Fish and Shellfish Immunology 95 (2019) 464–472

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Evaluation of commercial application of dietary encapsulated probiotic (*Geotrichum candidum* QAUGC01): Effect on growth and immunological indices of rohu (*Labeo rohita,* Hamilton 1822) in semi-intensive culture system

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ARTICLE INFO

Keywords:
G. candidum
Probiotic
L. rohita
Encapsulated
Growth
Immunity
Health status

ABSTRACT

Encapsulated probiotic administration can be a nutritional strategy to improve the growth performance and immune status of fish. Here commercial application of encapsulated *G. candidum* was evaluated as a feed supplement to fingerlings of *L. rohita* reared in earthen ponds under semi-intensive culture conditions. Fingerlings with an average body weight of 20 ± 2.34 g were distributed randomly in three groups and experiment was conducted in triplicate. The control group (P0) was fed 35% protein basal diet while the two treated groups, P1 and P2 were fed basal diet supplemented with 10^9 CFU g^{-1} un-encapsulated (free) and encapsulated *G. candidum*, respectively, for eleven weeks. Results indicated significantly ($P < 0.05$) improved growth rate, intestinal enzyme activities (protease, amylase and cellulase) and hemato-immunological indices (RBCs, Hb, HCT, WBCs, MCHC, respiratory bursts and phagocytic activity, total protein, lysozyme, IgM), upregulation of heat shock protein HSP 70 gene in muscle, intestine and liver tissues and reduction of serum AST and ALT activities, total cholesterol and triglyceride in fish fed *G. candidum* supplemented diets (P1 and P2 groups) as compared to basal diet (P0 group). However, diet supplemented with encapsulated *G. candidum* showed the most significant ($p < 0.001$) positive effect in comparison to un-encapsulated probiotic. In conclusion, a pronounced effect of G. *candidum* especially in the encapsulated form on the growth, health status and immunity of *L. rohita* reared in semi intensive culture system, suggesting its application as a feed additive in practical/commercial semi-intensive earthen pond culture system.

1. Introduction

Over the last decade, with an increase demand of ecofriendly sustainable development of aquaculture practices, research on microorganisms to serve as potential probiotics for fish and other aquatic organisms has increased [1,2]. A number of microorganisms have been evaluated as probiotics in aquaculture to improve growth, enzymatic potential, nutrient digestibility and manipulation of host immune responses of several fish species in controlled conditions [1,3]. Concurrently, search is ongoing to select suitable microbes which could successfully be used as probiotics for practical/semi-intensive culture system [4,5].

Generally, probiotic work depends on biological and physiochemical factors such as size and age of the culture aquatic species [6,7], culture condition and environmental factors [8]. Thus, one probiotic could not be used for all aquatic organisms [9], under all culture conditions. For instance, some microbes as a probiotic are beneficial for one fish species or aquatic animals, but are harmful to other species [10]. Similarly a particular probiotic may be effective in controlled conditions, but could be ineffective in extensive and semi-intensive practical aquaculture practices [5]. Although probiotics are gaining increased scientific and commercial interest in fin and shellfish aquaculture worldwide [2], only a few studies address their application in the semi-intensive culture of major carps [6,11].

In South Asian countries, especially Pakistan, Bangladesh and India, semi-intensive carp culture is very old and extensively used practice for obtaining fish [12]. The key characteristic of this system is the dependence on the combination of artificial and natural feed [13] and it is

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https://doi.org/10.1016/j.fsi.2019.11.011
Received 12 July 2019; Received in revised form 30 October 2019; Accepted 2 November 2019
Available online 04 November 2019
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managed by the addition of lime, organic, inorganic fertilizers and supplemented feed, etc. Among organic fertilizer, animal manure is commonly used to increase plankton (live food organisms) production [14,15]. Nevertheless, it is classified as hazardous organic matter, poses a risk to the water environment by introducing pathogenic bacteria [16] like *Pseudomonas, Aeromonas* species [17,18], and may cause various kind of diseases in aquatic organisms e.g., skin ulcerations, furunculosis, albinoderma, erythroderma etc. [19,20].

Recently, in aquaculture significant consideration has been given to the use of probiotics for the re-establishment of a disturbed microbiota with normal beneficial composition. It is well established that successful probiotic has a tendency to improve water quality [21,22], inhibit or out compete pathogenic microbes, to colonize the intestinal mucosal surface, improve survival, growth, immune response, disease resistance and nutritive values of fish (see reviews by Refs. [1,23]).

