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Evaluation of Growth, Non-specific Immunity and Enzymatic Profiles of *Labeo rohita* After Oral Administration of Glycolipid Surfactant Infused Feeds

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Abstract

Rhamnolipid, a potent bioactive compound extracted from *Pseudomonas sp*. has multifaceted biotechnological applications. The present investigation was undertaken to evaluate the impact of surfactant (rhamnolipid) on growth, non-specific immunity and enzymatic profiles of juvenile *Labeo rohita* incorporating graded doses through dietary supplementation. Randomly selected rohu juveniles were subjected to comprehensive analyses encompassing blood, serum, and liver tissue evaluations to discern the effects on growth, non-specific immune parameters, enzymatic activities, and organ modulatory changes. During the feeding trial, there was a discernible increase in the activities of myeloperoxidase, bacterial haemagglutination, respiratory burst, and hemolysis. Notably, lactate dehydrogenase levels remained within normal limits even at the highest dose T3 (36.0 mg kg^{-1} biomass day^{-1} (4X)), suggesting no systemic toxicity. Histopathological assessment of liver tissue showed no hepatic damage across all treatment groups, further confirming the compound's biocompatibility. Subsequent to the withdrawal period, *L. rohita* exhibited results in various assays comparable to those observed during the initial feeding period, underscoring the need for further in-depth investigations to elucidate the underlying mechanisms.

Introduction

Fish has gained increasing importance in the global food supply due to its high nutritional value and cost-effective protein content (Yousaf et al., 2025). Aquaculture-derived products, in particular, are recognized as rich sources of essential ω-fatty acids, minerals, and easily digestible proteins. With the rapid expansion of aquaculture, fish producers are compelled to either introduce new species or optimize existing culture methods to enhance the production efficiency of commercially important species (Nowosad et al., 2023).

Among these *Labeo rohita* (Hamilton, 1822), commonly referred to as rohu, is one of the principal carps found in India and is the dominant species in carp polyculture owing to its beneficial relationships with other species, rapid development, inclination of customers, and great marketability (Choudhary et al., 2021). The advancement of hatchery technologies and successful captive seed production has significantly contributed to the intensification of rohu farming. However, this intensification has simultaneously increased the prevalence of infectious diseases in culture systems, posing a major threat to sustainable aquaculture and leading to substantial economic losses. While antibiotics have traditionally been used to combat disease outbreaks, their effectiveness is short-lived, as prolonged use promotes the emergence of antibiotic-resistant bacterial strains. In addition, antibiotic residues in aquatic environments and fish tissues raise environmental and public health concerns, leading to stricter regulatory limitations on their use. These

challenges highlight the urgent need to explore alternative strategies for enhancing fish immunity and disease resistance, particularly through sustainable, non-antibiotic approaches (Abdel-Latif et al., 2022a, Rutkowska et al., 2024).

The banning of antibiotics in several countries, coupled with growing concerns over environmental and food contamination, has intensified the search for sustainable alternatives that can support optimal productivity in aquaculture systems (Seal et al., 2013; Park et al., 2016). From a toxicological standpoint, the continued use of chemical agents such as pesticides including deltamethrin, diazinon, and cypermethrin has been shown to cause detrimental effects in fish, including immunosuppression, oxidative stress, and hematological alterations (Farag et al., 2021). Similarly, chlorpyrifos exposure has been reported to induce oxidative stress, immunotoxicity, genotoxicity, hepato-renal impairment, and marked histopathological changes (Mokhbatly et al., 2020). Additional studies have revealed that herbicides like penoxsulam and atrazine can also lead to immune suppression, oxidative damage, and disruptions in renal and hepatic function in exposed fish (Hany et al., 2022).

