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IMPROVING GROWTH AND IMMUNITY IN *Labeo rohita* USING PROBIOTIC MICROSPHERES

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Abstract: Microspheres are increasingly used in aquaculture for fish growth and immunity. *In vivo* efficacy of *Geotrichum candidum* capsules by using alginate and combined with starch and xanthan with a coated chitosan layer. *Labeo rohita* fingerlings were equally distributed in 10 groups. Control was fed basal diet and experimental fish was fed probiotic, *G. candidum* 10^9 CFU/g, remaining eight groups were fed microspheres-based diet prepared with alginate, starch, xanthan and chitosan in different ratios. A significant impact of probiotic microspheres was found on growth, feed conversion, enzymatic activities, immunology, lipid profile of fish as compared to fish given basal diet. Chitosan microcapsules of *G. candidum* resulted in higher performance as compared to fish given free probiotic. Among chitosan microcapsules, Alg-C showed a significantly positive impact on all immunological parameters, followed by Alg-C. Chitosan and alginate are best to improve the bioavailability and targeted release of probiotic in GIT tract of *L. rohita*. Therefore, present study proposes a sustainable solution for improving physiological responses of *L. rohita* by feeding microspheres.

Keywords: microspheres, *G. candidum*, *L. rohita,* polymer, alginate, probiotic encapsulation, chitosan

1. Introduction

Probiotics are supplied as a live complement in fish feed to assist the target host. Commonly, the probiotic prevents the pathogenic microbe by improved immunity, survival, growth and increased feed consumption, promote anti-mutagenic in the aquaculture (Harikrishnan et al., 2010; Andani et al., 2012). Today, probiotic widely being utilized for attaining a sustainable aquaculture, but many challenges are still associated with ingestion of probiotic. Probiotic live cells viability during processing, packing and conversion through the host gut are not consistent to provide the anticipated positive effect (Cordero et al., 2015; Nousheen et al., 2025). The lowest viable cells number range from 108-109 CFU each day dose-1 (Ramos et al., 2018). Thus, there is great attention toward improved viability of microbes to be administered in fish diet.

Microencapsulation is among one of most relevant to provide improved survival during processing conditions, storage stations and entry in gut when given in diet (Cordero et al., 2015). The aim is to create microcapsules of probiotic to protect those from harsh conditions during feed processing and storage (Tripathi & Giri, 2014) after consumption transit to intestinal tract (Sun and Griffiths, 2000) as well as at higher oxygen levels (Sunohara et al., 1995). Encapsulation protects microbes from the different

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condition like lower pH, higher acidic stations, bile salts (Lee et al., 2000), digestion (Sousa et al., 2013), temperature shock and anti-microbial agent (Sultana et al. 2000). It is proposed that microencapsulation gives protection to the probiotics from interfacial inactivation (Pinpimai et al.,2015; Nazzaro et al., 2012).

Probiotic microcapsules have been reported to provide improve results as compared to free form. For instance, dietary alginate along with skim milk microcapsule composed of *S. cerevisiae* and *L. rhamnosus* gave the most prominent results on weight gain percent, FCR and SGR of tilapia (Pirarat et al., 2015). Alginate microcapsule of *S. putrefacien* showed higher viability/survival in the gut of sole fish (Rosas ledesma et al., 2012). Additionally, probiotic strain Pdp 11 microspheres provided significant rise in IgM levels in seabream (*Sparus aurata*) as compared to unencapsulated bacteria (Cordero et al. 2015).

