Assessing Glycolipid Surfactant-Enriched Diets on Growth, Immunity and Enzymatic Activity in *Catla catla* (Hamilton, 1822)

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Abstract
Rhamnolipid, a bioactive compound derived from Pseudomonas sp. Kmka-114, aimed to assess the effects on growth, non-specific immunity and enzymatic profiles in juvenile Catla catla through graded dietary supplementation. Juvenile catla were randomly selected for thorough evaluations of blood, serum and liver tissues to determine the influence on growth metrics, non-specific immune responses, enzymatic activity and organ modulation. Throughout the feeding trial, there was a significant increase in the activities of myeloperoxidase, lactose dehydrogenase and alkaline phosphatase activity in 300 mg kg^{-1} biomass day^{-1} (6X).

Histopathological analysis of liver tissues at this concentration demonstrated no hepatic cell damage, indicating the surfactant's safety for the fish. Following a withdrawal period, Catla catla displayed a stable assay result, highlighting the necessity for further detailed studies to clarify the underlying mechanisms.

Keywords: *Catla catla*, Rhamnolipid, Non-specific immunity, Histology, Mangrove.

Introduction

Catla catla (Hamilton, 1822), the fastest growing Indian major carp cultured throughout the Indian sub-continent[15]. Interspecies interaction makes it easy for cultivating as a polyculture mode along with species like mrigala *Cirrhinus mrigala* and rohu *Labeo rohita*[16]. It holds a central role as preferable carp due to its rapid growth, consumer preference and high market value. The expansion of catla farming has been facilitated by advancements in hatchery technologies and the large-scale production of captive catla seed. However, the transition towards more intensive aquaculture practices has led to a rise in disease outbreaks in culture ponds, posing a significant challenge to sustainable freshwater aquaculture and contributing to considerable economic losses.

In the light of increasing concerns regarding the use of antibiotics in aquaculture, particularly the emergence of antibiotic-resistant bacteria, there is a heightened interest in alternative disease control strategies. The prohibition of antibiotics in several countries, alongside concerns over environmental contamination and food safety, has driven the search for substitutes that can sustain optimal productivity in aquaculture systems[32]. There is a constant search for immunostimulants compounds which can increase resistance to infectious diseases by enhancing both specific and non-specific defence mechanisms.

This has led to growing attention towards biosurfactants (BS), a class of surface-active compounds naturally produced by microorganisms. Microbial surfactants, are gaining attention for their wide range of applications, in pharmaceutical and cosmetic products for their antimicrobial, antiviral properties, gene transfection, immunomodulation and cancer therapy[37]. These compounds contain both hydrophilic and hydrophobic moiety[1]. They are preferred due to their bio-degradable and environmental nature. They are bio-compatible, possessing great stability in extreme condition such as temperature, pH and salinity[8]. Biosurfactants, inherently superior to chemically synthesized surfactants, are characterized by lower toxicity, biodegradability and greater environmental compatibility across various ecosystems[36].

The global market for surface-active agents is experiencing steady growth, with an annual increase of 6.75% and is projected to reach $3.21 billion by 2025[3]. The use of bio-surfactant is restricted due to its non-economical production. This can be commercialized by testing various cheap substrates for higher yield. Deshpande and Daniels[12] have pioneered in using animal fat waste for sophorolipid production from *Candida bambicola*.

Recent studies examined advances in understanding fish immune responses to bacterial stress which can significantly influence immune function. This innate immunity being the fundamental defence mechanism is offering protection against sudden exposure to environmental pathogens[26]. Compounds averting in compromise with this parameter can reveal mechanism of host interaction dealing with pathogens. The generated information holds the potentiality to act as a precautionary in preventing or dealing outbreak.

Although microbial surfactants have garnered increasing attention, the effects of a specific biosurfactant (rhamnolipid) on immunity and key enzymatic activities are undivulged. Keeping the above in view, the present study has been undertaken to understand to address this knowledge gap.

The experiment was focused on C*atla catla*, one of the major Indian carp. The study was conducted under optimal conditions, administering varying doses of rhamnolipid (50, 150 and 300 mg/kg) followed by a 10-day withdrawal period. Given the importance of catla cultivation and its vulnerability to ectoparasite infestations during grow-out culture, a systematic evaluation was performed. The impact of rhamnolipid was assessed on immune responses, enzyme activities and liver tissue following feed supplementation.