In light of these adverse effects, attention has turned toward biosurfactants (BS) a group of surface-active compounds naturally synthesized by microorganisms as a safer and more environmentally compatible alternative (Pardhi et al., 2022; Puyol et al., 2021). Inherently superior to chemically synthesised surfactants, biosurfactants are less toxic, biodegradable, and ecologically acceptable in a variety of environmental settings (Rincon et al., 2020). The global surface-active agents market is witnessing steady annual growth of 6.75% with an estimation to be a $3.21 billion market by 2025 (Anestopoulous et al., 2020). Various renewable sources can be used to produce microbial surfactants gaining attention for their diverse applications, such as anti-biofilm, antimicrobial activities, gene transfection, vaccine delivery, immunomodulation, and cancer therapy (Hruzova et al., 2020; Aleksic et al., 2017; Saimmai et al., 2019; Zakharova et al., 2019; Ceresa et al., 2019; Coelho et al., 2020).

Despite the growing interest in microbial surfactants, there remains limited understanding of the specific effects of rhamnolipid a prominent biosurfactant on immune responses and key enzymatic activities in fish such as *Labeo rohita* when administered through feed (Pardhi et al., 2022; Puyol et al., 2021). Given the significance of rohu in South Asian countries viz., Bangladesh, Nepal, Pakistan, and India and its susceptibility to ectoparasite infestations during grow-out culture (Jena et al., 2009), a systematic evaluation of rhamnolipid's impact on immunity, enzyme activities, and liver tissue after feed administration is needed. To address this knowledge gap, the present study investigates the effects of dietary rhamnolipid at graded doses (9, 18, and 36 mg kg^{-1} biomass day^{-1}) and includes a 10-day withdrawal period to evaluate residual effects on immunity, enzyme profiles, and liver tissue integrity under controlled conditions.

Material and Methods

Extraction of Rhamnolipid

Pseudomonas aeruginosa Kmka-114 (Accession Number: PP474329) in the mid-exponential phase 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 duration of 9 days. The mineral salts medium used comprised 3.0 g KH_2PO_4, 7.0 g K_2HPO_4, 0.2 g $MgSO_4 \bullet 7H_2O$, and 1 g $(NH_4)_2SO_4$ per L, resulting in a C/N ratio of 55:1, as per the formulation by Santa Anna et al. (2001). 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 xg, 4°C) was used to collect the precipitate that resulted.

Experimental Condition

A total of 120 healthy rohu (*Labeo rohita*) fingerlings, devoid of any observable disease symptoms as determined through meticulous gross examination of skin, fins, and gills, were procured from the Harabhanga (Jirad) fish farm in Boudha, Odisha, India. After that, the 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 18±0.7 g. The surfactant-infused feeding trial spanned 30 days and involved three experimental groups

receiving different doses: T1: 9 mg kg^{-1} biomass day^{-1} (1X); T2: 18 mg kg^{-1} biomass day−1 (2X), and T3: 36 mg kg^{-1} biomass day^{-1} (4X). Water quality parameters were closely monitored and kept within ideal values during the trial, following APHA (2012) recommendations and regular operating procedures. Feeding of the experimental fish occurred twice daily at 8:00 am and 17:00 pm. 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 rohu fingerlings, providing valuable insights into its potential effects and withdrawal dynamics. During the experiment, measurements were made of the following parameters for each group: pH, dissolved oxygen, total alkalinity, total hardness, ammonia, nitrite, nitrate, and water temperature.

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 (0.25 mm) 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%. 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 per L of water-soluble clove oil (Dabur) 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

Utilising the following formulae, the growth performances were assessed in terms of, initial body weight (IBW), final body weight (FBW), weight gain ratio (WGR), specific growth rate (SGR), feed conversion ratio (FCR), and survival rate (SR):

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

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

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

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

Table 1. Composition of medicated feed and control feed for *Labeo rohita* (grams/kg)

Feed ingredients	Control	T1 (9 mg/kg of fish biomass/day)	T2 (18 mg/kg of fish biomass/day)	T3 (36 mg/kg of fish biomass/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	19.7	19.4	18.8
Biosurfactant (BS)	0	0.3	0.6	1.2

*****As in case of T1, 1 kg biomass was provided with 30 gm of medicated feed (3% BW/day), that contained 9 mg Biosurfactant.

