immune response and protection in rainbow trout to *Aeromonas salmonicida* bacterin following prior immersion in immunostimulants, *Fish and Shellfish Immunology*, 3(1): 51-58.
<https://doi.org/10.1006/fsim.1993.1005>
- Khattabya., Shalabyame., Sharafsm., El Marakbyhi. and Rizkallaeh., 2004, The physiological changes and growth performance of the Nile tilapia *Oreochromis niloticus* after feeding with Biogen® as growth promoter. *Egyptian Journal of Aquatic Biology and Fisheries*, 8: 145-158.
- Kono M., Shimizu C. and Matsui T., 1987, Effect of chitin, chitosan, and cellulose as diet supplements on the growth of Cultured Fish, *Nippon Suisan Gakkaishi*, 53(1): 125-129.
<https://doi.org/10.2331/suisan.53.125>
- Kresno S.B., 2001, *Imunologi: Diagnosis dan Prosedur Laboratorium* (Jakarta: Universitas Indonesia).
- Kumar B. and Benerjee V., 1990, Effect of sub lethal toxicity of seven on blood parameters in *Clarias batrachus* (L.), *Himalayan Journal of Environment and Zoology*, 4: 166-172.
- Kumar R., Mukherjee S.C., Ranjan R. and Nayak S.K., 2008, Enhanced innate immune parameters in *Labeo rohita* (Ham.) following oral administration of *Bacillus subtilis*, *Fish & Shellfish Immunology*, 24(2): 168-172.
<https://doi.org/10.1016/j.fsi.2007.10.008>
- Kumar V., Sahu N.P., Pal A.K. and Kumar S., 2006, Immunomodulation of *Labeo rohita* juveniles due to dietary gelatinized and nongelatinized starch, *Fish & Shellfish Immunology*, 23: 341-353.
<https://doi.org/10.1016/j.fsi.2006.11.008>
- Landini G.F., Schwantes A.R. and Schwantes M.L., 2002, *Astyanax scabripinnis* (Pisces: Characidae) hemoglobins: structure and function, *Brazilian Journal of Biology*, 62: 595-599.
<https://doi.org/10.1590/S1519-69842002000400006>
- Lin S., Pan Y., Luo L. and Luo L., 2011, Effects of dietary β -1, 3-glucan, chitosan or raffinose on the growth, innate immunity and resistance of koi (*Cyprinus carpio koi*), *Fish & Shellfish Immunology*, 31(6): 788-794.
<https://doi.org/10.1016/j.fsi.2011.07.013>
- Liua B., Wana J., Gea X., Xiea J., Zhou Q., Miaob L., Renb M. and Panb L., 2016, Effects of dietary Vitamin C on the physiological responses and disease resistance to pH stress and *Aeromonas hydrophila* infection of *Megalobrama amblycephala*, *Turkish Journal of Fisheries and Aquatic Sciences*, 16: 421-433.
https://doi.org/10.4194/1303-2712-v16_2_22
- Lu Y., Liang X.P., Jin M., Sun P., Ma H.N., Yuan Y. and Zhou Q.C. 2016, Effects of dietary vitamin E on the growth performance, antioxidant status and innate immune response in juvenile yellow catfish (*Pelteobagrus fulvidraco*), *Aquaculture*, 464: 609-617.
<https://doi.org/10.1016/j.aquaculture.2016.08.009>
- Malla F.A., Sharma G. and Singh S., 2009, Chlorpyrifos pesticide toxicity on erythrocyte sedimentation rate in fish, *Channa punctatus* (Bloch), *Biology & Medicine*, 1(2): 54-55.
- Mansour A.T., Fayed W.M., Elkhayat B.K., Ali E., Omar M.A., Nour A.A.M. and Morshedy S.A., 2021, *Yucca schidigera* extract dietary supplementation affects growth performance, hematological and physiological status of European seabass, *Annals of Animal Science*, 21: 1043-1060.
<https://doi.org/10.2478/aoas-2021-0007>
- Maqsood S., Samoon M.H. and Singh P., 2009, Immunomodulatory and growth promoting effect of dietary levamisole in *Cyprinus carpio* fingerlings against the challenge of *Aeromonas hydrophila*, *Turkish Journal of Fisheries and Aquatic Sciences*, 9:111-120.
- Maqsood S., Singh P., Samoon M.H. and Balange A.K., 2010, Effect of Dietary Chitosan on non-Specific immune response and growth *Cyprinus carpio* Challenged with *Aeromonas hydrophila*, *International Aquatic Research*, 2: 77-85.