Material and Methods

Extraction of rhamnolipid: *Pseudomonas aeruginosa* Kmka-114 was isolated from the Karnataka Mangrove regions of India. In the mid-exponential phase, it was inoculated in 500 ml of sterile mineral salts medium and 3% (w/v) glycerol to start the synthesis of rhamnolipids. The flask was incubated at 30°C with continuous shaking at 200 rpm for a duration of 9 days. The mineral salts medium used comprised of 3.0 g KH_2PO_4, 7.0 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$ and 1 g $(NH_4)_2SO_4$ per liter, resulting in a C/N ratio of 55:1, as per the formulation by Santa Anna et al.[39]. The pH of the medium was adjusted to 7.0. At the end of incubation, the culture broths were centrifuged at 12,500 ×g for 30 minutes. After that, 6 M HCl was used to acidify the resultant supernatant to pH 3 and it was kept overnight at 4°C.

Centrifugation at 15,000 x g for 30 minutes at 4°C precipitated the surfactant, which was then dissolved in ethyl acetate. This mixture was washed once with pH 3 acidified water, dried with anhydrous sodium sulphate and then evaporated under vacuum. Following three ethyl acetate washes, the resultant oily residue was dissolved in 0.05 M sodium bicarbonate. After adding 6 M HCl to the aqueous solution to make it more acidic, rhamnolipids were extracted using ethyl acetate and then dried. Following acidification and an overnight incubation at 4°C, ethyl acetate was used to directly extract the supernatant four times. After being dried with anhydrous sodium sulphate, the mixed organic components were evaporated. After being dissolved in 0.05 M bicarbonate, the residue was filtered, acidified to pH 2 and kept at 4°C for the whole night. Finally, centrifugation (20,000 x g, 4°C) was used to collect the precipitate.

Experimental Condition: A total of 150 healthy catla (*Catla catla*) fingerlings, devoid of any observable disease symptoms as determined through meticulous gross examination of skin, fins and gills, were procured from the Parlakhamundi fish farm in Gajam, Odisha, India. A 500 L biofloc tanks with continuous aeration assistance were used to acclimatise the fingerlings to freshwater conditions for two weeks. The water temperature in the tanks was kept at a consistent 28 ± 0.5°C. During the acclimatization phase, a daily water exchange of 30% was executed and the fishes were provided with a basal diet constituting 3% of their average body weight (ABW). Each tank, including the control group, was stocked with thirty fishes having an average weight of 17 ± 26 g.

The surfactant-infused feeding trial spanned 30 days and involved three experimental groups receiving different doses: 50 $mgkg^{-1}$ biomass day^{-1} (BS1) (1x concentration), 150 $mgkg^{-1}$ biomass day^{-1}(BS2) (3x concentration) and 300 $mgkg^{-1}$ biomass day^{-1}(BS3) (6x concentration). Water quality parameters were closely monitored and kept within ideal values during the trial, following APHA[5] recommendations and regular operating procedures. Feeding of the experimental fish occurred twice daily at 8:00 hr and 17:00 hr. The surfactant-infused diet was administered for the entire 30-day trial period according to the prescribed graded levels, followed by a subsequent 10-day withdrawal period during which only basal feed was provided. This experimental design aims to comprehensively investigate the impact of surfactant infusion on the health and performance of catla fingerlings, providing valuable insights into its potential effects and withdrawal dynamics.

Diet preparation: In the feed laboratory, raw materials were meticulously prepared using locally sourced feed ingredients. Carboxymethyl cellulose (CMC), which acts as a binder, was pre-blended with a mineral and vitamin mixture that was obtained from fish farms. The dry ingredients, excluding the vitamin and mineral mixtures, underwent sieving through a 60-mesh sieve, followed by precise weighing and mixing in accurate proportions. The addition of distilled water facilitated the attainment of a homogeneous mixture while maintaining the crude protein level at approximately 30%.

Table 1
Composition of experimental and control feed.

Composition of medicated feed and control feed (mg kg^{-1}).				
Feed ingredients	**Control**	**BS1 (mg/kg of fish biomass per day)**	**BS2 (mg/kg of fish biomass per day)**	**BS3 (mg/kg of fish biomass per day)**
Fish meal	50	50	50	50
Soya meal	280	280	280	280
GNOC	260	260	260	260
Rice bran	200	200	200	200
Corn flour	150	150	150	150
Oil mix(ml)	40	40	40	40
Vitamin and mineral mixture	20	19.95	19.85	19.7
Biosurfactant (BS)	0	0.05	0.15	0.3

Subsequently, the dough was autoclaved, cooled to room temperature and then infused with the vitamin-mineral mixture and biosurfactant.