Several polymers and other coating material have already been used in food industry i.e. alginate, chitin, pectin and chitosan (Tee et al., 2014) only or in various combinations such as alginate-chitosan, alginate-gelatin and alginate-skim milk microsphere for encapsulation of probiotic bacteria (Cordero et al., 2015; Zhao et al., 2012). Most of encapsulating materials when used alone, may form a loose network resulting in the leakage of its core materials which limit beneficial effect claimed by probiotics (Zhang et al., 2011). Yet, this issue may be resolved either by the use diverse polymers combined or by coating probiotic microcapsule with natural or synthetic polymer i.e. chitosan. Previously alginate have been used for encapsulating microbes i.e. bacteria and yeast (Chandramouli et al., 2004; Gouin, 2004; Riaz and Masud, 2013). Alginate microcapsules have few drawbacks such as it is chemically not very stable as well as it disintegrates at a low pH and in presence of higher monovalent-ions and few other condition (Krasaekoopt et al., 2003; Lee et al., 2004). Therefore, scientist use alginate combined with other mixtures like glycerol, starch,10 % skim milk, chitosan and xanthan etc (Lee et al., 2004; Huq et al., 2013; Pinpimai et al., 2015) and suggested that other supporting material/compound/polymer is required to improve the physio-chemical structure of microspheres (shape, surface morphology, pH and temperature, stability, and release etc). Alginate along with chitosan is gaining more attention due to electrostatic interactions of carboxyl (COOH-) group of alginate with the amine (NH_2) of chitosan forms a strong matrix which reduce its permeability (Anal and Stevens, 2005; Ren et al., 2017), limiting leakage of the core material (Zhou et al., 2000; Chavarri et al.,2010), providing more stability against the gastric harsh condition i.e. pH, bile secretion, enzyme of host organisms (Krasaekoopt et al., 2003) and improve availability of microbes at desired site (Kumari et al., 2013). Additionally, chitosan also possesses exceptional properties for aquaculture systems, because it may act as growth enhancer and immune-stimulator, so it may also provide defense against certain microbes (Baños et al., 2006; Jose et al., 2012). *G. candidum* use in *L. rohita* feed enhances growth, gut health, and immunity (Amir et al. 2018), but faces challenges in feed stability and gut colonization. Chitosan-coated *G. candidum* microcapsules improved enzyme activity, immunity and survival in semi-intensive systems in rohu (Amir .et al., 2024). Therefore, examining various polymer combinations inclusion in fish feed with alginate, chitosan, starch, and xanthan will further explore their efficacy in this novel study

The viability and the bioavailability of local probiotic, *G. candidum* in gut of *L. rohita* would be checked by alginate and chitosan encapsulation with various combinations. This study will assess various formulation of *G. candidum* for dietary supplementation in fish feed to enhance the growth indices and health profile of *L. rohita*.

2. Materials and Methods

In this study, eleven-week experiment was set up to know the *in vivo* efficacy of *G. candidum* microcapsules of alginate or combined with starch & xanthan and then coated with chitosan alone or nano-chitosan in comparison to free fungal strain.

2.1 Probiotic

The probiotic, *G. candidum*, was cultivated as described previously Amir et al. (2024) by using several polymers including alginate (Alg), chitosan (Alg.C), with nano chitosan (Alg.CN), alginate & starch encapsulated (Alg.S), alginate & starch coated with chitosan (Alg.S.C), alginate& starch coated with nano chitosan (Alg.S.CN), alginate & xanthan (Alg.X), coated with chitosan (Alg.X.C), alginate & xanthan encapsulated and coated with nano-chitosan (Alg.X.CN). Additionally, the fungal probiotic cell density was calculated by standard method already reported by Nikoskelainen et al. (2003).

2.2 Probiotic supplementation in feed

The basal diet with 35% (crude protein) was designed and divided in eleven group i.e. Control (G0) free of probiotic while others were fortified with probiotic *G. candidum* (10^9 CFU g^{-1}), G1 free or non-encapsulated *G. candidum*, while other experimental groups were augmented with *G. candidum* microspheres with Alg (G2), Alg.C (G3), Alg.CN (G4), Alg. S (G5), Alg.S.C (G6), Alg.S.CN (G7), Alg.X (G8), Alg.X.C (G9), Alg.X.CN (G10). (Nikoskelainen et al. 2003; Amir et al., 2023). The feed was newly prepared after two weeks to avoid any variation in probiotic cell density.

2.3 Experiment Fish management

Fish was transferred in well aerated oxygen-filled polythene bags and after tempering were shifted in the five fiberglass tanks (capacity: 360 Gallon) having flow through system. Fish were adjusted for five-day period and provided 35% crude protein feed.