Nowadays, wide varieties of microbes are in use as probiotics for improving aquaculture production especially in Asia and South America [1,4,24–26]. Yet there are certain challenges associated with the selection and intake of probiotics like specificity of probiotic to species and culture condition [5], the viability of live probiotic cells during the food processing, long term storage and transition through the gastrointestinal tract of the host [27]. Therefore, currently, encapsulated probiotics are gaining importance for improving survival during processing, storage and target release when administered orally as well as research is in progress to evaluate the efficiency of particular probiotic with respect to species and culture system. Here an attempt has been made to assess the efficiency and practical application of local strain of yeast *Geotrichum candidum* QAUGC01 (Accession number KTC280407) by feeding free and encapsulated probiotic supplemented diets to fingerlings of *Labeo rohita* reared in earthen ponds under semi-intensive culture condition, an extensively used practice in Pakistan.

G. candidum is eukaryotic filamentous yeast like fungus, that can be isolated from soil, water, baker's dough, husks of fermentation, bread, milk, milk products and various plant substrates [28,29]. Its enzymatic potential i.e., release of α amylases, cellulases, β-glucanases, xylanases, proteases, lipases and phytases is well documented [28,30–32]. *G. candidum* QAUGC01 is a local strain isolated from dairy product yogurt. Its isolation, culture and *in vitro* enzymatic activity have been reported by Refs. [29,33]. Furthermore, the probiotic potential i.e., to colonize the GI tract of host, support the growth of lactic acid bacteria and competitively exclude the pathogenic bacteria as well as their products and influence the growth, immunity and disease resistance against pathogen of *L. rohita* under control and semi control condition at small scale has already been checked and reported [34–36]. It is well documented that *in vivo* efficiency of probiotics under control conditions, do not always provide the same results under practical semi intensive pond culture systems. According to Ref. [5], a probiotic may not be effective in all aquatic conditions, because the physiological and physico-chemical conditions of the host and environment could influence the efficiency of a particular probiotic. Therefore, before suggesting practical application, the effectiveness of *G. candidum* QAUGC01 under practical earthen ponds culture system was evaluated.

2. Materials and methods

2.1. Probiotic microorganism and encapsulation

G. candidum QAUGC01 isolated from commercial dairy product yogurt was cultured as described previously [35]. The cell culture suspension with a final viable count of 10^9 CFU ml^{-1} was used for encapsulation. The detailed procedure for encapsulation and *in vitro* and *in vivo* characterization of microcapsule of *G. candidum* is described previously by Ref. [37]. Briefly, *G. candidum* QAUGC01 culture suspension and sodium alginate solution (2% w/v) were mixed in 1: 3 ratio in order to get the final concentration of 10^9 CFU ml^{-1}. The resultant mixture was mixed thoroughly for 2–3 min. Then, 40 ml of calcium chloride dihydrate ($CaCl_{2.}$ $2H_2O$) solution (0.1 M) was mixed with 60 ml chitosan solution (0.4% w/v), to yield cationic solutions (gelling solution) of chitosan in calcium chloride [38,39]. The pH of solution was adjusted to 5.6 with sodium hydroxide. The gelling solution was taken in a beaker and placed on a mini orbital shaker set at uniform speed (90 rpm). Subsequently, the mixture of G. *candidum* with alginate was taken in a sterile syringe (10 ml) with 20G needle and drop-wise poured into a cationic gelling solution containing chitosan in $CaCl_2$. H_2O. The resultant chitosan coated capsules were allowed to remain in solution for hardening under gentle shaking. Then, microcapsules coated by chitosan were filtered and washed with deionized water in order to remove excess of chitosan. The microcapsules were dried in a petri dish and stored in sterilized petri dish at 4 °C until further use.

The practical application of chitosan coated alginate microcapsules of *G. candidum* in comparison to free (un-encapsulated) probiotic was evaluated by conducting 11 weeks feeding trial in semi intensive earthen pond culture system.