The vitamin and mineral mixture, obtained from fish farms, played a crucial role in fortifying the nutritional content of the feed. The final dough underwent a pressing process using a hand pelletizer to produce uniform-sized pellets with a diameter of 2 mm. The pellets underwent a 48-hour drying process in a hot air oven maintained at around 30°C. After drying, they were carefully sealed in airtight bags and kept for later use at 4°C, as outlined in table 1.

Sampling of Experimental Fish: On predetermined days (10, 20, 30 and 40) of the study, a systematic sampling strategy was used to evaluate the growth of fish. On each sampling day, five fish were chosen at random from each tank and their weights were recorded. Prior to blood collection, the fish were anaesthetized using 50 µl liter^{-1} of water-soluble clove oil to ensure human handling. Using a 2.0 ml hypodermal syringe, blood was extracted from the caudal vein. Blood samples ranging from 500 to 600 µl were taken and split into two distinct vials right away. EDTA, an anticoagulant, was present in a thin coating in one vial but not in the other.

Anticoagulant-filled vials were gently shaken to avoid clotting. By incubating the vials for about an hour and then centrifuging them at 3000 g for 15 minutes at 4°C, serum separation was accomplished. After using a micropipette to properly extract the resultant serum, it was kept at -20°C for further examination. This meticulous blood sampling and processing methodology ensures the preservation of blood components for subsequent assessments, contributing to the reliability of the growth-related data in the study.

Growth performance: At the beginning and at the end of the trial, body weight was measured for the fish after 1d of feed deprivation. The consumption of diet was recorded. The intial body weight (IBW), final body weight (FBW), weight gain ratio (WGR), specific growth rate (SGR) feed conversion ratio (FCR), daily feed intake (DFI) and survival rate (SR) (Table 2) were calculated as follows:

$$\text{SR}(\%) = 100 \text{ x} \frac{final\ number\ of\ fish}{initial\ number\ of\ fish}$$

$$\text{SGR}(\%) = 100 \text{ x} \frac{(\ln(final\ weight) - \ln\ (Initial\ weight)}{time\ period}$$

$$\text{FCR}\ (\%) = \frac{Feed\ intake}{final\ weight - initial\ weight}$$

$$\text{WGR}\ (\%) = 100 \text{ x} \frac{[Final\ wet\ weight\ (g) - Initial\ wet\ weight\ (g)}{Intial\ wet\ weight\ (d)}$$

$$\text{DFI}\ (\text{g/d}) = \frac{Feed\ consumption\ per\ fish\ (g)}{trial\ period\ (d)}$$

$$\text{FCR}\ = \frac{Feed\ intake\ (g)}{weight\ gain\ (g)}$$

Assessment of Non-Specific immune parameters: Numerous antibacterial substances, including proteins and enzymes including lysozyme proteolytic enzymes, complement proteins immunoglobulins, lectins and C-reactive proteins, are present in fish blood. In this study, the analysis of non-specific immune parameters including serum lysozyme, myeloperoxidase (MPO), nitroblue tetrazolium (NBT) assay, serum glucose, albumin and total protein was done.

Myeloperoxidase (MPO) Activity: MPO evaluation was conducted using Quade and Roth's approach[34]. 15 µl of fish serum was combined with 135 µl of Hank's balanced salt solution (devoid of Mg^{2+} and Ca^{2+}) and was added to microtiter plate wells. 50 µl freshly 20 mM 3,3',5,5'-tetramethyl benzidine were added. After incubating for two minutes at room temperature, 4 M sulfuric acid was added to halt it. Utilising a UV-VIS Spectrophotometer, the optical density at 450 nm was determined.

Respiratory Burst (RBT) Activity: The assay was determined as per Anderson and Siwicki[2]. 100 µl of heparinized blood was mixed with 0.2% NBT (nitroblue tetrazolium) solution of 100 µl and incubated for 30 minutes at 25°C. The NBT solution was prepared using 1x PBS (Phosphate buffer saline). After the reaction time, 50 µl of the suspension was taken out, mixed with 1 ml dimethyl formamide solution (Sigma-Aldrich) to solubilize formazan product. The tube was centrifuged at 6000 x g for 10 min and the reduction of NBT by intracellular superoxide radicals was determined. The supernatant OD was measured using a UV-VIS Spectrophotometer at 540 nm.