2.4 Experiment design

A complete randomize trial was conducted in triplicate at open-air facility during the months of June till August. Active fingerlings (avg. wt, 15.63±0.35gram) with no illness symptoms were randomly stocked in thirty-three fiberglass tanks. Fish was fed twice a day (9:00 am and 4:00 pm) at 3% body weight. Furthermore 2 hrs after feeding, unconsumed feed was collected for determination of feed conversion ratio (FCR). To avoid deterioration of water quality parameters, daily fecal matters were removed through manual siphoning and about 10-20% replaced with freshwater.

2.5 Sample collection

At the end, fish were starved for about one day before sampling. It was removed separately weighed by using top loading balance and counted for determining the weight of separate fingerlings. The survival rate (%), weight gain (% WG) and final biomass was assessed.

2.6 GUT enzyme essay

For gut enzyme, fish were anesthetized with MS- 222, dissected by placing on cool gel packs by adopting aseptic methods for enzyme concentration assessment reported earlier (Amir et al 2024)

2.7 Hematology and Immunological essay

For complete blood count, fish were captured and the blood was collected from the caudal peduncle vein. The heparin processed blood was used for CBC count i.e. red blood cells ($\times 10^6 \mu$/L), hemoglobin (gd/L), WBC ($x10^3 \mu L^{-1}$). Serum was kept at 4˚C storage for further analysis of AST, total serum protein and lysozyme activity, total-cholesterol, triglyceride, high density lipoprotein HDL, LDL, ALT activity and ALP activity.

2.8 Statistical analysis

Data obtained for growth, survival, enzyme essay, hematology and immunology for experimental groups were evaluated by using one–way ANOVA test. After significant variances were found comparison among the means were made using post hoc test i.e. LSD test. All the outcomes were statistically assessed at the significance of 0.05. For graph of data software, GraphPad Prism.5 was used.

3. Results and Discussion

3.1 Growth of fish

The diet consumption of control and *G. candidum* microspheres designated a significant consequence on growth of *L. rohita*. It was recorded that total weight, weight gain and percent weight gain (%) showed significantly higher ($p<0.001$) values in free form and dietary microspheres of *G. candidum* in comparison to the control feed. It was observed that final weight, weight gain, weight gain (%), and final biomass increased considerably ($p<0.001$) in G4 (Alginate-chitosan nanoform) diet followed by G3 (Alginate-chitosan), G7 (Alginate-starch-chitosan nanoform), G6 (Alginate-starch-chitosan), G10 (Alginate-xanthan-chitosan nanolayered), and G9 (alginate-Starch-chitosan) respectively (Fig 1). However, *G. candidum* encapsulated without chitosan coating, alginate-starch (G5), Alginate- xanthan (G8) and alginate alone (G2) could not display any remarkable increase in final weight, weight gain (%) and final biomass over the other forms of *G. candidum* microspheres with chitosan coating. Greater

survival (100%) was found in all diet groups with free and various *G. candidum* microspheres as compared to the control group (96%).

3.2 GI tract enzymatic activities

The GI tract of *L. rohita* fingerlings fed control, free- and diverse forms of *G. candidum* microcapsule showed significantly higher digestive enzyme activities (ANOVA, Tukey post hoc, $p<0.001$). ANOVA shown that there was significant increase in overall gut enzyme activities and increased protease and cellulase were observed in G4 followed by G3, G7, G6, G8 and G7 respectively as compared to the control diet (Fig 2). Amylase activity also showed higher values in G4 followed by G3, G7, G6 and G7 respectively when compared to the control group.

3.3 Haematology and Immunology

The trials showed enhanced status of hematology of all probiotic-supplemented fish as compared to the control group. Furthermore, outcomes also shown that *G. candidum* microcapsules improved erythrocytes, leukocytes, hemoglobin and hematocrit value as compared to free *G. candidum* (Fig. 3). It was also discovered the most-significant effect of probiotic microspheres with chitosan coated on RBC, WBC, Hb and Hct of G4, G3, G7, G6, G10 and G9 group as compared to probiotic without any chitosan coat (G2, G5 and G8). It was also evident that significant rise in RBC, WBC, Hct, Hb, and decreased in MCH, MCV, and MCHC in G4 group followed by G3 (Fig 3).