2.2. Probiotic supplementation in the feed

The feed ingredients were purchased from a local feed mill (Oryza organics private limited™). Feed pellets (2 mm) with crude protein (35%, Table 1) were prepared fortnightly and stored as reported earlier [35]. The prepared pelleted diet was divided into three groups. First control group (P0) pellets were sprayed with PBS (2.5 ml of PBS g^{-1} feed) without any probiotic while pellets of other groups P1 and P2 were top dressed with free and encapsulated *G. candidum* respectively at final concentration of10^9 CFU g^{-1}. To avoid extreme variation in microbial count, fortnightly fresh diets were prepared and stored at 4 °C.

2.3. Fish collection and management

About nine hundred fingerlings of *L. rohita* (average body weight 20 ± 2.24 g) were purchased from Government Fish Hatchery Manawan Lahore, Pakistan. Fish were transported in well aerated oxygen filled plastic bags to the Fisheries and Aquaculture facility and transferred into tanks having flow through system. They were acclimated for two days and during this period were fed 35% crude protein diet.

2.4. Experimental design

A completely randomized experiment was designed and conducted in replicate of three in earthen ponds during the month of June-to August by adopting a semi-intensive culture system. The culture system was maintained with the aid of organic and inorganic fertilizers, lime and prepared diet.

Table 1
Formulation and proximate composition of the basal diet.

Ingredients	Inclusion level (g/100 g)
Soybean meal	36
Gluten 60	37
Wheat bran	10
Fish meal	12
Fish oil	1
Vitamin and mineral mix	2
CMC[a]	2
Total	**100**
Proximate composition (%)	
Crude Protein	35.34
Crude Lipid	8.95
Ash	7.86

[a] Carboxymethyl cellulose, used as binder.

2.5. Earthen pond preparation

All ponds at Fisheries and Aquaculture facility were rectangular in shape with an average size of 120 m^2 and a depth of 1.45 m. Before the initiation of the experiment, all earthen ponds located adjacent to each other were sun dried. Ponds were treated with calcium carbonate at the rate of 125 kg ha^{-1} and fertilized with cow dung at the rate of 3333.33 kg ha^{-1} [11] to enhance pond productivity. Animal manure was spread evenly on the pond bottom and exposed to the sun for several days. After the application of manure, the ponds were half filled with water obtaining from the nearby freshwater Rumli stream. However, when water became fertile, more water was added and maintained level up to 1.3 m. Throughout the experiment, pond productivity and water level was maintained with the addition of fertilizers (animal manure and Diammonium phosphate (DAP)) and water. Occasionally, the productivity of each pond was checked by using sacchi disc, reading < 30 cm indicate the requirement of fertilizers.

About 720 fingerlings of *L. rohita* with no sign of infection were shifted evenly to the nine earthen ponds. Fingerlings were again acclimated in their respective tanks for two days. Afterwards nine earthen ponds, each with 80 fish were randomly assigned a treatment group (240 fish/group). Feeding trial was started by providing respective diet to each group i.e. devoid of any probiotic to P0 group while free and encapsulated *G. candidum* supplemented diets to P1 and P2 groups respectively. Fingerlings were provided their respective prepared diet twice a day (8:00 and 16:00) at 3% of the body weight for a period of eleven weeks.

During feeding trials, daily water temperature (°C), DO level (mg L^{-1}) and pH value were checked with a multiparameter (HI-9828 HANNA Instruments. Inc. Woonsocket, USA) while total ammonia was checked weekly. The DO level and temperature showed fluctuation during feeding trial, with DO ranging from 4.5 to 6.2 mg L^{-1} and temperature from 25.6 to 28.6 °C while other parameters showed no noticeable difference, pH (7.2 ± 0.52), total ammonia < 0.5). All experimental ponds were in the same vicinity, adjacent to one another and under similar environmental conditions, thus no noticeable difference in water quality parameters between groups was observed.

2.6. Growth performance

After the eleven week feeding trial, fish were starved for 24 h before harvesting. On the day of sampling, the outlet of the pond was opened and water was drained into a fish collecting basin. Fish of each pond were collected separately, weighed and the number was counted to evaluate the growth performance using standard formulas.

2.7. Intestinal enzyme activity

For intestinal enzyme analysis, 6 fish per tank (18 fish/group) were immediately anesthetized with MS-222 (0.10 g L^{-1} buffered with sodium bicarbonate), aseptically dissected at low temperature (using ice bag) by adopting a standard aseptic method and their gastrointestinal-tracts (GI tract) were carefully removed. The GI tract of 2 fish from the same pond were pooled (3 samples/pond or 9 samples/group), snap-frozen in the liquid nitrogen and immediately saved at 20 °C for the determination of intestinal enzymes activities. The activities of amylase, protease, and microbial origin cellulase were determined using respective standard methods as reported previously [35].