Assessment of Non-Specific İmmune Parameters

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 were done.

Myeloperoxidase Activity

The evaluation of myeloperoxidase activity was conducted using Quade and Roth's approach (1997). In short, 15 µL of fish serum was combined with 135 µL of Hank's balanced salt solution (which is devoid of Mg^{2+} and Ca^{2+}). Next, 50 µL of hydrogen peroxide (5 mM) and 20 mM 3,3',5,5'-tetramethyl benzidine were added. After incubating the process 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 Activity

50 µL of heparinized blood and 50 µL of 0.2% NBT solution were incubated for 30 minutes at 25°C to measure respiratory burst activity. The reduction of nitroblue tetrazolium (NBT) by intracellular superoxide radicals is reflected in this activity. The combination was then centrifuged, and a UV-VIS Spectrophotometer was used to measure the optical density of the supernatant at 540 nm (Anderson and Siwicki, 1994).

Lysozyme Assay

Lysozyme activity was measured using lyophilized *Micrococcus lysodeikticus* (Sigma-Aldrich, USA) in accordance with Ellis's directions (1990). 130 µL of newly prepared *Micrococcus lysodeikticus* solution was mixed with 10 µl of fish serum and 0.02M sodium citrate buffer. After adding the bacterial solution, the optical density was measured at 450 nm at 24°C both right away and an hour later. The lysozyme activity was calculated and represented in units mL^{-1} 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 (Triffany et al., 1972). Bradford (1976) and Doumas techniques with BCG dye were used to quantify the contents of total protein and albumin, respectively (Doumas et al., 1972). The resultant values were used to calculate globulin levels by subtracting albumin content from total protein.

Estimation of Enzyme Activity

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 (Michell et al., 1970).

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.

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 (Ashish and Banalata, 2008).

Data Analysis

Each result is displayed as mean ± standard error (SE) after undergoing a one-way analysis of variance (ANOVA). 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±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 28.00±0.5°C. Growth analysis revealed no significant differences between the experimental and control fish groups ($P>0.5$) (see 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, most parameters returned to levels similar to

the control on the 40th day, following a 10-day withdrawal of rhamnolipid.

The respiratory activity of the all the treated groups was significantly higher compared to the control, while the T3 concentration group exhibited a sharp incline in the 20th day which showed a decline on the 30th day (P<0.05). Myeloperoxidase activity remained elevated across all groups until the day of the feeding trial. Myeloperoxidase activity in the post-feed infusion stages was nearly identical to the control fish. At the post-feed stages on the 40th day, no drastic changes were observed across all experimental groups compared to the control.

A significant elevation in serum albumin level was observed in fishes during and post feed infusion stages. Protein level during feed infusion or on withdrawal the level remains the same as of control groups. A drop in glucose level in T1 (1X) group as compared to a significant drop in T2 (2x) and T1 (4x) group was observed during and post feed withdrawal stages (Table 3).

During the course of the 30-day feeding study, the treated fish showed slightly elevated serum enzymatic parameters (P<05) for lactate dehydrogenase but spike in alkaline phosphatase (Figure 2). Moreover, it was discovered that upon withdrawal, the control fish's mean serum enzymatic parameters values fell within a comparable range. Fish liver slices from the control and all the treated groups are displayed histologically in Figure 2. The fish livers taken from the control feed and biosurfactant feed diets showed regular gross morphology, well-defined cell nuclei, and hepatocytes

Table 2. Survival and growth performance and feed utilization of *Labeo rohita*

	0-40 days experiment			
Parameters	Control	T1	T2	T3
IBW(g)	18.81425	18.54700	18.85010	18.58030
FBW (g)	37.58000	41.41500	42.24830	42.30170
WGR (%)	99.76480	123.29750	124.12800	127.66900
SGR %	1.72964	2.00833	2.01762	2.05681
FCR (%)	1.83206	1.56334	1.55513	1.5488
SR (%)	100.00000	100.00000	100.00000	100.00000