Serum AST activity showed significant difference in fish, while Post hoc LSD showed significantly lesser AST in the probiotic fed fish vs control0 fish and *G. candidum*, microcapsule fed fish in contrast to free-probiotic fed rohu. Additionally, G3, G4, G6, G7, G9 and G10 group of fish fed with probiotic microcapsules with chitosan coat displayed most significant decrease in AST as compared to G5, G8 and Y2 group of *L. rohita* fed diet microcapsules minus chitosan coat. Generally, G4 displayed the lowermost and statistically comparable serum AST. while control showed the uppermost value (Fig. 4). It was observed that significantly lower ALT was found in probiotic fed groups as compare to control fish. *G. candidum* microcapsules fed fish had lower ALT as compare to free probiotic fed fish (G1). Likewise, lower level of ALT was observed in (G3, G4, G6, G7, G9 and G10), probiotic microcapsules with chitosan coating. Overall, the lowest AST activity was observed in G4 fish followed by G3. But, Control showed the maximum ALT followed by G1 (Fig. 3). Largely, the utmost ALT was detected in G4 followed by G3> G7>G6>G10>G9> G5>G2>G1 while control displayed the lowest activity (Fig. 3). The proportional assessment of *G. candidum* microcapsule shown the most distinct decrease in cholesterol and triglyceride in fish fed encapsulated probiotic with chitosan coat (G3, G4, G6, G7, G9 and G10) as compared to plain microcapsule in G5, G8 and G2 (Fig 4).

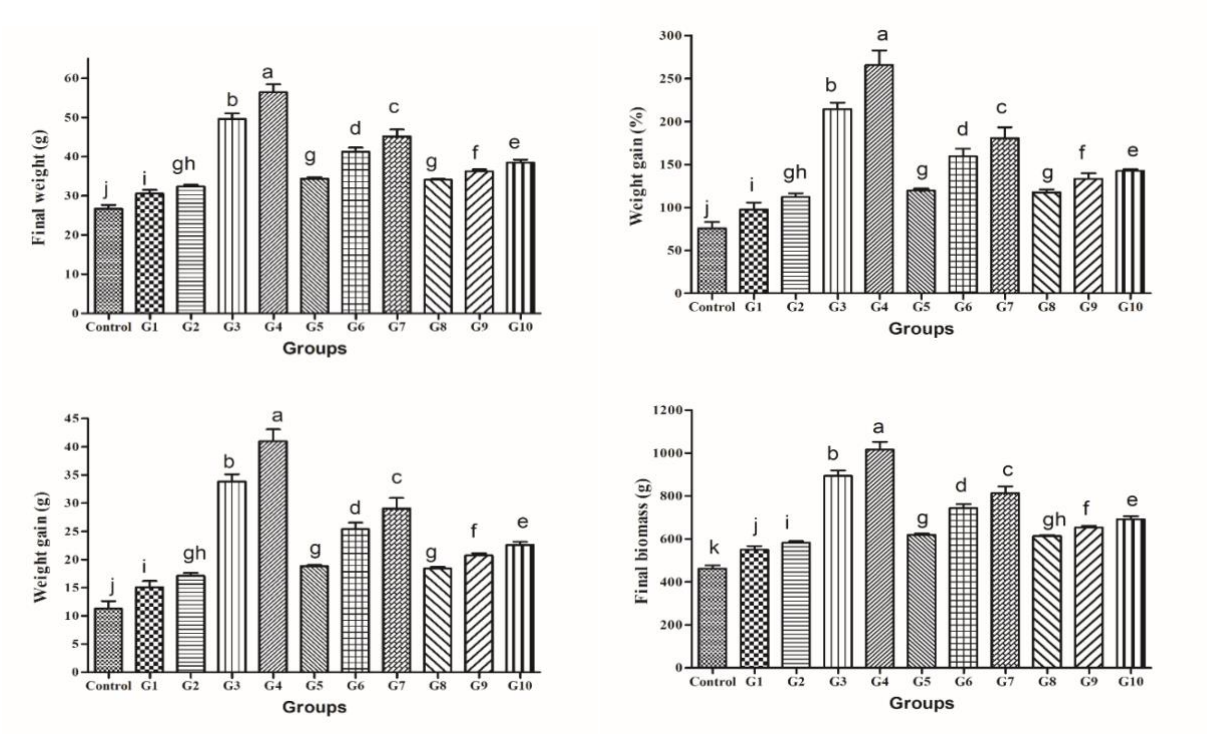

Fig 1: Weight gain (g), weight gain (%), final biomass (g) and final biomass (%) of L. rohita fed with control, free and G. candidum microspheres supplemented diet. Each bar shows the value as average ± SD, n=3.