2.8. Hematological indices

At random 9 fish from each pond were selected, anesthetized with freshly prepared MS-222 (0.10 gL^{-1} buffered with sodium bicarbonate) and their blood was drawn from the caudal vein with the help of 3 ml sterile syringe (24G, Shifa® Changzhou Tangda Medical App. Co., LTD). Blood samples were collected in lavender top K2 VACUETTE® EDTA tubes (LiuyangSanli Medical Technology Development Co., LTD). For enough volume, blood of 3 fish from each pond was pooled in same ETDA tubes (3 samples/pond or 9 samples/group). The blood was used for hematological indices i.e. WBCs ($\times 10^3 \mu L^{-1}$), RBCs ($\times 10^6$ μL^{-1}), Hb (g dL^{-1}), HCT (%), MCH (pg), MCHC (g dL^{-1}) and MCV (fL) determination [11].

2.9. Immunological and biochemical indices

For serum collection blood sample from 15 fish per pond (45 fingerling/group) was also collected from the caudal vein by using 2 ml heparinized syringe (24G) in red top EDTA VACUETTE® tubes. For obtaining enough sample, blood of five fingerlings from same pond was collected in the same blood collection tube (9 sample/treatment). Blood samples were centrifuged for 5 min at 3000 rpm and separated serum was decanted in microtube and stored at 4 °C.The analysis of total serum proteins, immunoglobulins (IgM), lysozyme, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity was measured by using respective standard methods reported earlier by Ref. [11]. While fresh heparinized blood was used for measuring the phagocytic and respiratory burst activity [40]. Total cholesterol and triglyceride assays were performed by adopting standard procedures reported by Ref. [41].

2.10. Gene expression

For evaluating, HSP 70 gene expression in each group, nine fish from each pond (27 fish/group) were dissected and their liver, muscle and intestine tissues were collected. In order to get enough sample (about 50 mg), samples of three fish from same pond were pooled (nine sample per treatment) and preserved in RNA later and stored at −80 °C for further use.

2.10.1. Extraction of total RNA and cDNA synthesis

Total RNA from each sample was extracted by using High pure RNA tissue kit (Roche, Basel, Switzerland). The purity of extracted RNA was measured by NanoDrop- ND1000 (Tech. Inc., Wilmington, USA). The RNA was converted into cDNA for qPCR using RevertAid First strand cDNA synthesis kit (Thermo Fisher Scientific, Lithuania). The extracted RNA (1 μg) was incubated at 70 °C for 5 min with Random Hexamer primers and then cooled at room temperature for 10 min in order that primers can anneal appropriately to the RNA. Subsequently, RNAs inhibitor, RT-buffer, RT-enzyme and dNTPs were added in the mixture and again incubated at 25 °C for 5 min followed by 42 °C for 60 min and for 3 min at 95 °C in a thermal cycler (Mastercycler, Gradient Eppendorf, USA). The prepared cDNA was immediately stored at −20 °C for further analysis.

2.10.2. PCR conditions for gene expression

For studying the relative expression of heat shock protein HSP70 gene in the tissues of experimental groups, quantitative real time (qPCR) was conducted. List of primers used are presented in Table 2. PCR conditions and cycle number were optimized first for HSP 70 gene before further analysis. The working conditions are followings, temperature cycle at 95 °C for 10 min along 40 cycles of amplification and

Table 2
Primers used for expression study.

Gene	Product size (bp)	Primer sequence	Reference
HSP 70	114	Forward CACAATCACCAACGATAAGGG Reverse TTGGCAGACACCTTTTCACGC	[80]
β-actin	200	Forward AGACCACCTTCAACTCCATCATG Reverse CCGATCCAGACAGAGTATTTACGC	[80]

quantification, (denaturation at 95 °C for 15 s; 1 min annealing at 62 °C and elongation at 95 °C for 15 s), along a slow heating at 60 °C and finally a cooling step to 42 °C. After qPCR, cycle threshold (C_T) was calculated. The value was calculated using method of real time machine (Roche: Light Cycler, LC-480, Germany) called AbsOuant 2nd derivative Max. Negative control containing RNA template was also run with samples to avoid from the possibility of genomic DNA contamination. The ratio of HSP 70 gene was normalized to β-actin (Housekeeping gene) expression. The advance relative-quantification E-method was used for efficiency correction with relative quantification of mRNA. While, $2_T^{-\Delta\Delta C}$ comparative threshold, C_T method was applied for calculating the gene expression level.