Table 2
Survival and growth performance and feed utilization of *Catla catla*

0-40 days experiment				
Parameters	**Control**	**BS1**	**BS2**	**BS3**
IBW(g)	17.14 ± 0.25	17.48 ± 0.192	17.09 ± 0.25	17.3 ± 0.175
FBW (g)	41.42 ± 0.25	42.98 ± 0.36	43.46 ± 0.28	45.64 ± 0.25
WG (%)	99.76 ± 0.47	98.47 ± 0.70	114.69 ± 0.53	126.12 ± 0.90
SGR %	1.72 ± 0.096	1.71 ± 0.0376	1.90 ± 0.0717	2.03 ± 0.0930
DFT	1.22 ± 0.0611	1.25 ± 0.06716	1.26 ± 0.079	1.30 ± 0.076
FCR	2.02 ± 0.002	1.97 ± 0.022	1.92 ± 0.0344	1.84 ± 0.068
SR (%)	100.00000	100.00000	100.00000	100.00000

Lysozyme Assay: It is a turbidometric assay carried out following Sankaran and Gurnani[38] with modification. 130 µl of newly prepared *Micrococcus lysodeikticus* (Sigma-Aldrich, USA) solution was mixed with 25 µl of fish serum. The bacterial suspension was made in 0.02M sodium acetate buffer with a concentration of 0.2 mg/ml. After adding the bacterial solution, the optical density was measured at 450 nm at 24°C both right away and an hour later. A standard curve was prepared using lysozyme from chicken egg-white (Himedia MB098). The lysozyme activity was calculated and represented as µg/ml based on the reduction in absorbance.

Biochemical Parameters - Blood Glucose, Total Protein, Albumin: Commercial kits from Coral Clinical Systems in India were used to measure the amounts of albumin, total protein and blood glucose. For blood glucose estimation, the Trinder method was used in conjunction with the glucose oxidase measurement[47]. Bradford[9] method was followed to generate a standard curve using a known concentration of bovine serum albumin (BSA) (Himedia MB083) to measure the serum protein. Doumas techniques with BCG (bromocresol green) dye were used to quantify the contents serum albumin[14]. The resultant values were used to calculate globulin levels by subtracting albumin content from total protein.

Estimation of Enzyme Activity

a. **Alkaline Phosphatase Activity Assay:** The alkaline phosphatase activity (ALP) in fish serum was determined in compliance with the manufacturer's instructions using a ready-to-use kit (Bio Vision, USA). To see the yellow colour shift (λ max = 405 nm) brought on by alkaline phosphatase dephosphorylation, p-nitrophenyl phosphate (pNPP) was employed as the phosphatase substrate[28].
b. **Lactate Dehydrogenase Activity Assay:** Lactate dehydrogenase (LDH) activities in fish serum were determined using a ready-to-use kit (Sigma-Aldrich, USA) in compliance with the provided instructions.
c. **Tissue Preparations:** At the conclusion of the experiment, fish from all groups were sacrificed and liver tissues from the control and highest biosurfactant-infused treated group were processed for histopathological examination. Sections fixed in paraffin were stained with hematoxylin-eosin and examined under a light microscope to record and examine histological alterations[7].

Statistical Analysis: All the data displayed were representation as the arithmetic mean ± standard error (SE) after undergoing a one-way analysis of variance (ANOVA) in SPSS (23.0 version, USA). Significance was considered at $p < 0.05$. This comprehensive and rigorous approach to data collection and analysis ensures the reliability and validity of the findings in elucidating the impact of biosurfactant infusion on various physiological and immune parameters in fish.

Results

Water quality parameters were systematically assessed every alternate day, maintaining optimal conditions across all tanks throughout the entire experimental period. Specifically, pH was sustained at 7.0 ± 0.4, dissolved oxygen at 5 ± 0.83 ppm, total alkalinity at 103 ± 2 ppm, total hardness at 94 ± 1 ppm, ammonia at 1.6 ± 0.09 ppm, nitrite at 0.03 ± 0.003 ppm, nitrate at 0.3 ± 0.06 ppm and water temperature at 28 ± 0.5°C. Growth analysis revealed slightly higher differences between the experimental and control fish groups ($p > 0.5$) (Table 2). Presentation of non-specific immune parameters is depicted in figure 1.

Notably, myeloperoxidase, respiratory burst and lysozyme activities exhibited significant variations ($p < 0.05$) in treated fish compared to the control. However, the parameters level returned to similar in all treated group with a minor difference to the control on the 40thday. The respiratory activity of all the treated groups till 20th day of the experiment was significantly higher compared to the control. The concentration group exhibited a decrease until after 20th day ($p < 0.05$) and remained nearly same even after withdrawal. Myeloperoxidase activity significantly varied across all groups until the 20thday of the feeding trial. In the post-feed infusion stages, all groups were nearly identical but elevated compared to the control. At the withdraw stages i.e. 40th day, a stability in data was observed with less to no drastic changes observed across all experimental groups compared to the control.