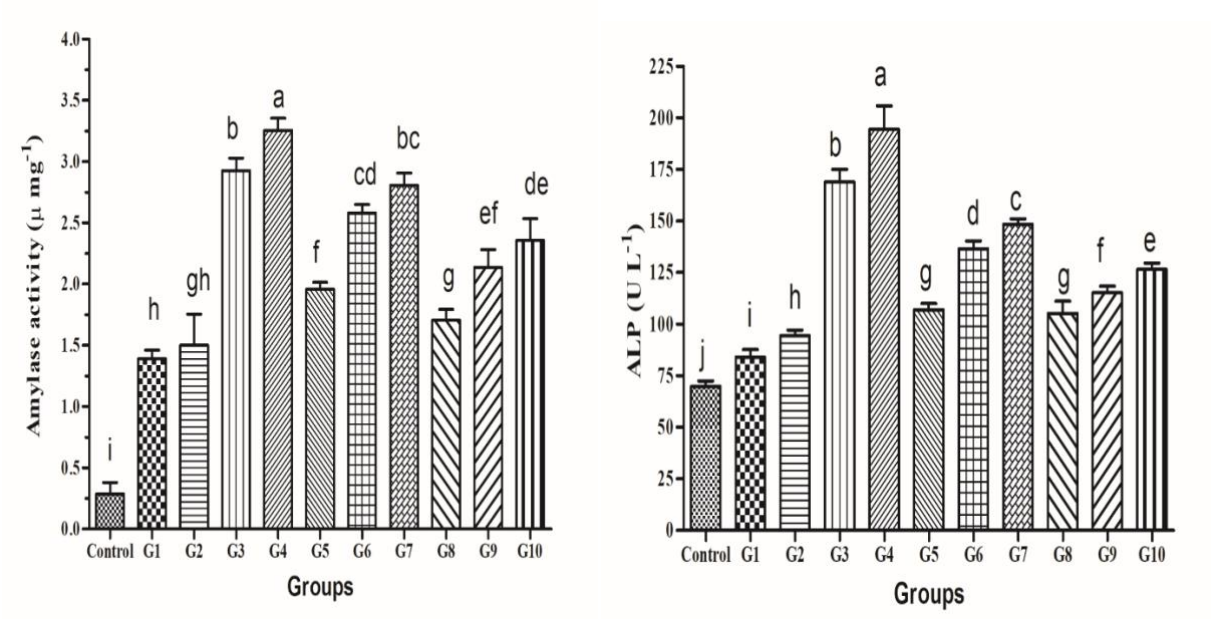

Fig 2: Amylase and Alkaline phosphatase (ALP) of rohu (L. rohita) fed with control, free and G. candidum microspheres supplemented diet (n=9). Each bar shows the value as average ± SD, n=9.