- Martins M.L., Tavares-Dias M., Fujimoto R.Y., Onaka E.M. and Nomura D.T., 2004, Haematological alterations of *Leporinus macrocephalus* (Osteichthyes: Anostomidae) naturally infected by *Goezia leporini* (Nematoda: Anisakidae) in fish pond, Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 56: 640-646.
<https://doi.org/10.1590/S0102-09352004000500011>
- Mehrad B., Jafaryan H. and Taati M.M., 2012. Assessment of the effects of dietary vitamin E on growth performance and reproduction of zebrafish, *Danio rerio* (Pisces, Cyprinidae), Journal of Oceanography and Marine Science, 3(1): 1-7.
<https://doi.org/10.5897/JOMS11.022>
- Muahiddah N. and Diamahesa W.A., 2023, The use of garlic (*Allium sativum*) as an immunostimulant in Aquaculture, Journal of Fish Health, 3(1): 11-18.
<https://doi.org/10.29303/jfh.v3i1.2751>
- Mudjiman A., 2011, Makanan Ikan (Fish Feed/Fish Food). Penebar Swadaya, Jakarta, Indonesia.
- Mulero V., Esteban M.A., Munoz J. and Meseguer J., 1998, Dietary intake of levamisole enhances the immune response and disease resistance of the marine teleost gilthead seabream (*Sparus aurata* L.), Fish & Shellfish Immunology, 8(1): 49-62.
<https://doi.org/10.1006/fsim.1997.0119>
- Narnaware Y. and Baker B., 1996, Evidence that cortisol may protect against the immediate effects of stress on circulating leukocytes in the trout, General and Comparative Endocrinology, 103(3): 359-66.
<https://doi.org/10.1006/gcen.1996.0131>
- Niki L., Albright L.J. and Evelyn T.P.T., 1991, Influence of seven immunostimulants on the immune response of Coho salmon to *Aeromonas salmonicida*, Diseases of Aquatic Organisms, 12(1): 7-12.
<https://doi.org/10.3354/dao012007>
- Nolle N., Genschick S., Schwadorf K., Hrenn H., Brandner S. and Biesalski H.K., 2020, Fish as a source of (micro)nutrients to combat hidden hunger in Zambia, Food Security, 12(6): 1385-1406.
<https://doi.org/10.1007/s12571-020-01060-9>
- Novian F., Grandiosa R. and Pratiwi D.Y., 2023. Growth Performance and Health Status of Giant Gouramy (*Osphronemus gouramy* Lac.) Seed feeded with a combination of chitosan and probiotic supplements (sinbiotic). Jurnal Ruaya: Jurnal Penelitian dan Kajian Ilmu Perikanan dan Kelautan, 11(1): 52-63.
<https://doi.org/10.29406/jr.v11i1.4754>
- NRC., 2011, National Research Council, Division on Earth, Life Studies, Committee on the Nutrient Requirements of Fish and Shrimp, Nutrient requirements of fish and shrimp, National Academies Press.
- Nuryati S., Maswan N.A., Alimuddin S., Sumantadinata K., Pasaribu F.H., Soejoedono R.D. and Santika A., 2010, Gambaran darah ikan mas setelah divaksinasi dengan vaksin DNA dan diuji tantang dengan koi herpes virus, Jurnal Akuakultur Indonesi, 9(1): 9-15.
<https://doi.org/10.19027/jai.9.9-15>
- Padua S.B., Martins M.L., Menezes-Filho R.N. and Nagata M.M., 2015, Uso de Aquate FishTM durante a formação de juvenis de tilápia: aumento da resistência contra doenças, melhoria na integridade intestinal e no desempenho produtivo. Revista Aqua Feed, pp 4-8.
- Payung C.N., Tumbol R.A. and Manoppo H., 2017, Dietary ginger (*Zingiber officinale*) enhance resistance of Nile tilapia (*Oreochromis niloticus*) against *Aeromonas hydrophila*, AACL Bioflux, (4): 962-968.
- Peng L.I. and Gatlin III D.M., 2009, Dietary vitamin E requirement of the red drum *Sciaenops ocellatus*, Aquaculture Nutrition, 15: 313-319.
<https://doi.org/10.1111/j.1365-2095.2008.00596.x>
- Perutz M.F., 1978, Hemoglobin structure and respiratory transport, Scientific American, 239: 92-125.
<https://doi.org/10.1038/scientificamerican1278-92>
- Pulungan L.A., Riauwaty M. and Lukistyowati I., 2022, Hematology of pangasianodon hypophthalmus that were fed with containing fermented red ginger (*Zingiber officinale* var. rubrum) to prevent the motile *Aeromonas septicaemia* disease, Asian Journal of Aquatic Sciences, 5(2): 291-300.
<https://doi.org/10.31258/ajaoas.5.2.291-300>
- Purbomartono C., Habibah U., Wahyuningtyas R.A., Husin A. and Samadan G.M., 2021, Growth and immunity of African catfish (*Clarias gariepinus*) with dietary inclusion of ginger (*Zingiber officinalis*) and turmeric (*Curcuma domestica*), AACL Bioflux, 14(3): 1365-1372.
- Qian Y.X., Chen H.Q. and Sun J.F., 2002, Effects of starvation on hematological and blood biochemical indices in cultured *Lateolabrax japonicus*, Journal of Fishery Sciences of China, 9(2): 133-137.
- Rairakhwada D., Pal A.K., Bhatena Z.P., Sahu N.P., Jha A. and Mukherjee S.C., 2007, Dietary microbial levan enhances cellular nonspecific immunity and survival of common carp (*Cyprinus carpio*) juveniles, Fish & Shellfish Immunology, 22: 477-486.
<https://doi.org/10.1016/j.fsi.2006.06.005>
- Ramesh D., Vinothkanna A., Rai A.K. and Vignesh V.S., 2015, Isolation of potential probiotic *Bacillus* spp. and assessment of their subcellular components to induce immune responses in *Labeo rohita* against *Aeromonas hydrophila*, Fish & Shellfish Immunology, 45: 268-276.
<https://doi.org/10.1016/j.fsi.2015.04.018>
- Reda R.M. and Selim K.M., 2015, Evaluation of *Bacillus amyloliquefaciens* on the growth performance, intestinal morphology, hematology and body composition of Nile tilapia, *Oreochromis niloticus*, Aquaculture International, 23: 203-217.
<https://doi.org/10.1007/s10499-014-9809-z>
- Refaey M.M., Mehrim A.I., Zenhom O.A. and Mansour A.T., 2022, Effect of fatty acids manipulation on survival and physiological response of hybrid red tilapia under chronic cold stress, Aquaculture, 561: 738663.
<https://doi.org/10.1016/j.aquaculture.2022.738663>
- Riggs A., 1976, Factors in the evolution of hemoglobin function. Federation Proceedings, 35(10): 2115-2118.