The data developed from the study showed serum biochemical parameters of catla fed with different level of surfactin shown in table 3. Serum albumin levels of BS1 and BS2 treated groups were observed higher compared to the BS1 which is nearly equal to the control group. Protein level at the compound withdrawal stage seems to have no relevant difference among each group. A significant drop in glucose level was observed in 3x and 6x group.

During the course of the feeding study, compared to the control group, the activities of the ALT and LDA of surfactin supplement groups were significantly different ($p < .05$). The treated BS3 group fish showed major elevation in serum enzymatic parameters. Lactate dehydrogenase activity of BS3 remained elevated and similar throughout the study. BS2 group levels were high compared to the BS1 and control. BS1 group came back to equal level with control on compound withdrawal. A difference in alkaline phosphatase activity was observed in BS 2 and BS3. Both the group activity came crashing to a comparable range on withdrawal (Fig. 2). Moreover, it was discovered that upon withdrawal, the BS2, BS3 treatment group fishes mean LDH and AKP values were higher to control.

Histological observation: Fish liver slices from the control and BS3 groups are displayed histologically in fig. 2. The BS3 was selected due to the elevated parameters compared to others. In control group, the hepatocytes are found normal

with intact architecture. The hepatic sinusoids were abundantly present. In BS3 group, normal hepatocytes were observed with absence of vacuoles. But no haemorrhage, congestion and cellular hypertrophy was observed in the treated group tissue. A few nuclear pyknosis were observed in both the groups which can be considered a natural tendency occurring during the maturation of RBCs and WBCs.

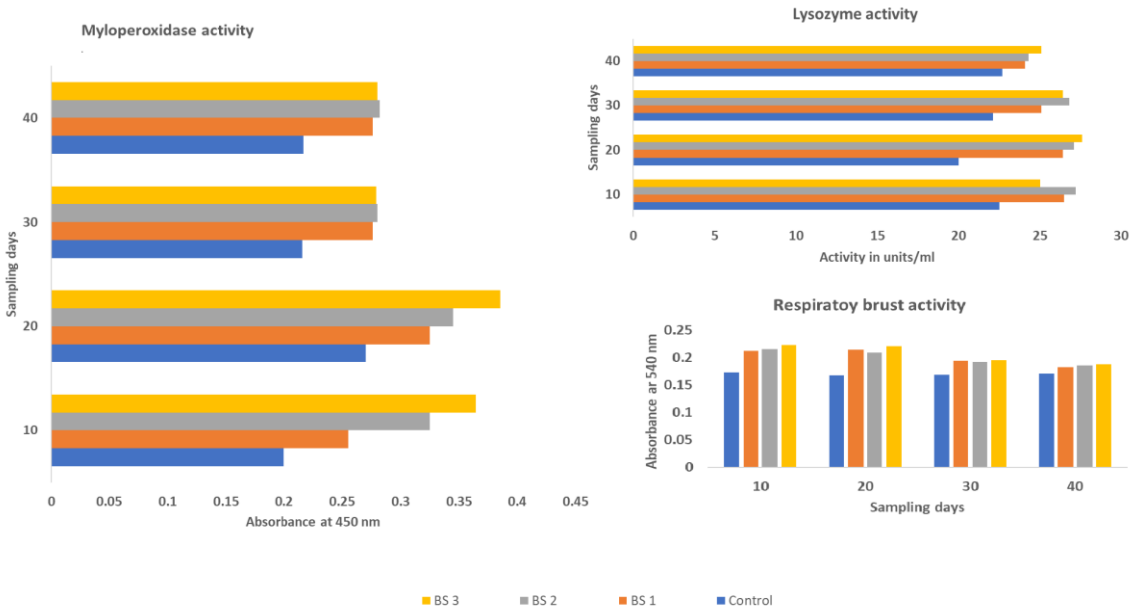

Figure 1: *Catla catla* Myeloperoxidase activity, Lysozyme activity and Respiratory burst activity

Table 3
Effect of surfactant on total serum protein, albumin, globulin and glucose in *C. catla* fingerlings.