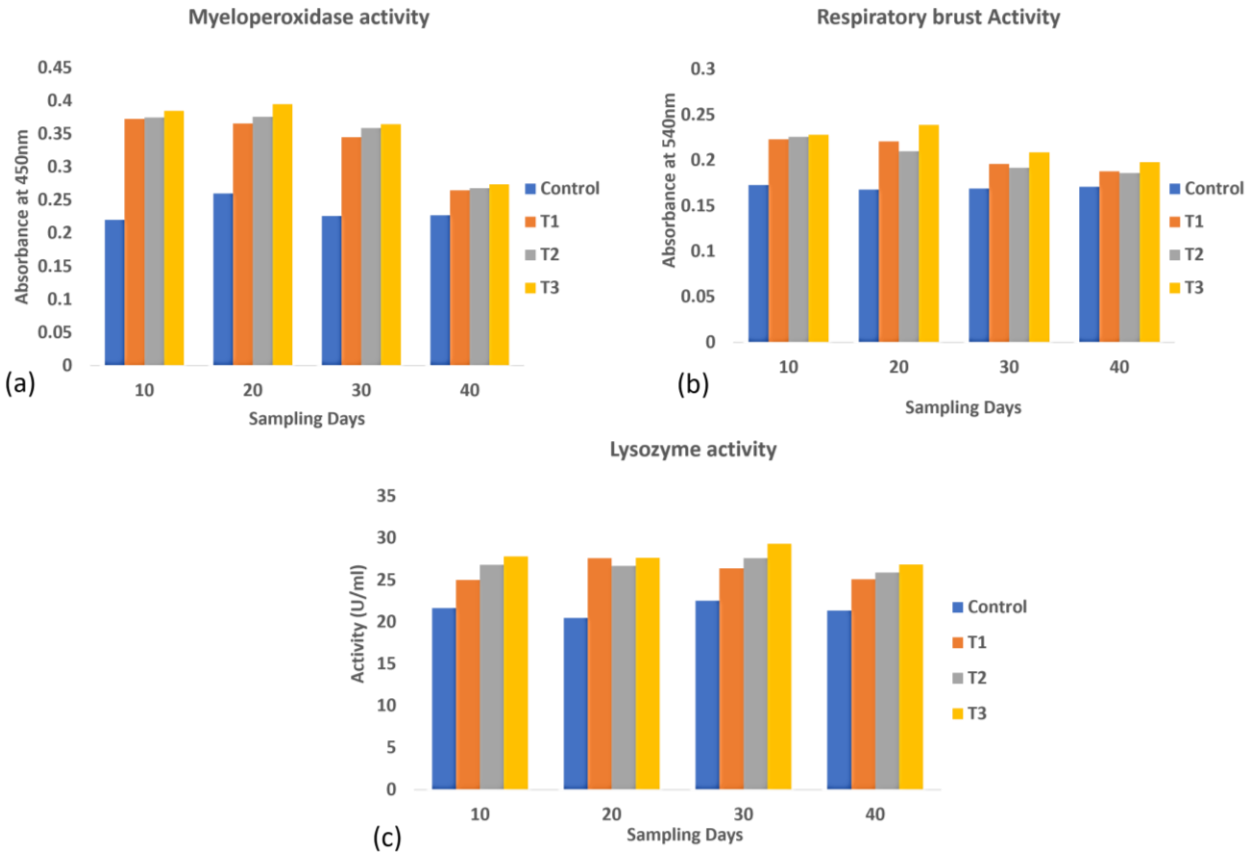

Figure 1. Non-specific immune parameters of *Labeo rohita* (Hamilton, 1822): (a) Myeloperoxidase activity; (b) Respiratory burst activity; (c) Lysozyme activity recorded in control and biosurfactant-treated groups at each sampling day. The experimental period comprised Days 10, 20, and 30 (biosurfactant supplementation phase) and Day 40 (10 days post-dosing). Data are presented as mean±SE (n=5 fish per group per time point).

with a normal shape. In control group, the hepatocytes are found normal with intact architecture. The hepatic sinusoids were abundantly present. In experimental group, normal hepatocytes were observed with absence of vacuoles Figure 3.