- Ringo E., Lovmo L., Kristiansen M., Bakken Y., Salinas I., Myklebust R., Olsen R.E. and Mayhew T.M., 2010, Lactic acid bacteria vs. pathogens in the gastro-intestine of fish: a review. *Aquaculture Research*, 41:451-467.
<https://doi.org/10.1111/j.1365-2109.2009.02339.x>
- Ringo E., Olsen R.E., Vecino J.G., Wadsworth S. and Song S., 2012, Use of immunostimulants and nucleotides in aquaculture: a review. *Journal of Marine Science Research & Development*, 2(1): 1000104.
<https://doi.org/10.4172/2155-9910.1000104>
- Rodriguez A., Cuesta A., Ortuno J., Esteban M.A. and Meseguer J., 2003, Immunostimulant properties of a cell wall-modified whole *Saccharomyces cerevisiae* strain administered by diet to seabream (*Sparus aurata* L.), *Veterinary Immunology and Immunopathology*, 96(3-4): 183-192.
<https://doi.org/10.1016/j.vetimm.2003.07.001>
- Rohani M.F., Tarin T., Hasan J., Islam S.M. and Shahjahan M., 2023, Vitamin E supplementation in diet ameliorates growth of Nile tilapia by upgrading muscle health, *Saudi Journal of Biological Sciences*, 30(2): 103558.
<https://doi.org/10.1016/j.sjbs.2023.103558>
- Ronald R., 2001, *Fish Pathology*. 4th edition. Wiley-Blackwell, 590 pages.
- Rozi R., Mukti A.T., Samara S.H. and Santanumurti M.B., 2018, Pengaruh pemberian kitosan dalam pakan terhadap pertumbuhan, sintasan dan efisiensi pemanfaatan pakan nila (*Oreochromis niloticus*), *Jurnal Perikanan Universitas Gadjah Mada*, 20(2): 103-111.
<https://doi.org/10.22146/jfs.38868>
- Saheli M., Islami H.R., Mohseni M. and Soltani M., 2021. Effects of dietary vitamin E on growth performance, body composition, antioxidant capacity, and some immune responses in Caspian trout (*Salmo caspius*). *Aquaculture Reports*, 21: 100857.
<https://doi.org/10.1016/j.aqrep.2021.100857>
- Sahoo P. and Mukherjee S., 2001, Effect of dietary β -1,3 glucan on immune responses and disease resistance of healthy and aflatoxin B1-induced immunocompromised rohu (*Labeo rohita* Hamilton), *Fish & Shellfish Immunology*, 11(8): 683-695.
<https://doi.org/10.1006/fsim.2001.0345>
- Sakai M., 1999, Current research status of fish immunostimulants, *Aquaculture*, 172(1-2): 63-92.
[https://doi.org/10.1016/S0044-8486\(98\)00436-0](https://doi.org/10.1016/S0044-8486(98)00436-0)
- Salam M.A., Rahman M.A., Paul S.I., Islam F., Barman A.K., Rahman Z., Shaha D.C., Rahman M.M. and Islam T., 2021, Dietary chitosan promotes the growth, biochemical composition, gut microbiota, hematological parameters and internal organ morphology of juvenile *Barbonymus gonionotus*, *PLoS One*, 16(11): e0260192.
<https://doi.org/10.1371/journal.pone.0260192>
- Sanchez-Martínez J.G., Rábago-Castro J.L., Vázquez-Sauceda M.de.la.L., Pérez-Castañeda R., Blanco-Martínez Z. and Benavides-González F., 2017, Effect of β -glucan dietary levels on immune response and hematology of channel catfish *Ictalurus punctatus* juveniles, *Latin American Journal of Aquatic Research*, 45(4):690-698.
<https://doi.org/10.3856/vol45-issue4-fulltext-5>
- Saputra A., Budiardi T., Samsudin R. and Rahmadya N.D., 2018, Growth performance and survival of snakehead *Channa striata* juvenile with different stocking density reared in recirculation system. *Jurnal Akuakultur Indonesia*, 17(2): 104-112.
<https://doi.org/10.19027/jai.17.2.104-112>
- Sarker U.K., Hossain M.I., Hossain M.M., Sarkar R., Rahman M.M., Abdullah-Al-Mamun M., Alam M.M., 2023, Effects of major immunostimulant (Betamune) on health and production of Nile tilapia *Oreochromis niloticus*. *International Journal of Fisheries and Aquatic Studies*, 11(3): 7-16.
<https://doi.org/10.22271/fish.2023.v11.i3a.2802>
- Selvaraj V., Sampath K. and Sekar V., 2005, Administration of yeast glucan enhances survival and some non-specific and specific immune parameters in carp (*Cyprinus carpio*) infected with *Aeromonas hydrophila*, *Fish & Shellfish Immunology*, 19(4): 293-306.
<https://doi.org/10.1016/j.fsi.2005.01.001>
- Setijaningsih L., Setiadi E. and Taufik I., 2021, The effect of garlic *Allium sativum* addition in feed to the growth performance and immune response of tilapia *Oreochromis niloticus*. In *IOP Conference Series: Earth and Environmental Science*, 744(1): 012072.
<https://doi.org/10.1088/1755-1315/744/1/012072>
- Shiau S.Y. and Yu Y.P., 1999, Dietary supplementation of chitin and chitosan depresses growth in tilapia, *Oreochromis niloticus* $\times$ *O. aureus*, *Aquaculture*, 179(1-4): 439-446.
[https://doi.org/10.1016/S0044-8486\(99\)00177-5](https://doi.org/10.1016/S0044-8486(99)00177-5)
- Simic M.G. and Karel M., 1980, *Autoxidation in Food and Biological Systems*. Plenum Press, New York, NY.
<https://doi.org/10.1007/978-1-4757-9351-2>
- Siwicki A.K. and Korwin-Kossakowski M., 1988, The influence of levamisole on the growth of carp (*Cyprinus carpio*) larvae. *Journal of Applied Ichthyology*, 4(4): 178-181.
<https://doi.org/10.1111/j.1439-0426.1988.tb00559.x>
- Smith V.J., Brown J.H. and Hauton C., 2003, Immunostimulation in crustaceans: does it really protect against infection?. *Fish & Shellfish Immunology*, 15(1): 71-90.
[https://doi.org/10.1016/S1050-4648\(02\)00140-7](https://doi.org/10.1016/S1050-4648(02)00140-7)
- Sudiono J., 2014, *Sistem kekebalan tubuh (The Immune System)*, Penerbit Buku Kedokteran EGC, Jakarta, pp 52-54.

- Suprayudi M.A., Indriastuti L. and Setiawati M., 2006, Pengaruh penambahan bahan-bahan imunostimulan dalam formulasi pakan buatan terhadap respon imunitas dan pertumbuhan ikan kerapu bebek (*Cromileptes altivelis*), Jurnal Akuakultur Indonesia, 5(1): 77-86.
<https://doi.org/10.19027/jai.5.77-86>
- Talpur A.D., Ikhwanuddin M.H.D. and Bolong A.A., 2013, Nutritional effects of ginger (*Zingiber officinale* Roscoe) on immune response of Asian seabass, *Lates calcarifer* (Bloch) and disease resistance against *Vibrio harveyi*, Aquaculture, 400-401: 46-52.
<https://doi.org/10.1016/j.aquaculture.2013.02.043>
- Taveekijakarn P., Miyazaki T., Matsumoto M. and Arai S., 1996, Study on vitamin E deficiency in amago salmon, Bulletin of the Faculty of Bioresources Mie University, 16: 17-24.
- Udo I.U., Etukudo U. and dan Anwana U.I.U., 2018, Effects of Chitosan and Chitosan Nanoparticles on Water Quality, Growth Performance, Survival Rate and Meat Quality of the African Catfish, *Clarias gariepinus*. Nanoscience, 1(1): 12-25.
<https://doi.org/10.31058/j.nano.2018.11002>
- Viarengo A., Lowe D., Bolognesi C., Fabbri E. and Koehler A., 2007, The use of biomarkers in biomonitoring: A 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms, Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 146: 281-300.
<https://doi.org/10.1016/j.cbpc.2007.04.011>
- Wassef E., El Masry M.H. and Mikhail F.R., 2001, Growth enhancement and muscle structure of striped mullet, *Mugil cephalus* L., fingerlings by feeding algal meal-based diets. Aquaculture Research, 32: 315-322.
<https://doi.org/10.1046/j.1355-557x.2001.00043.x>
- Wojtaszek J., Dziewulska-Szwajkowska D., Łozińska-Gabska M., Adamowicz A. and Dżugaj A., 2002, Hematological effects of high dose of cortisol on the carp (*Cyprinus carpio* L.): cortisol effect on the carp blood, General and Comparative Endocrinology, 125(2): 17683.
<https://doi.org/10.1006/gcen.2001.7725>
- Wu Z.X., Pang S.F., Chen X.X., Yu Y.M., Zhou J.M., Chen X., Pang L.J., 2013, Effect of *Coriolus versicolor* polysaccharides on the hematological and biochemical parameters and protection against *Aeromonas hydrophila* in allogynogenetic crucian carp (*Carassius auratus gibelio*), Fish Physiology and Biochemistry, 39: 181-190.
<https://doi.org/10.1007/s10695-012-9689-y>
- Xu C.M., Yu H.R., Li L.Y., Li M., Qiu X.Y., Fan X.Q., Fan Y.L. and Shan L.L., 2022, Effects of dietary vitamin C on the growth performance, biochemical parameters, and antioxidant activity of Coho Salmon *Oncorhynchus kisutch* (Walbaum, 1792) postsmolts, Aquaculture Nutrition, 6866578.
<https://doi.org/10.1155/2022/6866578>
- Yossa R., Levesque S., Groman D.B. and Lora J.H., 2018, Preliminary evaluation of purified lignin and hemicellulose as prebiotics candidates for Atlantic Salmon, *Salmo salar* L., Journal of Applied Aquaculture, 30(3): 256-271.
<https://doi.org/10.1080/10454438.2018.1439795>
- Zainun Z., 2007, Pengamatan Parameter Hematologi Pada Ikan Mas yang Diberi Imunostimulan, Buletin Teknisi Litkayasa Akuakultur, 6(1): 45-49.
<https://doi.org/10.15578/blta.6.1.2007.45-49>
- Zaki M.A., Salem M.E.S., Gaber M.M. and Nour A.M., 2015, Effect of chitosan supplemented diet on survival, growth, feed utilization, body composition & histology of sea bass (*Dicentrarchus labrax*), World Journal of Engineering and Technology, 3(4): 38-47.
<https://doi.org/10.4236/wjet.2015.34C005>
- Zhao M., He H., Guo D., Zhang X., Jia L., Hou T. and Ma A., 2022, Chitosan oligosaccharides-tripolyphosphate microcapsules as efficient vehicles for desalted duck egg white peptides-calcium: fabrication, entrapment mechanism and in vivo calcium absorption studies, Food Science & Technology, 154: 112869.
<https://doi.org/10.1016/j.lwt.2021.112869>
- Zhou Q., Wang L., Wang H., Xie F. and Wang T., 2012, Effect of dietary vitamin C on the growth performance and innate immunity of juvenile cobia (*Rachycentron canadum*), Fish & Shellfish Immunology, 32(6): 969-975.
<https://doi.org/10.1016/j.fsi.2012.01.024>
- Zhu K., Shi S., Cao Y., Lu A., Hu J. and Zhang L., 2019, Robust chitin films with good biocompatibility and breathable properties, Carbohydrate Polymers, 212: 361-367.
<https://doi.org/10.1016/j.carbpol.2019.02.054>