40 days experiment				
	Serum protein (g/dl)	**Serum albumin (g/dl)**	**Globulin (g/dl)**	**Serum glucose (mg/dl)**
Control	2.50 ± 0.12	1.00 ± 0.05497	1.5 ± 0.06	120.3 ± 0.04
BS 1	2.54 ± 0.06	1.07 ± 0.03463	1.67 ± 0.026	118.4 ± 0.0053
BS 2	2.59 ± 0.096	1.52 ± 0.0127	1.07 ± 0.083	94.12 ± 0.0968
BS 3	2.71 ± 0.03	1.67 ± 0.025	1.04 ± 0.005	84.02 ± 0.045

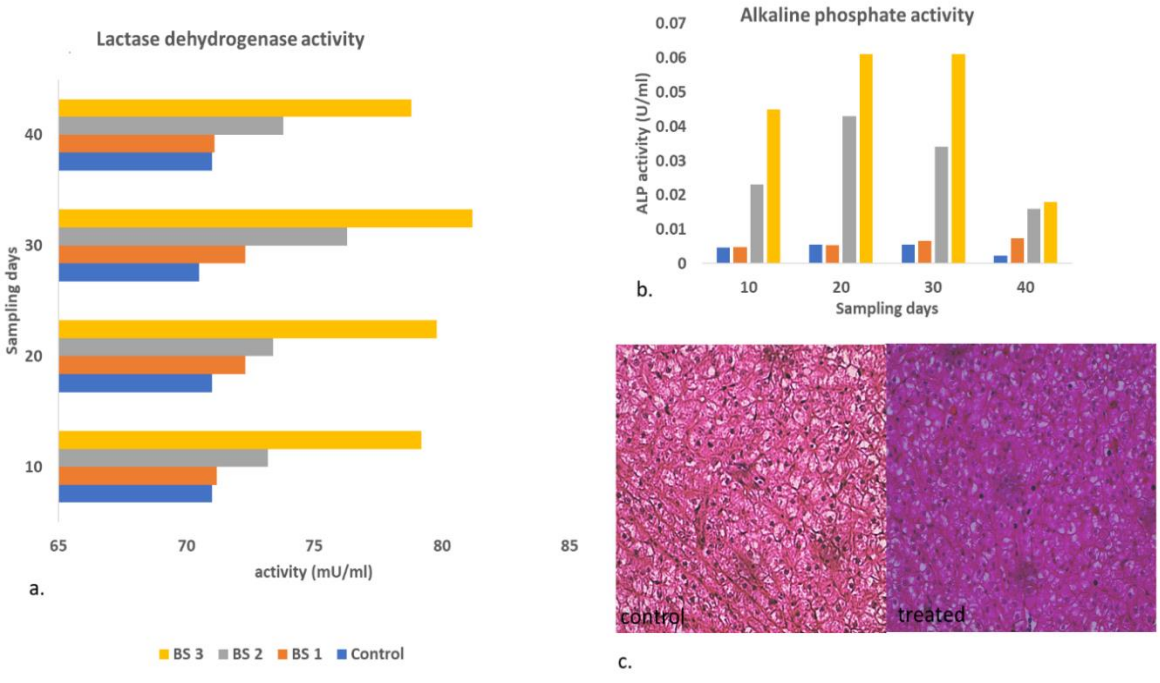

Figure 2:(a) Lactase dehydrogenase, (b) Alkaline phosphatase activity, (c) Histopathology.

Discussion

With the increasing focus on utilizing bacterial secondary metabolites for disease prevention in aquaculture, the industry is expanding rapidly and becoming highly lucrative. This study investigates the immunomodulatory and growth-promoting properties of biosurfactants (BS) derived from *Pseudomonas sp.* in fish. A review of existing literature reveals a significant gap in research on metabolites isolated from *Pseudomonas sp*. in the littoral region of Karnataka being used as fish feed supplements. The pursuit of enhanced growth in farmed food fish within specific culture periods drives farmers and entrepreneurs to explore diverse feed additives. In this study, fish were administered varying doses of the biosurfactant (50,150,300 mg/kg) through feed to evaluate its potential effects. Slightly higher growth variations were observed in the higher dose group compared to the control. Similar findings were reported by Shi et al[45] and Shao et al[43].

The growth rate of tilapia (*Oreochromis niloticus*) was higher when supplied with surfactant concentrations ranging from 12.5 to 100 mg/kg. Shi et al[44] observed that a combination of 100 mg/kg NT-antimicrobial lipopeptide, including iturins and fengycins along with surfactin, resulted in a higher yield. Currently, the mechanisms underlying surfactin-promoted growth are not clearly understood. Based on earlier reports, it can be hypothesized that the enhanced growth performance of various animals might be associated with the antimicrobial activity and the role of antimicrobial peptides as potent immune regulators[50,52]. Additionally, this effect may be influenced by unexamined factors related to water quality and host microbiomes, which were beyond the scope of this investigation.