2.11. Statistical analysis

Before running statistical analysis, data were assessed for variance homogeneity and normality distribution by using Bartlett and Shapiro-Wilk's tests. All data were represented as mean ± SD. Significant differences in growth performance, survival, intestinal enzymatic activity, hematology and immunological parameters as well as HSP 70 gene expression between control and *G. candidum* fed experimental treatment groups were identified by using one–way ANOVA. Once significant differences were identified, then the comparison among the means was evaluated by LSD post hoc test using computerized statistical software package for social sciences (SPSS, Software version 20, Inc. Chicago, USA). All results were statistically evaluated at the 5% level of significance. Mean values along with their SD were plotted using GraphPad Prism 5 software.

3. Results

3.1. Growth performance

A diet supplemented with encapsulated probiotic *G. candidum* showed significant positive effects on growth performance and survival of *L. rohita* fingerlings reared in ponds under semi intensive culture conditions (Table 3). ANOVA indicated significant differences in final weight ($F_{2, 9} = 40$, $p < 0.001$), weight gain ($F_{2, 9} = 42.6$, $p < 0.001$), weight gain percent ($F_{2, 9} = 24.6$, $p < 0.001$), final biomass ($F_{2, 9} = 47.2$, $p < 0.001$), SGR ($F_{2, 9} = 22.3$, $p < 0.001$) and survival ($F_{2, 9} = 89.6$, $p < 0.001$) between P0, P1 and P2. The Post hoc LSD test indicated improved growth performance of probiotic treatment groups (P1 and P2) as compared to control (P0). Furthermore, all possible pairwise comparisons indicated that among probiotic fed groups, higher weight gain, final biomass and SGR was observed in P2group followed by P1. Similarly, the highest survival (99%) was observed in P2 group followed by P1 (96%) while P0 group showed lowest survival (89%) during the experimental period.

Table 3
Comparative effect of free and encapsulated *G. candidum* supplemented diet on growth indices of *L. rohita* fingerlings, after 11 weeks rearing in earthen ponds under semi-intensive culture conditions (n = 3).

	Groups			Pooled SEM	F ratio	p-value
	P0	P1	P2			
IBW (g)	20.2^a	19.6^a	20.4^a	0.71	0.323	0.736
FBW (g)	59.3^c	73.3^b	90.2^a	2.40	40.0	0.001
Weight gain (g)	39.1^c	53.6^b	69.7^a	2.34	42.6	0.001
Weight gain (%)	194^c	272^b	341^a	14.7	24.6	0.002
[1]SGR (% body weight/day)	1.41^c	1.73^b	1.95^a	0.058	22.3	0.002
Initial biomass (g)	1617^a	1574^a	1628^a	60.1	0.220	0.809
Final biomass (g)	4252^c	5650^b	7192^a	214	47.2	0.001
Survival (%)	89.0^c	96.0^b	99.0^a	0.53	89.6	0.001

One way ANOVA followed by LSD post hoc test shows a pairwise comparison between groups. Means with different superscripts in same rows are significantly different at $p < 0.05$. P0, fed basal diet devoid of *G. candidum*, while P1 and P2, fed diet supplemented with free (un-encapsulated) and encapsulated *G. candidum*.[1]SGR = specific growth rate.

Table 4
Comparative effect of free and encapsulated *G. candidum* supplemented diet on intestinal enzyme activities (U mg^{-1}) of *L. rohita* fingerlings after 11 weeks rearing in earthen ponds under semi intensive culture conditions (n = 9).

	Groups			Pooled SEM	F ratio	p-value
Intestinal enzymes (U mg^{-1})	P0	P1	P2			
Protease activity	1.03^{cB}	1.69^{bB}	2.53^{aB}	0.05	207	0.001
Cellulase activity	0.47^{cC}	0.95^{bC}	2.07^{aC}	0.04	346	0.001
Amylase activity	1.61^{cA}	2.03^{bA}	2.97^{aA}	0.08	135	0.001
F ratio	14.0	104	48.8	–	–	–
P value	0.001	0.001	0.001	–	–	–

One way ANOVA followed by LSD post hoc test shows pairwise comparison between and within groups. Means with different lowercase superscripts in same rows are significantly different between groups, while uppercase superscripts in same column are significantly different within group at $p < 0.05$. P0, fed basal diet devoid of *G. candidum*, while P1 and P2, fed diet supplemented with free (un-encapsulated) and encapsulated *G. candidum*.