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Review and Progress

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Safety and Automation in Singapore's Local Fish Production: Opportunities and Challenges for Sustainable Aquaculture Development

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Abstract Singapore aims to increase its local food production to 30% by 2030, with a strategic focus beginning on fish and shrimp production. Challenges from rising labour costs and limited space for sustainable aquaculture present significant obstacles. This process review highlights current technologies for fish sorting and harvesting and assesses the potential for automation in onshore fish farming. It also examines the fish farming process, scrutinises safety practices and standards, and applies a risk assessment framework for selected aquaculture activities, notably for feeding and harvesting activities, and offers insights into the global aquaculture industry, offering a strategic standpoint for increasing local fish production, reinforcing food safety, and support the sustainability of the aquaculture sector. To achieve the 30% local food production target by 2030, Singapore needs to address geographic limitations, supply-chain dependence, labour costs, and energy consumption. Thus, embracing urban environments, ensuring product freshness, and implementing advanced technologies like auto-feeders, IoT, and recirculating systems are important approaches for supporting the expansion of the local aquaculture industry. Alongside these opportunities, this review also considers capital cost, energy intensity, workforce competency, maintenance burden, and cybersecurity exposure as practical constraints on adoption. This review is expected to provide a practical plan to integrating automation technologies, safety management practices, and sustainable production strategies in urban aquaculture development.

Keywords Food safety; Local produce target; Fish farming processes; Automation in aquaculture; Fish farming industry; Onshore fish farming

1 Introduction

Food security concerns and potential disruptions to global supply chains have encouraged Singapore to increase local food production from 10% to 30% by 2030. Fish and shrimp are an important source of this food, but the main challenges are labour cost and the space available for sustainable aquaculture. This study sets out to understand the processes of fish farming and to identify where automation may be applied across breeding, rearing and transportation. A key objective is to identify and understand existing technologies and solutions for fish sorting and harvesting. A third aim is to characterise the onshore fish-farming industry in Singapore, identifying the key players in the aquaculture value chain and the automation opportunities in onshore farming. The research follows a structured approach, beginning with the challenges and opportunities in aquaculture both locally and globally, then examining the fish-farming process, reviewing the prevailing safety practices and standards, and culminating in a risk assessment of two pivotal activities: feeding and harvesting. The insights are intended to strengthen understanding of the aquaculture industry in Singapore and worldwide, and to inform strategies for increasing local fish production, reinforcing food safety and supporting the sector.

Internationally, the aquaculture sector has moved rapidly towards what is variously described as smart, precision or digital aquaculture. Reviews of Internet of Things (IoT)-based smart aquaculture management systems describe sensor networks, software interfacing, artificial intelligence, cloud computing and data storage as the constituent technologies of contemporary farm monitoring, and identify water quality management, feeding behaviour monitoring and animal health surveillance as the dominant application areas (Yadav et al., 2023). Parallel work on the energy performance of land-based systems has drawn attention to the fact that the water-efficiency gains of

recirculating aquaculture systems (RAS) are obtained at a substantial electrical energy cost, and that reported energy intensities vary widely with system design and species (Badiola et al., 2018). Together, these strands indicate that automation in aquaculture is best understood not as a uniform improvement but as a set of trade-offs between productivity, resource use, capital intensity and operational risk.

Unlike previous discussions, which focus mainly on aquaculture production technologies or on their environmental performance, this review integrates automation, occupational safety and workplace risk management within the specific context of Singapore's local fish production. Occupational safety and health in aquaculture has been characterised internationally as an under-reported area with a hazard profile spanning safety, physical, chemical, biological, ergonomic and psychosocial categories (Lloyd's Register Foundation, 2021), yet it is rarely treated alongside automation adoption in the same analysis. Bringing the two together is the intended contribution of this review, and it is directly relevant to land-scarce, high-regulation urban settings where automation is being adopted quickly and where the workforce composition is changing as a result. This project investigates the safety practices relevant to aquaculture both offshore and onshore. The relevant safety standards for aquaculture are reviewed, and a risk assessment is performed for a typical aquaculture process.

The following are the major tasks of the project.

- 1 Understand the aquaculture challenges and opportunities in Singapore and overseas
- 2 Study and review the process of fish farming
- 3 Conduct a review of safety practice and standards in fish farming
- 4 Conduct a risk assessment for the activities, feeding and harvesting phase

2 Methods

2.1 Review design

This work is a structured narrative review with an applied risk assessment component. It was not registered as a systematic review and does not claim systematic-review status: the evidence base combines peer-reviewed literature with regulatory instruments, national standards and industry documentation, and the latter categories are not indexed in a way that permits exhaustive, reproducible retrieval. The review design was instead structured to be transparent about what was consulted and on what basis material was retained.

The review proceeded in four sequential stages, corresponding to the four tasks stated in Section 1: (i) characterisation of aquaculture challenges and opportunities in Singapore and internationally; (ii) description of the fish farming process and the production systems in use; (iii) review of safety practices and standards applicable to aquaculture work; and (iv) application of a formal risk assessment methodology to two representative activities, feeding and harvesting, in both manual and automated modes.

2.2 Information sources

Three categories of source were consulted.

Peer-reviewed literature. Searches were conducted in ScienceDirect, Scopus and Google Scholar. Search terms were combined from three groups: production system terms ("aquaculture", "fish farming", "recirculating aquaculture system", "RAS", "onshore", "offshore"); technology terms ("automation", "auto-feeder", "Internet of Things", "IoT", "smart aquaculture", "precision aquaculture"); and safety terms ("occupational safety and health", "risk assessment", "hazard", "injury"). Singapore-specific searches additionally used "Singapore" and "30 by 30". Records were screened by title and abstract, and retained records were read in full.

Regulatory and standards material. National and international instruments governing aquaculture practice and workplace safety were consulted directly from the issuing bodies, including the Singapore Food Agency, the Singapore Standards Council, the Ministry of Manpower and the Workplace Safety and Health Council in Singapore, and equivalent bodies in Prince Edward Island (Canada), Malaysia and Western Australia. These sources are not indexed in academic databases and were located through the issuing organisations' own publication registers.