The condition of aquatic organisms in natural communities is directly related to water quality. The productivity level of fish in cultivable water bodies depends on ecological conditions, growth and food availability. A good yield from natural food sources is always based on planktonic production systems and limnetic ecology. Further studies can focus on the mechanisms by which surfactin promotes growth. The present study also revealed distinct results in enzymatic and serum biochemical parameters compared to the control. This can also be considered in relation to growth, as observed in the tilapia study by Zhai et al[53]. Non-specific immune parameters, crucial for defending against a broad spectrum of pathogens, were analyzed, revealing significant alterations in respiratory burst activity, myeloperoxidase activity and lysozyme activity in treated fish compared to the control group.

Myeloperoxidase, an important enzyme produced by neutrophils during respiratory burst, utilizes hydrogen peroxide generated during this process to produce hypochlorous acid, which has potent microbicidal action[11,21]. Notably, these enhancements in immune responses persisted for over 10 days following the withdrawal of the biosurfactant, gradually returning to baseline levels comparable to control fish. This observation aligns with previous studies on commercial antibiotics in sea bream which highlight the immunomodulatory effects of biosurfactants, depending on dosage, administration method, duration of exposure and developmental stage of the fish[46].

Fish metabolic and physiological health can be evaluated through biochemical markers associated with serum enzymes[54]. Monitoring these parameters enables the detection of pathophysiological changes related to nutrition. This study specifically examined liver enzymes including alkaline phosphatase (ALP) and lactate dehydrogenase (LDH), which are indicators of stress and tissue damage. The findings revealed a modest yet significant increase in these enzyme levels up to 30th of the experiment, followed by a decline during the withdrawal phase. The observed rise in LDH activity in *Catla catla* may be due to the conversion of pyruvate to lactate, resulting in elevated lactic acid levels in the blood.

Tissue injury can lead to altered plasma enzyme activity, as enzymes may leak from damaged cells, resulting in changes in serum enzyme levels. Similar effects have been observed in rat models by Pari and Gnanasoundari[33], as well as in *Pangasianodon* hypophthalmus exposed to high doses of oxytetracycline (OTC)[27], as reported by Manna et al[27]. In our study, no significant adverse effects were observed, indicating no apparent damage to internal organs. During the withdrawal period, a decrease in enzyme levels suggested that the organ function changes were reversible. It is crucial to strictly adhere to administration protocols, as higher doses may lead to irreversible organ changes. These findings highlight the importance of careful management when administering substances that affect serum enzyme activity in fish to maintain physiological health and prevent potential organ damage.

Moreover, we observed significant increases in total blood albumin levels in the BS2 and BS3 therapy groups, along with a slight rise in serum protein concentrations. Notably, a decline in globulin and glucose albumin levels remained significant in the BS2 and BS3 groups even after the withdrawal period.

The investigation looked at how surfactants affect fish behavior by examining liver tissue. The study found no significant changes in either behavior or liver health. This aligns with previous research showing that biosurfactants from different bacteria strains are effective against aquatic pathogens. When *Labeo rohita* fish were given food supplemented with *Bacillus subtilis*, their blood protein, albumin and globulin levels increased[30]. Similarly, cats fed meals with *Bacillus amyloliquefaciens* for 4 or 8 weeks showed higher serum and mucus protein levels[4]. Another study found that Asian seabass had higher muscle protein levels after receiving a probiotic combination[23]. These findings suggest an improved immune response in the fish,

as indicated by increased serum protein, albumin and globulin levels[50].

The observed increase in total serum protein levels supports the production of defense components in sufficient amounts. Detailed assessments of liver tissue showed no changes indicative of detoxification processes[6,17,31]. The fish displayed normal swimming behavior throughout the study, with no balance disruption or unusual surfacing behavior. There were no signs of lethargy or excessive mucus secretion. Unclarified but the moment of fish fed with higher concentration, the movement seems to be faster. The observation was stated with difficulty in sampling from these BS2 and BS3 tanks.