Discussion

With recent focus on using bacterial secondary metabolites for disease prevention in aquaculture species, aquaculture stands out as a quickly growing and attractive business. The investigation of the immunomodulatory and growth-promoting properties of biosurfactants (BS) isolated from *Pseudomonas sp.* in fish is the focus of this work. Surprisingly, a literature survey reveals a dearth of evidence concerning metabolites isolated from *Pseudomonas* in the littoral region of Karnataka being employed as fish supplements. The pursuit of enhanced growth in farmed food fish within defined culture periods propels farmers and entrepreneurs to explore various feed supplements. To assess the potential of the biosurfactant compound, fish were administered varying doses ranging from 50-200 µg/mL through feed. Slight growth variations were noted in the higher dose group compared to the control, potentially influenced by unexplored factors related to water and host microbiomes in this study.

Non-specific immune parameters, crucial for combating diverse pathogens, were investigated, revealing significant variations in respiratory burst activity, myeloperoxidase activity, and lysozyme activity in treated fish compared to the control. Interestingly, these immune parameter enhancements persisted for over 10 days after compound withdrawal, gradually returning to levels akin to control fish. This observation aligns with findings from other studies using commercial antibiotics on sea breams, emphasizing the immunomodulatory effects of the biosurfactant, contingent on dose, administration route, duration, and fish stage (Serezli et al, 2005). Fish metabolic and physiological health may now be evaluated using biochemical measures linked to serum enzymes (Mondal et al, 2020). Present study concentrated on liver enzymes, specifically alkaline phosphatase (ALP) and lactate dehydrogenase (LDH), which function as stress markers and tissue damage biomarkers. According to this research, fish had a little but substantial increase in these enzymes up to the 30th day of the experiment, after which they had a decrease during the withdrawal phase. The conversion of pyruvate into lactate, which raises the blood's lactic acid concentration, may be the cause of the observed increase in LDH activity in *Labeo rohita*.

When injury occurs in tissues abundant in enzymes, several enzymes leak from damaged cells, leading to changes in plasma enzyme activities. Similar occurrences related to serum enzyme activities were documented in rat studies by Pari and Gnanasoundari (2006). Manna et al (2021) also noted an elevation in LDH enzyme activity in *P. hypophthalmus* when

Table 3. Biochemical parameters of *Labeo rohita* (Hamilton, 1822): (a) Total protein; (b) Serum albumin; (c) Serum glucose levels in treatment and control groups during the experimental period (Days 10, 20, and 30). The experimental duration represents only the biosurfactant supplementation (dosing) phase. Data are presented as mean±SE (n=5 fish per group per time point)

	30 days experiment (*Labeo rohita*)			
	Serum protein (g/dl)	Serum albumin (g/dl)	Globulin (g/dl)	Serum glucose (mg/dl)
Control	2.61±0.0346	0.97±0.0646	1.6±0.037	115.5±0.71
T1	2.55±0.001	1.03±0.00748	1.51±0.0035	106.5±0.09
T2	2.6±0.00423	1.37±0.0127	1.23±0.00423	97.93±0.0926
T3	2.65±0.006	1.57±0.025	1.0±0.086	87.2±0.0627