Industry and operator documentation. Publicly available descriptions of production systems operated by licensed Singapore farms were used to characterise the technologies in current local use. Two operators were selected as illustrative cases, one offshore and one onshore, on the basis that they are licensed by the Singapore Food Agency, operate at commercial rather than pilot scale, and have publicly documented their production technology. These cases are illustrative of the systems in use and are not presented as a representative sample of the sector.

2.3 Selection criteria

Material was retained where it met all of the following criteria: published in English; directly concerned with aquaculture production systems, aquaculture automation, or occupational safety and health in aquaculture or a closely comparable primary industry; and either applicable to tropical or land-constrained production settings, or providing a standard or framework transferable to such settings.

Material was excluded where it addressed fish biology, genetics, nutrition or disease without reference to production process, workforce or safety; where it concerned capture fisheries rather than aquaculture, except where the occupational safety content was explicitly transferable; where it was promotional material without a verifiable technical or regulatory basis; and where a more recent authoritative version of the same standard or dataset was available.

No formal quality appraisal instrument was applied to individual studies, and no meta-analysis was undertaken. This is a limitation of the review and is discussed in Section 4.7.

2.4 Risk assessment framework

The risk assessment reported in Section 3.5 follows the three-step methodology set out in the Code of Practice on Workplace Safety and Health Risk Management issued by the Workplace Safety and Health Council in Singapore (WSH Council, 2011), supported by the risk management guidance of the Ministry of Manpower (Ministry of Manpower, n.d.). The three steps are hazard identification, risk evaluation and risk control.

Hazards were identified by decomposing each activity into its constituent work steps and classifying the hazards encountered at each step under the categories used in the international aquaculture occupational safety literature: safety, physical, chemical, biological, ergonomic and psychosocial (Lloyd's Register Foundation, 2021). Risk was evaluated using the semi-quantitative matrix specified in the Code of Practice, in which severity and likelihood are each rated on a five-point scale and multiplied to give a Risk Priority Number (RPN), which is then classified as low, medium or high risk. The matrix and classification bands are reproduced in Figure 1.

Risk controls were assigned following the hierarchy of controls, in which elimination and substitution are preferred to engineering controls, engineering controls to administrative controls, and administrative controls to personal protective equipment. Where the risk assessment tables in Section 3.5 record personal protective equipment as an existing control, the additional control column identifies the higher-order measure recommended. Two activities, feeding and harvesting, were selected for assessment because they are performed at high frequency across the production cycle, they occur in both manual and automated modes in Singapore farms, and they involve direct worker interaction with water, live stock and moving machinery.

The risk assessments presented are generic worked examples applied to a representative aquaculture process. They were not conducted at a named facility, and the ratings should be read as illustrative of the method rather than as an audit of any particular farm. A site-specific assessment would be required before the ratings could be relied upon operationally.

Classification of Risk (Risk Matrix) (‘RPN’ column)

Likelihood	Rare (1)	Remote (2)	Occasional (3)	Frequent (4)	Almost Certain (5)
Severity					
Catastrophic (5)	5	10	15	20	25
Major (4)	4	8	12	16	20
Moderate (3)	3	6	9	12	15
Minor (2)	2	4	6	8	10
Negligible (1)	1	2	3	4	5

Risk level	Risk Acceptability	Recommended Actions
Low Risk	Acceptable	- No additional risk control measures may be needed. - Frequent review and monitoring of hazards are required to ensure that the risk level assigned is accurate and does not increase over time.
Medium Risk	Tolerable	- A careful evaluation of the hazards should be carried out to ensure that the risk level is reduced to as low as reasonably practicable (ALARP) within a defined time period. - Interim risk control measures, such as administrative controls or PPE, may be implemented while longer term measures are being established. - Management attention is required.
High Risk	Not acceptable	- High Risk level must be reduced to at least Medium Risk before work commences. - There should not be any interim risk control measures. Risk control measures should not be overly dependent on PPE or appliances. - If practicable, the hazard should be eliminated before work commences. - Management review is required before work commences.

Severity Scoring (‘S’ column)

Level	Severity	Description
5	Catastrophic	Fatality, fatal diseases or multiple major injuries.
4	Major	Serious injuries or life-threatening occupational disease (includes amputations, major fractures, multiple injuries, occupational cancer, acute poisoning).
3	Moderate	Injury requiring medical treatment or ill-health leading to disability (includes lacerations, burns, sprains, minor fractures, dermatitis, deafness, work-related upper limb disorders).
2	Minor	Injury or ill-health requiring first-aid only (includes minor cuts and bruises, irritation, ill-health with temporary discomfort).
1	Negligible	Not likely to cause injury or ill-health.

Likelihood Scoring (‘L’ column)

Level	Likelihood	Description
1	Rare	Not expected to occur but still possible.
2	Remote	Not likely to occur under normal circumstances.
3	Occasional	Possible or known to occur.
4	Frequent	Common occurrence.
5	Almost Certain	Continual or repeating experience.

Figure 1 Risk matrix and classification of risk (WSH Council, 2011)

3 Results

3.1 Aquaculture challenges and opportunities in Singapore and global contexts

According to Funge-Smith and Phillips (2001), there are four types of aquaculture systems (Table 1). Singapore uses water-based systems, traditional and modern coastal net-cage farms and close-containment floating farms, and land-based systems, including tank-based culture, open-pond methods and indoor multi-tier systems (World Aquaculture Society, 2020; Singapore Food Agency, 2022a).

Table 1 Four types of Aquaculture systems

No.	Aquaculture system
1	Water-based systems in cages and pens, found in inshore and offshore settings.
2	Land-based systems: rain-fed ponds, irrigated channels, tanks and flow-through systems.
3	Recirculating aquaculture systems (RAS): enclosed systems and open pond-based recirculation.
4	Integrated farming systems: livestock-fish and combined agriculture-fish systems in farms and irrigation ponds.

Since the pandemic, the island nation has adopted a “30-by-30” target to achieve food security by producing 30% of its nutritional needs by 2030. The two types of aquaculture system used in Singapore are offshore and onshore farming (World Aquaculture Society, 2020). Singapore’s aquaculture industry comprises 110 licensed land- and sea-based fish farms and currently supplies about 10 percent of local fish consumption (Singapore Food Agency, 2022b). Of this, roughly 85 percent comes from marine aquaculture (offshore cage farming) and 15 percent from freshwater aquaculture in inland waterways (Shen et al., 2021). Marine coastal farms are located in the Johor Straits - Punggol, Serangoon/Loyang, Lim Chu Kang and Pulau Ubin producing species such as grouper, seabass, snapper and milkfish (Singapore Food Agency, 2022c); freshwater farms produce Asian seabass, milkfish, grey mullet, snappers, golden pompano, hybrid groupers and shrimp (Shen et al., 2021).

Non-food species fall into three groups: aquaculture feed (microalgae, macroalgae, phytoplankton, zooplankton and other planktonic organisms); ornamentals (fish, corals, shrimp and molluscs); and organisms used in flow-through or recirculating systems to improve marine environmental conditions (Leal et al., 2018). Two main factors limit local production to about 10 percent: limited coastal area and species range, and heavy reliance on global supply chains. To reach the 30% target, Singapore must convert these challenges into opportunities.