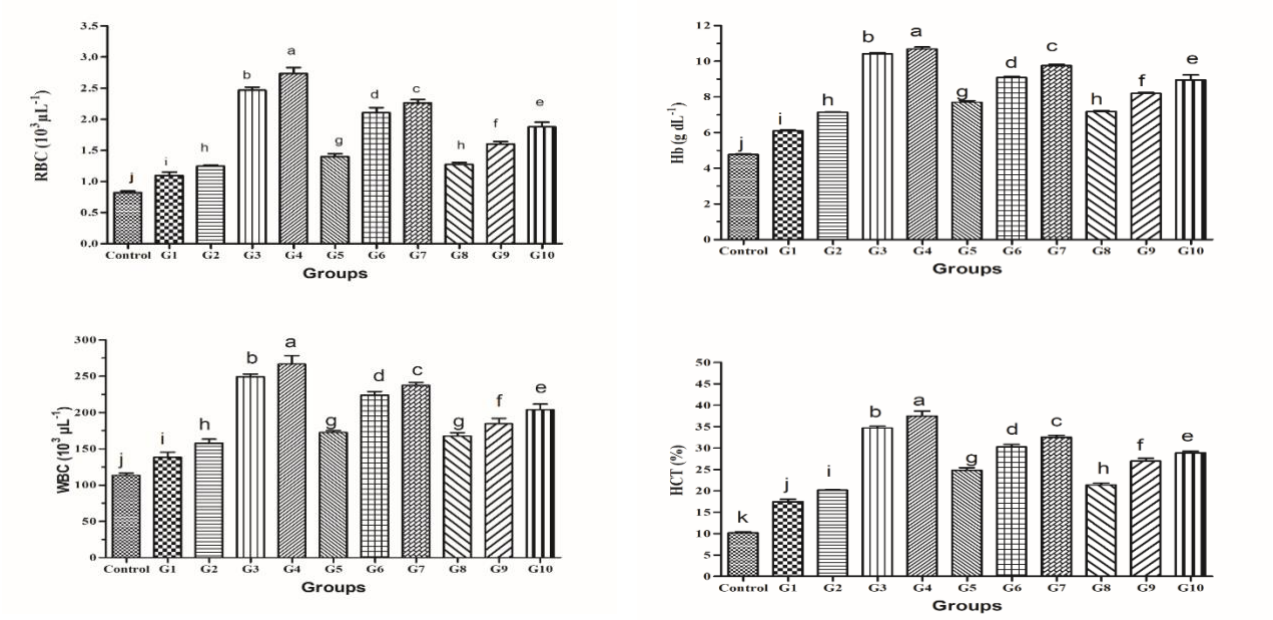

Fig 3: RBC (red-blood cells), WBC (white-blood cells), Hb (Hemoglobin) and HCT (hematocrit) count of L. rohita fed with control, free and G. candidum microspheres supplemented diet (n=9). Each bar shows the value as average ± SD, n=9.