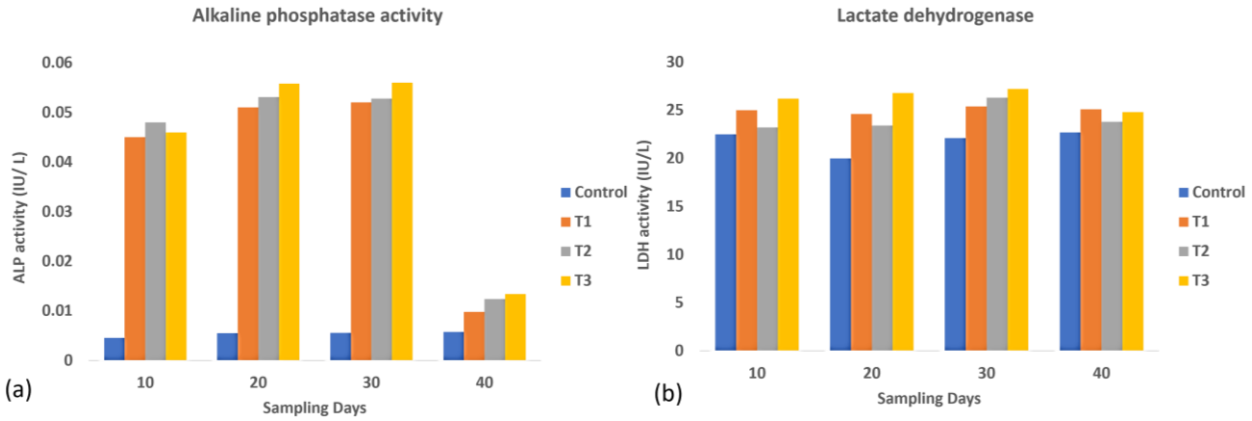

Figure 2. Enzyme activity of *Labeo rohita* (Hamilton, 1822): (a) Alkaline phosphatase; (b) Lactate dehydrogenase recorded in control and treatment groups during the experimental period. The study comprised Days 10, 20, and 30 (biosurfactant supplementation phase) and Day 40 (10 days post-dosing). Data are presented as mean±SE (n=5 fish per group per time point).

subjected to a higher dose of OTC. Importantly, present study revealed no alarming outcomes, suggesting no evident harm to internal organs. During the withdrawal period, a decline in enzyme levels indicated a reversible alteration in organs. Adhering strictly to administration protocols is essential, as higher doses may induce irreversible changes. These findings highlight the critical role of careful management when administering substances that could influence enzyme activities in fish serum, ensuring the preservation of physiological health.

There were increases in total blood protein, albumin, and globulin levels; the T3 treated group had the highest serum protein levels. Albumin content remained significantly higher in T3 and T4 groups even after withdrawal. These results, which highlight the inherent response increase, are consistent with earlier research on rohu and other fish species on supplemental diets.

The investigation explored the impact of surfactants on fish behaviour and liver histology, revealing no discernible changes in either behaviour or liver pathology. This observation aligns with prior research illustrating the efficacy of biosurfactants derived from different bacterial strains against diverse aquatic pathogens. When rohu (*L. rohita*) were fed a diet enriched with *B. subtilis*, there was a comparable increase in blood protein, albumin, and globulin levels (Nayak et al., 2007). Further, when *B. amyloliquefaciens*-supplemented meals were given to cats for 4 and 8 weeks, Das et al, (2013) observed increased serum and mucus protein levels in the cats. After feeding a combination of probiotics to Asian seabass *Lates calcarifer*, Lin and colleagues recently observed significantly higher protein levels in the muscle (Lee et al., 2017). This study revealed increased amounts of serum protein, albumin, and globulin, which is likely connected to fishes' higher innate response. (Wiegertjes et al.,1996). The creation of these defence components in sufficient amounts is corroborated by the rise in serum's total protein level. The impact of surfactants on fish behaviour and liver histology was thoroughly examined, with histopathological assessment confirming no alterations in the liver indicative of detoxification (Arellano et al., 1999; Olojo et al, 2005; Figueiredo et al., 2007). Fish exhibited normal swimming behaviour, with no signs of equilibrium loss or surfacing observed. Notably, there were no indications of lethargy or mucous secretion during any period.