3.2 Species for food and non-food farmed in Singapore

The Singapore Food Agency regulates and licenses land- and sea-based farms (Singapore Food Agency, 2022b). Diseased fish represent a commercial risk, and stocks are affected when processes are not robust. The farming cycle for *Lates calcarifer* (Asian seabass) comprises five stages, fertilised egg, larva, fry (juvenile), fingerling and broodstock completed over up to five years; males are typically harvested at about two years and nine months, while females are retained longer to continue the cycle (FAO, 2009; Shen et al., 2021). Offshore, Singapore Aquaculture Technologies Pte Ltd operates floating tank-based farms (a 3,000 m² floating tank) using data- and AI-driven Recirculating Aquaculture Systems (RAS) for disease prevention and performance monitoring (Singapore Aquaculture Technologies, 2022). Onshore, Apollo Aquaculture Group Pte Ltd uses land-based RAS to recycle and reuse water with 24/7 monitoring and control; its modular three-tier design enables high-rise farming and can be dismantled and re-established away from the coastline, including in urban areas (URA Singapore, 2017).

Waste falls into three categories; solid, dissolved and pathogenic (Miller and Semmens, 2002). In a well-managed farm, roughly 30% of feed becomes solid waste (Miller and Semmens, 2002).

3.3 Challenges and opportunities

There are two main reasons for the 10 percent limited aquaculture production contributing to local food supply. Firstly, limited coastal regions and species for farming, second being heavily reliant from global fish food supply chains. Hence to achieve 30% production local food supply, the Island nation recognises Challenges and Opportunities from the current food production environment (Table 2).

3.4 Study and review of the fish farming process

Singapore Food Agency regulates and provides licenses to fish farms both land and sea based in the island nation. The list of licensed farms is available from the Singapore Food Agency (2022b). Diseased fish is a business/commercial risk and fish stocks are impacted when processes are not robust.

Table 2 Description of Challenges and Opportunities

Description of Challenges and Opportunities	
Challenges	Opportunities
Global Supply Chain Issues (Food Security)	Increase Local Fish Production
Limited coastal regions	Utility in Urban Environments
Labour cost and Skilled Workforce	Freshness and Longevity
Energy cost	Deployment of Technologies such as Auto-feeder and Internet-of-Things (IoT), water monitoring systems and Recirculating Aquaculture Systems (RAS)

Fish farming process for *Lates calcarifer* Asian Seabass comprises 5 cycles completed up to 5 years. Male Asian Seabass is intended for harvest within 2 years and 9 months, and females are kept longer to continue the 5 cycles from Fertilisation of egg, Larva, Fry (Juvenile), Fingerling and broodstock (FAO, 2009; Shen et al., 2021).

3.4.1 Farming system offshore

Two main system types are seen in Singapore's offshore farming: the 3,000 m² floating tank and the recirculating aquaculture system (Singapore Aquaculture Technologies, 2022). In the Island-nation Singapore Aquaculture Technologies (SAT) Pte Ltd employs smart farming pioneering floating tank-based fish farms with data and artificial Intelligence driven RAS (Recirculating Aquaculture System). Enabling disease prevention and performance monitoring systems for multifactorial data analysis.

3.4.2 Farming system onshore

In Singapore's onshore farming there are also two systems, the fish transfer pump and the manual feed process (URA Singapore, 2017). Apollo Aquaculture Group Pte Ltd uses a Land-based food fish culture system to prevent disease in fish food. The RAS (Recirculating Aquaculture System) is also used in land-based fish farming operations. The intent is to recycle and reuse water resources, 24-hour monitoring system and control system is used in their farming environments. Innovation is essential and for this company, a prototype modular three-tier farm enables high-rise farming of fish. The modularity of the structure allows it to dismantle and set up a farm away from the coastline even in urban areas.

3.4.3 Waste removal and cleaning

There are three categories; Solids, Dissolvable and Pathogenic Waste (Miller and Semmens, 2002), in an effectively managed farm, approximately 30% of the feed used will become solid waste. Table 3 summarises each waste category and the corresponding cleaning or control approach (Miller and Semmens, 2002).

Table 3 Waste removal and cleaning

No.	Type of waste	Cleaning / control
1	Solid waste from uneaten feed and faeces; if not cleared, becomes total suspended and total dissolved solids.	Rapid capture reduces the dissolved organic load.
2	Dissolved waste (ammonia, nitrite, nitrate, phosphorus, organic matter); un-ionised ammonia, excreted through the gills, is the most toxic form.	Autotrophic bacteria convert ammonia to less toxic forms; providing a large surface area for bacterial growth is the most effective approach.
3	Pathogenic waste; farms can increase potentially pathogenic organisms in effluent.	Chlorination, ultraviolet radiation and ozonation; UV treatment occurs in a chamber and is not harmful downstream.

3.4.4 Review of safety practice and standards in fish farming

Safety standards in fish farming are unique and developed over years of research in global fish farming industries; these standards are non-exhaustive and adapted to the requirements and capacity of localities. There are no

specific policies that any one country must adhere to, and each nation would follow their own best practices. The Singapore Standard 670:2021 (Singapore Standards Council, 2021) titled Specification for good aquaculture practice is one example of aquaculture farm production in the Island nation; (a) Food safety and quality; (b) Animal health and welfare; (c) Environmental integrity; and (d) Socio-economic aspects. However, the article does not specify aquaculture occupational health and safety practices intended for this study (Table 4).

Examples of Safety standards used worldwide for aquaculture normative Safety Practice:

Table 4 Safety standards used in aquaculture worldwide

No.	Standard / code of practice	Summary
1	Aquaculture Safety Code of Practice, Prince Edward Island (Prince Edward Island Aquaculture, 2008)	Requires aquaculturists and workers to identify and control hazards; covers safe use of tools and machinery and safe work practices for onshore and offshore operations.
2	Guidelines on Occupational Safety and Health in Fishing and Aquaculture Operations, Malaysia (OSH Act 1994) (Department of Occupational Safety and Health, 2001)	Applies to fishing operations in Malaysia; assists the industry in preventing accidents, injury, disease and death.
3	Aquaculture Industry OHS Code, Western Australia (2002) (Aquaculture Council of Western Australia, 2002)	Requires workplaces to meet statutory requirements under the Marine Act, OSH Act and Fish Resources Management Act; defines responsibilities of licence holders, managers, contractors and operators.
4	Code of Practice for Diving at Work, Singapore (SS 511:2018) (WSH Council, 2021)	Covers offshore diving; not prescriptive but requires proper planning and assessment of diving operations and compressed-air breathing apparatus.
5	Occupational health and safety in the aquaculture industry: a global review (Lloyd's Register Foundation, 2021)	Reviews OHS incident evidence and intervention effectiveness; categorises hazards as safety, physical, chemical, biological, ergonomic and psychosocial.

3.5 Risk assessment for feeding and harvesting activities

The main activities involved in manual and automated aquaculture operations, including feeding, equipment maintenance, and harvesting, are summarized in Table 5.