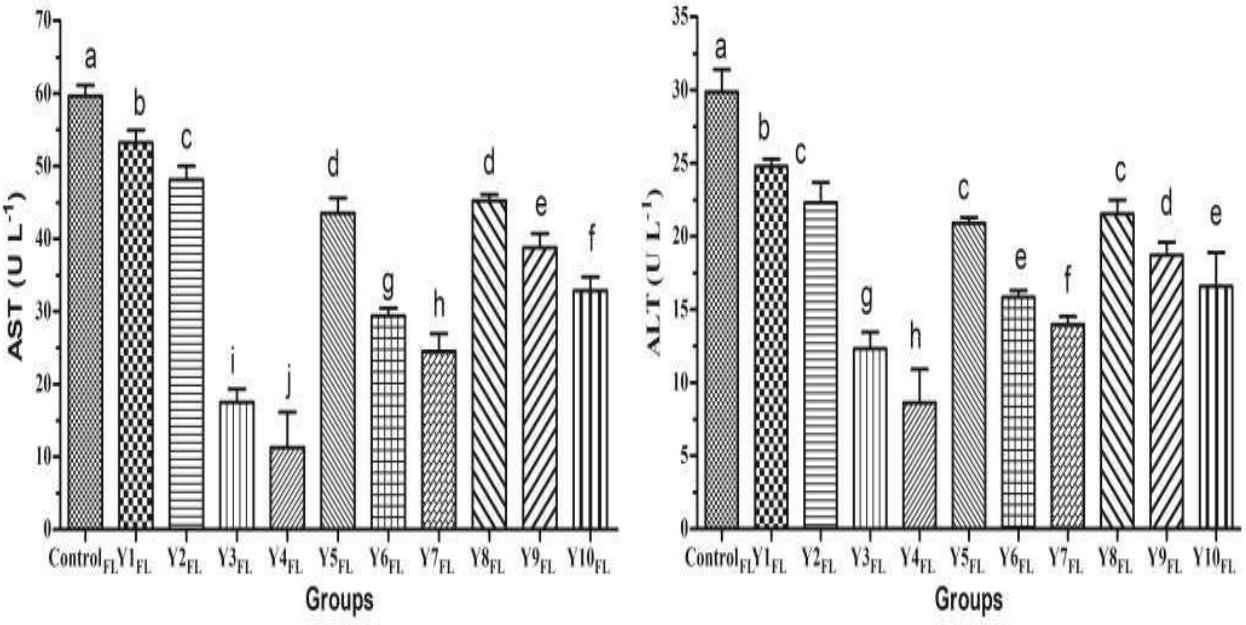

Fig 4: Aspartate amino-transferase (AST) and alanine amino-transferase (ALT) of L. rohita fed control, free and G. candidum microshperes supplemented diet (n=9). Each bar shows the value as average ± SD, n=9.

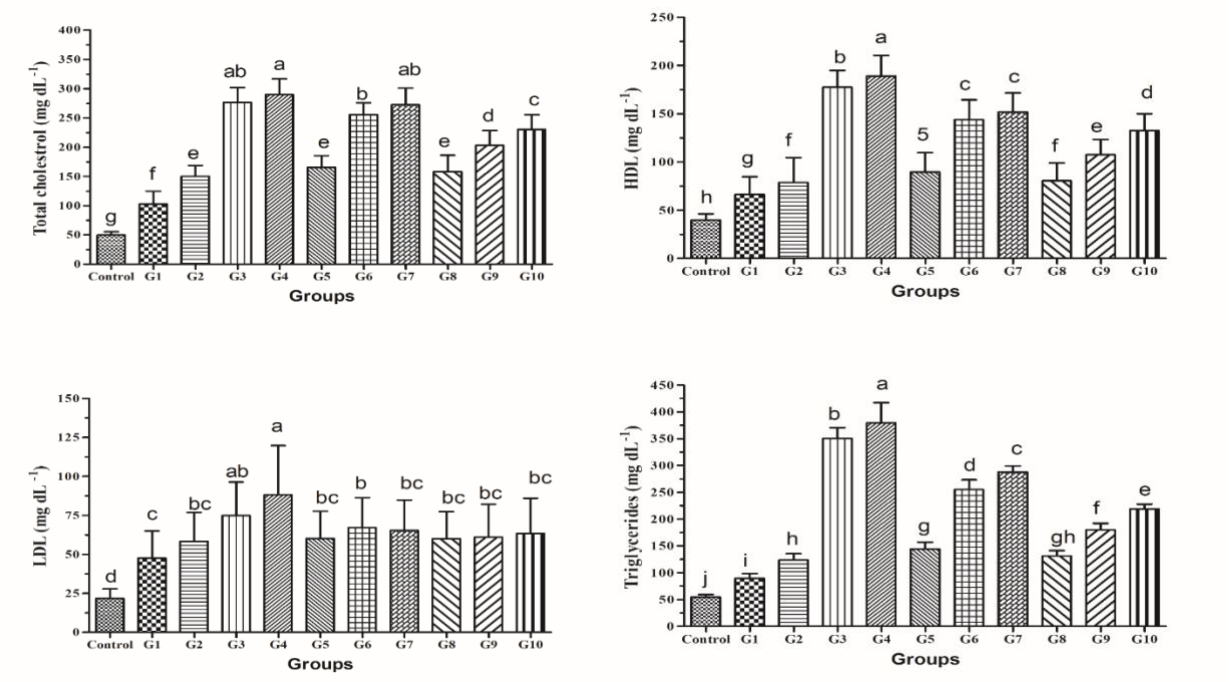

Fig 5: Total-Cholesterol, Low-density-lipoprotein (LDL), High density lipoprotein (HDL) and Triglyceride values of L. rohita fed with control, free and G. candidum microspheres supplemented diet. Each bar shows the value as average ± SD, n=9.