3.2. Intestinal enzyme activity

Digestion and absorption of the material can be inferred from the activity of intestinal enzymes in the gastrointestinal tract. Here, encapsulated *G. candidum* showed a more pronounced effect on the activities of intestinal enzymes of fingerlings *L. rohita* reared under semi intensive culture conditions (Table 4). ANOVA revealed significant differences in the activities of protease ($F_{2, 27} = 207$, $p < 0.001$), cellulase ($F_{2, 27} = 346$, $p < 0.001$) and amylase ($F_{2, 27} = 135$, $p < 0.001$) of P0, P1 and P2 groups. The pairwise comparison between groups indicated higher activities of all studied enzymes in probiotic fed groups (P1 and P2) as compared to control group (P0), while P2 group showed the highest activities ($p < 0.001$) of all three enzymes.

Moreover, comparison of protease, amylase and cellulase activity within the group, demonstrated a statistically significant difference in all studied enzymes in each group of fish, P2 ($F_{2, 27} = 48.8$, $P < 0.001$), P1 ($F_{2, 27} = 104$, $P < 0.001$) and control (P0) group ($F_{2, 27} = 14.0$, $p < 0.001$). Moreover, in all groups, activity of amylase was significantly higher followed by protease and cellulase respectively.

3.3. Hematological indices

The encapsulated *G. candidum* supplemented diet showed significant effect on hematology of *L. rohita* fingerlings reared in ponds under semi intensive culture conditions (Table 5). ANOVA revealed significant differences in erythrocytes ($F_{2, 27} = 104$, $p < 0.001$), leukocytes ($F_{2, 27} = 570$, $p < 0.001$), hemoglobin ($F_{2, 27} = 588$, $p < 0.001$), hematocrit ($F_{2, 27} = 89.9$, $p < 0.001$), MCV($F_{2, 27} = 8.66$, $p < 0.001$), MCH($F_{2, 27} = 12.7$, $p < 0.001$) and MCHC($F_{2, 27} = 3.90$, $p = 0.03$) of P0, P1 and P2 group. The Post hoc test showed higher values of RBCs, WBCs, Hb and HCT in probiotic fed groups (P2 and P1) as compared to control group. Furthermore, comparison among probiotic fed groups revealed significantly higher concentrations of RBCs, WBCs and Hb content in the P2 as compared to the P1 group. Conversely, significantly ($p = 0.001$) lower concentration of MCV observed in P2 group, as compared to P1 and P0 having statistically comparable concentration ($p = 0.14$). Similarly, P0 showed highest MCH concentration followed by P1 and then P2 group.

Table 5
Comparative effect of free and encapsulated *G. candidum* supplemented diet on hematological indices of *L. rohita* fingerlings after 11 weeks rearing in earthen ponds under semi intensive culture conditions (n = 9).

	Groups			Pooled SEM	F ratio	p-value
	P0	P1	P2			
RBC (10^6 µL)	1.03^c	1.52^b	2.21^a	0.06	104	0.001
WBC (10^3 µL)	140^c	179^b	224^a	1.76	570	0.001
Hb (g dL^{-1})	5.16^c	7.31^b	8.34^a	0.07	588	0.001
HCT (%)	20.1^c	26.2^b	32.1^a	0.63	89.9	0.001
MCV (fL or 10^{-15} L)	199^a	174^{ab}	145^c	9.14	8.66	0.001
MCH (pg)	51.1^a	48.6^{ab}	37.8^c	1.97	12.7	0.001
MCHC (g dL^{-1})	25.8^b	26.0^b	28.0^a	0.61	3.90	0.034

One way ANOVA followed by LSD post hoc test shows pairwise comparison between groups. Means with different superscripts in same rows are significantly different at $p < 0.05$. P0, fed basal diet devoid of *G. candidum*, while P1 and P2, fed diet supplemented with free (un-encapsulated) and encapsulated *G. candidum*.