Table 5 Activities for both onshore and offshore farming

Activity	Manual	Automated
Feeding	Personnel wear non-slip boots and gloves and approach a tank or cage with a container of feed (URA Singapore, 2017).	An auto-feeder, scheduled by personnel, dispenses feed into the tank or cage (Hatchery International, 2021).
Equipment maintenance	Structural design provides pipes, containment tanks, water drainage and recirculating systems (Singapore Aquaculture Technologies, 2022).	Machinery includes feed dispensers, material conveyors, RGB cameras for movement and disease monitoring, close-containment tanks and water-quality sensors (Singapore Aquaculture Technologies, 2022).
Harvest	Personnel wear non-slip boots and carry containers to pick and place live fish stock (Singapore Aquaculture Technologies, 2022).	A conveyor transfers live fish stock while fresh water cleans the fish (Farm-Futurism, 2022).

Risk management According to the Code of Practice on Risk Management:

The steps in Hazard Identification, Risk Evaluation and Risk Control specify the methodology and requirements of this Code of Practice (WSH Council, 2011). Severity, likelihood, Risk Priority Number and the classification of risk are shown in Figure 1. The resulting assessments for manual feeding and harvesting are presented in Table 6 and Table 7, and for machinery-assisted operations in Table 8 and Table 9. Severity, Likelihood, Risk Priority Number and Classification of Risk is found with the infographics (Figure 1) followed by the Risk Assessment Form (Ministry of Manpower, n.d.).

Table 6 Risk assessment for manual feeding activities in aquaculture

Company	NA
Conducted by (name, designation, date)	Y. van Ram Sri, Researcher, 20 Aug 2026
Process / location	Manual feeding in aquaculture
Approved by (name, designation, date)	Sri Yahweh van Ram, Researcher, 20 Aug 2026
Last review date	NA
Next review date	20-Jul-29

Table 7 Risk assessment for machinery-assisted aquaculture operations

No.	Work activity	Hazard	Possible accident / ill-health & persons at risk	Existing risk control	Risk (S × L = RPN, level)	Additional risk control	Action officer
1	Transporting fish feed	Ergonomic; lifting heavy loads	Soft-tissue injury (sprains, strains); operations staff	Train on proper lifting technique; strength training	3 × 3 = 9 (Medium)	Distribute load by dividing the weight	Operations Manager
2	Fish feeding	Physical; slippery/moist floor (slip, trip, fall)	Fractures and dislocated joints; operations staff	PPE non-slip boots; ventilators	3 × 2 = 6 (Medium)	Regular maintenance and inspection of premises	Operations Manager (on-going)
3	Fish feeding	Ergonomic; prolonged standing, repetitive strain	Soft-tissue injury (sprains, strains); operations staff	Adequate lighting; distribute load	3 × 3 = 9 (Medium)	Worker rotation; lifting-technique training	Operations Manager (on-going)
4	Fish feeding	Biological; inhalation of pathogenic wastewater	Legionella pneumophila infection; operations staff	Service ventilators every 6 months with anti-bacterial wash	2 × 4 = 8 (Medium)	Engineering control of aerosol generation at source; quarterly antibacterial treatment	Operations Manager
5	Storage of fish feed	Biological; contamination of feed	Diseased livestock; damaged goods	Dry, clean storage; monitor feed expiry	3 × 3 = 9 (Medium)	Monthly maintenance of storage facility	Operations Manager (on-going)
6	Shelving and storage of feed	Physical; falling object (feed container)	Head injury, potentially fatal; operations staff	Safety harness; protective helmet	4 × 2 = 8 (Medium)	Regular inspection; keep feed within designated shelving	Operations Manager
7	Harvesting	Needle-stick objects and sharp teeth	Incised/puncture wound, laceration, infection; harvesting staff	Cut-resistant gloves; adequate lighting	3 × 3 = 9 (Medium)	Handling tools to avoid direct hand contact; worker rotation	Operations & Admin Manager (on-going)
8	Transporting the harvest	Ergonomic; lifting heavy loads	Soft-tissue injury; operations staff	Train on lifting technique; strength training	3 × 3 = 9 (Medium)	Distribute load by dividing weight	Operations Manager
9	Transporting the harvest	Physical; slippery floor (slip, trip, fall)	Fractures and dislocated joints; operations staff	PPE non-slip boots; ventilators	3 × 2 = 6 (Medium)	Regular maintenance and inspection	Operations Manager (on-going)

Table 8 Risk assessment information for machinery-assisted aquaculture operations

Company	NA
Conducted by (name, designation, date)	Y. van Ram Sri, Researcher, 20 Aug 2026
Process / location	Machinery used in the aquaculture farming system
Approved by (name, designation, date)	Sri Yahweh van Ram, Researcher, 20 Aug 2026
Last review date	NA
Next review date	20-Jul-29

Table 9 Risk assessment matrix for machinery-assisted aquaculture operations

No.	Work activity	Hazard	Possible accident / ill-health & persons at risk	Existing risk control	Risk ($S \times L$ = RPN, level)	Additional risk control	Action officer
1	Feeding with a pre-programmed automated machine	Machine malfunction scatters feed → slip, trip, fall	Head injury, incised/puncture wound, laceration, infection; operations engineer	Emergency-stop button; dispenser maintenance; reset and re-upload programme	$3 \times 3 = 9$ (Medium)	Containment of dispensed feed at the discharge point; adequate lighting; feed monitoring	Operations Manager (monthly)
2	Harvesting: transfer-conveyor machine	Hand caught in conveyor while removing fish material	Amputated phalanges, crushed bones, joint dislocation; harvesting staff	Safety cage; emergency-stop button; safety signage	$3 \times 2 = 6$ (Medium)	Interlocked guarding and lockout-tagout procedure for clearing blockages; safe entry procedure	Area Manager (on-going)

4 Discussion

4.1 Safety management as an enabler of automation

The thirty-by-thirty vision to expand local fish production must account for the present-day cap of 10 percent of the existing fish production and possible fluctuating percentages where fish stock may be diseased, amounting to losses in farmed production and total output. Therefore, in order to revive the industry, the deployment of safe work practices through the study of aquaculture production processes are the enablers to enrich the workforce and prevent safety hazards from occurring in offshore and onshore aquaculture workplaces. The international aquaculture safety standards provide a lens into the aquaculture occupational health and safety practices from Prince Edward Island, Malaysia, Western Australia, Lloyd's Register Foundation and diving code of practice from Singapore. Code of practice is instrumental when conducting risk assessments for offshore and onshore aquaculture processes from manual labour to machine aided automated feed and harvest processes.

4.2 Economic barriers to adoption

The capital intensity of automated systems is a substantial barrier, particularly for the smaller operators that make up much of Singapore's licensed farm population. Multi-tier land-based facilities and instrumented floating tank systems require capital outlay well beyond that of traditional net-cage operations, and the payback period depends on sustained production volume and price realisation that a small operator may not be able to guarantee. Where automation is adopted incrementally, integration cost between subsystems supplied by different vendors becomes a further expense that is frequently underestimated at the planning stage. The technologies described in this review may therefore support productivity and sustainability gains, but the conditions under which those gains are realised are specific and are not met by every operator.

4.3 Energy consumption of recirculating systems

RAS delivers marked reductions in water exchange, but at a significant electrical energy cost. Reported energy intensities vary widely across published studies, reflecting differences in species, system scale, thermal regulation requirements and boundary definitions (Badiola et al., 2018). Water circulation and thermal control consistently account for the largest share of demand. For Singapore, where electricity is generated predominantly from imported natural gas, this energy intensity has direct implications for both operating margin and the carbon footprint of locally produced fish. A production target expressed in volume terms does not offer a guarantee to environmental improvement over imported products; the comparison depends on the energy profile of the production system and the emissions intensity of the electricity supply.