Several studies have found that *Bacillus sp.* lipopeptide biosurfactants can help to prevent white spot syndrome virus (WSSV) infections in prawns[13]. Biosurfactants derived from Pseudomonas sp. have shown antiparasitic properties against *Ichthyophthirius multifiliis*. These studies indicate that biosurfactants have the potential to enhance fish immunity. Effective doses (10-100 µg/mL) of these biosurfactants showed positive results in rainbow trout fingerlings without causing any adverse effects, confirming their antiparasitic properties[20]. The immunostimulatory efficacy of phospholipopeptide biosurfactants derived from *Staphylococcus hominis* has been assessed in *Oreochromis mossambicus*.

The study revealed that fish fed diets supplemented with biosurfactants exhibited heightened immune responses and increased disease resistance ($p < 0.05$)[35]. The findings suggest that biosurfactants isolated from Pseudomonas sp. have the potential to enhance aquaculture production by strengthening fish immunity. Additionally, the use of poly-3-hydroxybutyrate (PHB)[29], a bacterial synthesis product used as a feed additive in aquaculture, has shown an immunomodulatory role against WSSV infection in shrimp, positively influencing survival rates and highlighting the efficacy of such additives in bolstering immune responses.

The examination of surfactant effects on fish behaviour and liver histology yielded reassuring results, indicating no observable changes in behaviour or liver pathology. These findings contribute to the growing body of evidence supporting the effectiveness and safety of biosurfactants in aquaculture. Additionally, freshwater-reared *Litopenaeus vannamei* exposed to varying concentrations of phospholipids or cholesterol in their diet exhibited alterations in the expression of immune-related genes. It showed enhanced tolerance to *Vibrio alginolyticus* when supplemented with phospholipids[52].

Laranja et al[22] conducted a compelling study demonstrating improved protective effects against pathogenic *Vibrio campbellii* challenges in gnotobiotic *Artemia franciscana* following administration of a superior PHB-accumulating *Bacillus* strain JL47. It is interesting to note the similarities between research on lipopeptide biosurfactants targeting specific pathogens and the beneficial effects of rhamnolipid as a feed additive. It seems that biosurfactants may serve as a valuable tool in aquaculture to enhance immune responses and potentially increase production. Non-specific immunity plays a crucial role in fish defense modulation, aiding acquired immunity in maintaining a healthy and disease-resistant lifestyle in fish.

The study can be indirectly related to plant-based surfactants tested on fish species. For example, Hedrera et al[18] found that saponin is able to induce an immune response in zebrafish at a 3.3 g/kg inclusion level in feed. It is worth noting that Hedrera's study with the biosurfactants was incorporated directly into water and not into the feed. Therefore, our data yielded information on the response of the whole fish and not necessarily on intestinal-specific effects.

Plant extracts such as saponins are known to enhance non-specific immune responses and are effective against a wide range of pathogens[42]. Saponins have been found to stimulate the release of cytokines and interferon in shrimps. They are also known to improve the antiseptic and antimicrobial properties of hemolymph.

López et al[25] previously reported that saponins in the diet of Zebra fish act as an excellent immune booster. In an experiment conducted by Vasudava and Rina[48] on catla using seeds of *Achyranthes aspera*, the chemical constituents were found to be triterpenoid saponins containing oleanoic acid as the aglycone. The total serum protein level was higher on days 14 and 21 and lower on day 28 compared to the control, but none of these differences was significant. No significant differences were observed between the test and control groups, except for the globulin levels on day 21, where the test group was significantly higher than the control.

The inhibition of trypsin due to total protease inhibitors in serum peaked on day 21 and then decreased on day 28. Feeding *C. catla* with *A. aspera* incorporated diet enhanced specific and non-specific immune system factors, increasing the level of natural anti-proteases in the serum. This may provide defense against invading bacterial pathogens[48]. The results from the current trial indicate that dietary surfactin, when administered below a certain threshold, has the potential to enhance growth and influence enzyme and biochemical parameters.

However, the excessive supplementation of surfactin does not seem to offer any additional benefits. These findings align with previous research[24,41,43,53] and attribute the behaviour of surfactin to its surface activity. It is suggested that all the biological effects of surfactin stem from its surface activity. Its interaction with the cell membrane is significantly influenced by the concentration of surfactin. This underscores the necessity of a threshold concentration of surfactin in the bilayer for its solubilization[10,40].

Conclusion

This study provides critical insights into the potential utilization of biosurfactants as immunomodulatory agents within aquaculture systems. The subtle variations observed across multiple parameters highlight the imperative for continued research focused on optimizing dosages, refining administration protocols and investigating additional factors that may influence outcomes. As the aquaculture industry progresses, findings from such studies are pivotal for promoting sustainable and health-centric aquaculture practices. This emphasizes the necessity for further exploration and rigorous investigation in this domain.

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