3.4. Immunological and biochemical indices

Encapsulated *G. candidum* dietary supplementation positively affected the immunological and biochemical indices of *L. rohita* fingerlings (Table 6, Figs. 1–3). ANOVA revealed significant differences in serum total protein ($F_{2, 27} = 104$, $p < 0.001$), immunoglobulin ($F_{2, 27} = 603$, $p < 0.001$), lysozyme activity ($F_{2, 27} = 439$, $p < 0.001$), total cholesterol ($F_{2, 27} = 68.9$, $p < 0.001$), triglyceride level ($F_{2, 27} = 150$, $p < 0.001$), AST ($F_{2, 27} = 118$, $p < 0.001$) and ALT activity ($F_{2, 27} = 83.8$, $p < 0.001$), phagocytic activity ($F_{2, 9} = 146$, $p < 0.001$), phagocytic index ($F_{2, 9} = 194$, $p < 0.001$) and respiratory burst activity ($F_{2, 27} = 421$, $p < 0.001$) between P0, P1 and P2 groups. The Post hoc LSD test indicated higher values of total serum protein, immunoglobulin, lysozyme activity, phagocytic activity, phagocytic index and respiratory burst activity in probiotic fed groups (P1 and P2) as compared to control group (P0). Furthermore, all possible pairwise comparison showed the significant positive effect of encapsulated *G. candidum* followed by free probiotic (P2 > P1 > P0).

Additionally, lower levels of total cholesterol, triglyceride, AST and ALT activity were observed in probiotic fed groups (P2 and P1) as compared to control group (P0) of *L. rohita*. Furthermore, all possible pairwise comparison showed the lowest values of total cholesterol, triglyceride, AST and ALT activity in the P2, followed by the P1 group, while P0 group showed the highest levels (P2 < P1 < P0).

Table 6
Comparative effects of free and encapsulated *G. candidum* supplemented diet on immunological and biochemical indices of *L. rohita* fingerlings after 11 weeks rearing in ponds under semi intensive culture conditions (n = 9).

	Groups			Pooled SEM	F ratio	p-value
	P0	P1	P2			
Total serum protein (g dL^{-1})	2.39^c	3.26^b	4.02^a	0.080	104	0.001
Immunoglobulin (mg ml^{-1})	4.55^c	6.27^b	8.37^a	0.078	603	0.001
Lysozyme activity (µg ml^{-1})	7.51^c	10.52^b	11.4^a	0.097	439	0.001
Total cholesterol (mg dL^{-1})	218^a	132^b	78.1^c	8.54	68.9	0.001
Triglycerides (mg dL^{-1})	312^a	242^b	120^c	7.93	150	0.001
AST (U L^{-1})	110^a	86^b	62^c	2.20	118	0.001
ALT (U L^{-1})	32^a	20^b	10^c	1.18	83.8	0.001

One way ANOVA followed by LSD post hoc shows a pairwise comparison between groups. Means with different superscripts in same rows are significantly different at $p < 0.05$. P0, fed basal diet devoid of *G. candidum*, while P1 and P2, fed diet supplemented with free (un-encapsulated) and encapsulated *G. candidum*.

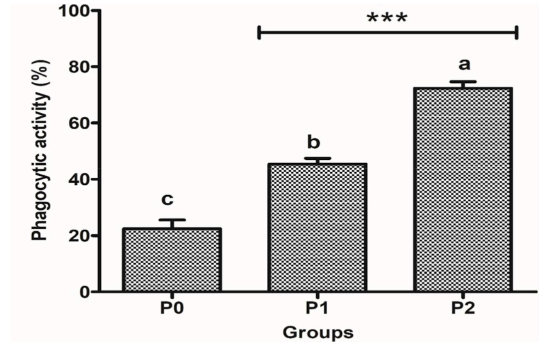

Fig. 1. Phagocytic activity of *L. rohita* fingerlings after 11 weeks feeding free (un-encapsulated) and encapsulated *G. candidum* supplemented diet. The bar shows the values as average ± SD, n = 3. ANOVA followed by LSD post hoc test represent comparisons between groups. Averages followed by different alphabets on bars are significantly different at $P < 0.05$. P0, fed basal diet devoid of *G. candidum*, while P1 and P2, fed diet supplemented with free (un-encapsulated) and encapsulated *G. candidum*.