4.4 Technical workforce requirements

Automation changes the composition of the aquaculture workforce as well as its size. Instrumented systems require competencies in sensor calibration, control system operation, data interpretation and mechanical maintenance that are not typically present in a workforce trained for manual husbandry. The occupational safety consequence is significant: a worker who does not understand the control logic of an automated feeder is poorly placed to recognise abnormal operation, and a worker who has not been trained in energy isolation is at elevated

risk when clearing a conveyor. Competency requirements should therefore be treated as an integral part of automation planning rather than as a downstream training matter, and safe operating procedures should be developed in parallel with system commissioning rather than after it.

4.5 Maintenance and system reliability

Highly automated closed systems concentrate dependency on a small number of critical subsystems. Failure of circulation, oxygenation or biofiltration in a densely stocked RAS can produce rapid stock loss, which represents both a commercial risk and a stressor on staff responding to the event. Maintenance access is itself a hazard-generating activity: the entanglement and crush risks identified in Table 7 arise principally during clearing and servicing rather than during normal operation. Reliability planning, including redundancy of critical plant, alarm escalation and defined out-of-hours response, is accordingly a safety matter and not solely a production matter.

4.6 Cybersecurity of IoT-based systems

Where feeding, aeration and water quality control are network-connected, the integrity and availability of the control network become determinants of both stock survival and worker safety. Unauthorised modification of a feeding schedule or a setpoint, or denial of access to monitoring data, could produce consequences equivalent to a mechanical failure. Sensor networks in aquaculture are frequently assembled from heterogeneous commercial components with varying security maturity, and are often deployed in physically exposed locations where device access cannot be tightly controlled. Segmentation of control networks from general business networks, authenticated access to setpoint changes, logging of control actions, and a documented manual fallback procedure are reasonable baseline measures. This intersection between process safety and information security is under-addressed in the aquaculture literature relative to its potential consequence.

4.7 Limitations of this review

Several limitations should be noted. The review is narrative rather than systematic; no protocol was registered, no formal quality appraisal instrument was applied, and the inclusion of regulatory and industry material means that exhaustive reproducible retrieval was not possible. The evidence base is drawn from secondary sources, and no farm-level operational or injury data were collected; the risk assessments presented are generic worked examples of the method rather than site-specific assessments. Aquaculture occupational injury data are in any case sparse and inconsistently reported internationally, which constrains any quantitative claim about the safety effect of automation. Finally, English-language restriction may have excluded relevant regional material, particularly from Southeast Asian producers whose operating context is closest to Singapore's. These limitations indicate the value of future work based on primary farm-level data, including operational energy use, injury and near-miss reporting, and structured comparison of hazard profiles before and after automation at the same facility.

5 Conclusion

There remain limited aquaculture safety publications relating to Singapore. This review indicates that automation technologies, including automated feeding systems, IoT-based monitoring and recirculating aquaculture systems, offer meaningful opportunities for strengthening Singapore's local fish production and for progressing towards the 30-by-30 target. Realising these opportunities depends on the coordinated management of the constraints identified: capital cost, energy consumption, workforce competency, maintenance and reliability, and the cybersecurity of connected control systems. Automation does not remove occupational risk from aquaculture work; it redistributes it, concentrating on a smaller number of higher-consequence hazards among technical staff while reducing the distributed ergonomic and biological burden on manual workers. Integrating automation with sustainable production strategies and with a risk management approach grounded in an established code of practice will be essential for achieving long-term aquaculture development goals.

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Author contributions

Yahweh Van Ram Sri conceived and designed the study, performed the literature review and risk assessment, prepared all tables and figures, and wrote the manuscript.

Conflict of Interest Disclosure

The author affirms that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

References

- Aquaculture Council of Western Australia, 2002, Aquaculture industry occupational health and safety code, Aquaculture Council of Western Australia.
- Badiola M., Basurko O.C., Piedrahita R., Hundley P., and Mendiola D., 2018, Energy use in recirculating aquaculture systems (RAS): a review, *Aquacultural Engineering*, 81: 57-70.
<https://doi.org/10.1016/j.aquaeng.2018.03.003>
- Department of Occupational Safety and Health, 2001, Guidelines on occupational safety and health in fishing and aquaculture operations, DOSH Malaysia.
- FAO, 2009, *Lates calcarifer*, in *Cultured Aquatic Species Fact Sheets*, edited by Crespi V. and New M., Food and Agriculture Organization of the United Nations.
- Farm-Futurism, 2022, How Israel became the most advanced in aquaculture and fish farming, Farm-Futurism.
- Funge-Smith S., and Phillips M.J., 2001, Aquaculture systems and species, Food and Agriculture Organization of the United Nations.
- Hatchery International, 2021, Smart feeding: automated feeder fundamentals, Hatchery International.
- Leal M.C., Rocha R.J.M., Rosa R., and Calado R., 2018, Aquaculture of marine non-food organisms: what, why and how, *Reviews in Aquaculture*, 10: 400-423.
<https://doi.org/10.1111/raq.12168>
- Lloyd's Register Foundation, 2021, Occupational safety and health in the aquaculture industry: a global review, Lloyd's Register Foundation.
- Miller D., and Semmens K., 2002, Waste management in aquaculture, West Virginia University Extension.
- Ministry of Manpower, n.d., Risk management, Ministry of Manpower Singapore.
- Prince Edward Island Aquaculture, 2008, Aquaculture safety code of practice, Workers Compensation Board of Prince Edward Island.
- Shen Y., Ma K., and Yue G.H., 2021, Status, challenges and trends of aquaculture in Singapore, *Aquaculture*, 533: 736210.
<https://doi.org/10.1016/j.aquaculture.2020.736210>
- Singapore Aquaculture Technologies, 2022, Farming technologies, Singapore Aquaculture Technologies.
- Singapore Food Agency, 2022a, Farming technologies, Singapore Food Agency (SFA).
- Singapore Food Agency, 2022b, List of licensed fish culture farms, Singapore Food Agency (SFA).
- Singapore Food Agency, 2022c, Food farms: coastal fish farming in Singapore, Singapore Food Agency (SFA).
- Singapore Standards Council, 2021, Singapore Standard SS 670:2021 - Specification for good aquaculture practice, Enterprise Singapore.
- URA Singapore, 2017, Farming innovations in Singapore: can we farm fish on land?, Urban Redevelopment Authority of Singapore.
- World Aquaculture Society, 2020, The Singapore aquaculture industry - contributing to Singapore's food security, World Aquaculture Society.
- WSH Council, 2011, Code of practice on workplace safety and health risk management, Workplace Safety and Health Council, Singapore.
- WSH Council, 2021, Workplace safety and health guidelines: inland/inshore commercial diving (SS 511:2018), Workplace Safety and Health Council, Singapore.
- Yadav A., Noori M.T., Biswas A., and Min B., 2023, A concise review on the recent developments in the Internet of Things (IoT)-based smart aquaculture practices, *Reviews in Fisheries Science and Aquaculture*, 31(1): 103-118.
<https://doi.org/10.1080/23308249.2022.2090228>



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