4. Discussion

Probiotic use in fish culture has few limitations i.e. tolerance to GI tract condition and easy storage, which obviously restrict its practical usage in aquaculture. Encapsulation appear as most suitable technique to improve efficacy of probiotic for sustainable expansion of aquaculture. However, microencapsulation be influenced by types and conc. of encapsulating agent, the process of microencapsulation, hardening solution, the size of microspheres along with the physiology of host animal (Papadimitriou et al., 2015; Jyothi et al., 2010). In this trial, zero mortality was recorded in *L. rohita* fed both type of diets (free and encapsulated *G. candidum*. Similarly, the substantial effect of free *G. candidum* probiotic in contrast to diet (without probiotic) on the survival (%), growth performance (body weight, body mass), GI tract enzyme (Protease and amylase), hematology (increased RBCs, WBCs, HB), immunity (greater total serum protein, IgM, Lysozyme) in *L. rohita*. Similar interpretations were recorded by Mohapatra et al., (2012), immunity (Gupta et al., 2014; Ullah et al., 2018), gut enzymes activity (Amir et al., 2023; Ullah et al., 2019) and resistance against pathogenic microbes (Amir et al., 2019; Ullah et al., 2018; Nousheen et al., 2026) of fin fish and shell fish.

Beyond improving probiotic delivery efficiency, encapsulation also support the probiotic action at biochemical level by protecting cells from environmental stressors and guarantee their continuous

release in the intestine (Rahiman et al., 2010). Thus, increased survival facilitates colonization, improves enzymatic digestion and immunity, which contribute to better feed utilization and fish health (Giri et al., 2014; Subharanjani et al., 2015). It was observed that *G. candidum* had favorable effect on the AST, ALT and ALP activities. ALT further indicates that liver cell damages and high serum cholesterol (Fakruddin et al., 2017: Amir et al., 2024). A decrease in AST among all probiotic fed groups show an improve in liver function as compared to control fish. It is well established already that ALT and ALP catalyze the production and de-amination of amino acids, thus in stress, in order to encounter the high energy requirments of the organisms, involve in the conversion of protein to carbohydrate (Waarde and Henegouwen, 1982). Similar results were obtained decreased in activities of plasma AST and ALT in *O. niloticus* (El-Rehman et al., 2009). Accordingly, decrease in the activities of hepatic enzyme (ALT, LDH, AST,) in response to probiotic, *B. licheniformis* (Adorian et al., 2018), yeast (0.5%) in *O. niloticus* (Hassaan et al., 2014) and *S. cerevisiae* in *S. galilaeus* (Abdel-Tawwab et al., 2006). ALP is poly-functional enzyme which plays role in the hepatic function as well as bone development while its activity could be controlled through feed (Lalles, 2010). ALP increase here may be due to increase retention of minerals or due to stimulation of phosphorus metabolism. It is already known that low ALP is an indicator of mineral deficiency in fish (Sugiura et al., 2004: Amir et al., 2024; Farooq et al., 2025). Similar results were noted in rainbow trout (*O. mykiss*) after exposure with *L. rhamnosus* by Panigrahi et al. (2010). Moreover, increase in ALP was found in *A. japonicas* after feeding yeast diet (Ma et al. 2013).

Previous studies on probiotics in *L. rohita* have primarily utilized various strains like *Bacillus cereus, Lactobacillus spp.,* and *G. candidum*, often delivered through direct feed, which improved 25% growth rate and 95% survival but suffered from low viability after pelleting (Hoseinifar et al., 2022, Amir et al., 2018). However, encapsulation formulations including alginate-chitosan microcapsules, yielded 99% efficiency and pH-temperature tolerance (Amir et al. 2023 and 2024) compared to xanthan used in carp enabling better gut colonization (Van Doan et al., 2021).

It was determined in this study that alginate with nano-coated chitosan and chitosan bulk form with alginate is the best microspheres for improved target release of *G. candidum* in the gut of *L. rohita*. In short, microcapsules of *G. candidum* may be used in fish farm to get higher fish production in sustainable way. Future research should explore the efficacy of *G. candidum* microcapsules in other freshwater fish species, as well as their long term impact beyond 11-week duration on growth. Also, estimating the cost and large scale application of probiotic microsphere delivery will be vital for evaluating its inclusion for commercial aquaculture application.

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