Prominent analogies with prior studies on Bacillus sp. lipopeptide biosurfactants preventing the white spot syndrome virus (WSSV) in prawns (Donio et al., 2018) and *Pseudomonas*-derived biosurfactants exhibiting antiparasitic effects against *Ichthyophthirius multifiliis*. Emphasizing the potential of biosurfactants to boost fish immunity, the investigation found that effective doses

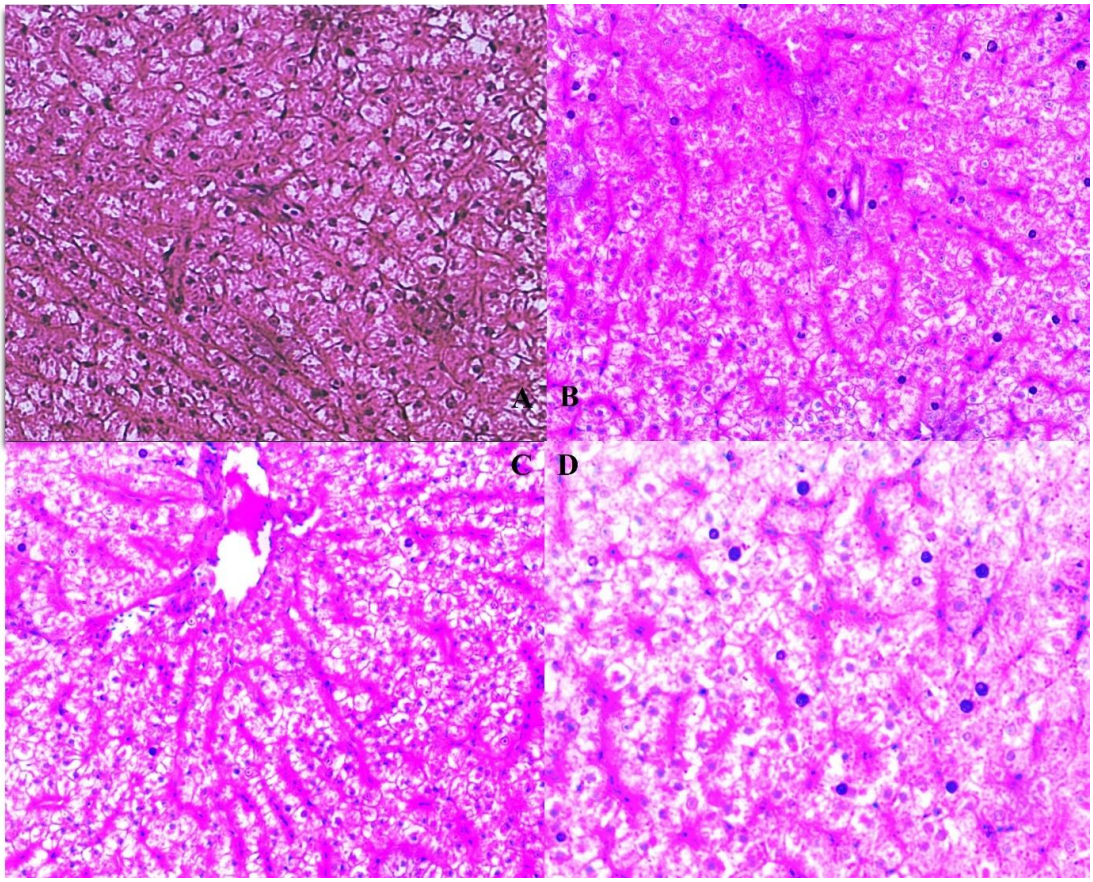

Figure 3. Histopathological alterations in liver tissues of *Labeo rohita* (Hamilton, 1822): (A) Control group displaying normal hepatocytes with intact sinusoidal architecture; (B–D) experimental groups fed diets supplemented with increasing biosurfactant concentrations, showing predominantly normal hepatic architecture.

(10 to 100 µg/mL) mirrored the results of this investigation and shown no negative effects in rainbow trout fingerlings, confirming the biosurfactant's antiparasitic properties (Jubury et al., 2018). The immunostimulatory efficacy of phospholipopeptide BS from Staphylococcus hominis was evaluated in Oreochromis mossambicus (Rajeswari et al., 2016). Fish that were fed BS-infused diets showed higher immune responses and illness resistance ($P<0.05$). These findings imply that biosurfactants that were separated from *Pseudomonas sp*. hold the potential to enhance aquaculture production by fortifying fish immunity. The exploration of poly-3-hydroxybutyrate (PHB) (Monica et al., 2017), a product of bacterial synthesis used as a feed additive in aquaculture, reveals its immunomodulatory role against WSSV infection in shrimps, positively impacting survival rates and showcasing the potential of such additives in strengthening immune responses. The scrutiny of surfactant effects on fish behaviour and liver histology provides reassuring results, indicating no observed changes in behaviour or liver pathology. These findings contribute to the growing body of evidence supporting the effectiveness and safety of biosurfactants in aquaculture. Freshwater reared *Litopenaeus vannamei* (Yan et al., 2020) on exposure with different concentration of phospholipids or cholesterol in their diet showed effect in expression of immune related gene. Shrimp showed better tolerance towards *Vibrio alginolyticus* when supplemented with phospholipid in feed. Laranja et al., (2018) in an intriguing investigation showed improved the protective effects against a pathogenic *Vibrio campbellii* challenge in gnotobiotic *Artemia franciscana* on administration of a superior PHB-accumulating *Bacillus strain* JL47. Based on the similarities between research on lipopeptide biosurfactants that target particular pathogens and the beneficial effects of rhamnolipid as a feed additive, biosurfactants may be used in aquaculture to improve immune responses and potentially increase production.

Conclusion

The present investigation underscores the promising role of rhamnolipid, a biosurfactant derived from *Pseudomonas* sp., as a dietary supplement capable of modulating immune responses in *Labeo rohita* without inducing physiological or histopathological harm. The observed enhancement in key non-specific immune markers reflecting the compound's immunostimulatory potential. These responses were dose-dependent and sustained throughout the feeding trial, affirming the efficacy of rhamnolipid in activating innate immune defence mechanisms in fish. Importantly, the absence of adverse alterations in lactate dehydrogenase levels, even at the highest supplementation dose, indicates that the metabolic stress imposed by rhamnolipid is minimal. Moreover, histological evaluations of liver tissues revealed no signs of hepatocellular damage, reinforcing the biosurfactant's biocompatibility and safety for long-term dietary use. The return of immune and enzymatic parameters to near-baseline levels after the withdrawal period further suggests that the effects of rhamnolipid are reversible and non-cumulative, which is a desirable trait for feed additives used in commercial aquaculture systems. Together, these findings provide compelling evidence that rhamnolipid can be a viable, eco-friendly alternative to synthetic immunostimulants or antibiotics in aquaculture. By enhancing disease resistance without compromising organ health or inducing toxicity, it aligns well with the principles of sustainable and health-conscious aquafarming. However, the subtle variations across different concentrations, along with post-withdrawal dynamics, point to the necessity of further research. Future studies should aim to elucidate the precise immunological and metabolic pathways modulated by rhamnolipid, explore its synergistic effects with other functional feed components, and assess its long-term impact under field conditions. Fine tuning the dosage and administration schedule will be critical for translating these findings into practical applications, ultimately contributing to improved fish health, productivity, and environmental sustainability in the aquaculture sector.

Ethical Statement

The study was approved by the Faculty of Pharmaceutical department insititutional review board of Siksha O Anusandhan University, Odisha, India. All trials were not need to provide ethic documentation.

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Author Contribution

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Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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