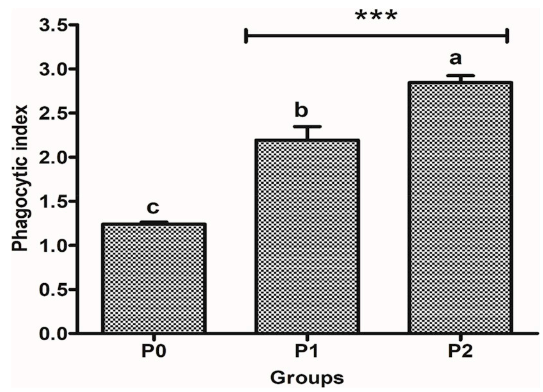

Fig. 2. Phagocytic index of *L. rohita* fingerlings after 11 weeks feeding free (un-encapsulated) and encapsulated *G. candidum* supplemented diet. The bar shows the values as average ± SD, n = 3. ANOVA followed by LSD post hoc test represent comparisons between groups. Averages followed by different alphabets on bars are significantly different at $P < 0.05$. P0, fed basal diet devoid of *G. candidum*, while P1 and P2, fed diet supplemented with free (un-encapsulated) and encapsulated *G. candidum*.

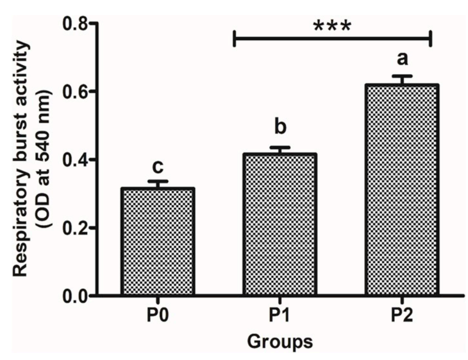

Fig. 3. Respiratory burst activity of *L. rohita* fingerlings after 11 weeks feeding free (un-encapsulated) and encapsulated *G. candidum* supplemented diet. The bar shows the values as average ± SD, n = 9. ANOVA followed by LSD post hoc test represent comparisons between groups. Averages followed by different alphabets on bars are significantly different at $P < 0.05$. P0, fed basal diet devoid of *G. candidum*, while P1 and P2, fed diet supplemented with free (un-encapsulated) and encapsulated *G. candidum*.

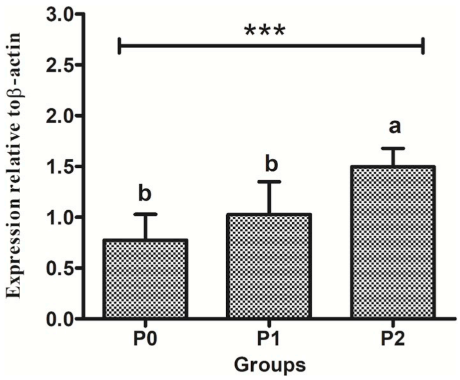

Fig. 4. HSP 70 gene expression in the muscle of *L. rohita* fingerlings after 11 weeks feeding free (un-encapsulated) and encapsulated *G. candidum* supplemented diet. The bar shows the values as average ± SD, n = 9. ANOVA followed by LSD post hoc test represent comparisons between groups. Averages followed by different alphabets on bars are significantly different at $P < 0.05$. P0, fed basal diet devoid of *G. candidum*, while P1 and P2, fed diet supplemented with free (un-encapsulated) and encapsulated *G. candidum*.

3.5. Gene expression

The encapsulated in contrast to free (un-encapsulated) *G. candidum* supplemented diet showed the most significant positive effect on HSP 70 gene expression in different tissues of *L. rohita* fingerlings (Figs. 4–6). One way ANOVA revealed significant differences in HSP 70 gene expression in the muscle ($F_{2, 27}$ = 17.9, p = 0.001), intestine ($F_{2, 27}$ = 51.7, p = 0.001) and liver ($F_{2, 27}$ = 71.3, p = 0.001) of fish from P0, P1 and P2 groups. The Post hoc LSD test revealed up-regulation of HSP 70 gene in probiotic fed groups (P1 and P2) as compared to control group (P0). Furthermore, all possible pairwise comparison showed significantly higher HSP 70 expression in muscle, intestine and liver tissues of P2 group as compared to the P1 group of fish. The muscle tissues of P0 and P1 groups showed statistically comparable and lower expression related to HSP 70 protein (LSD post hoc, p = 0.121, Fig. 4) as compared to P2 group.

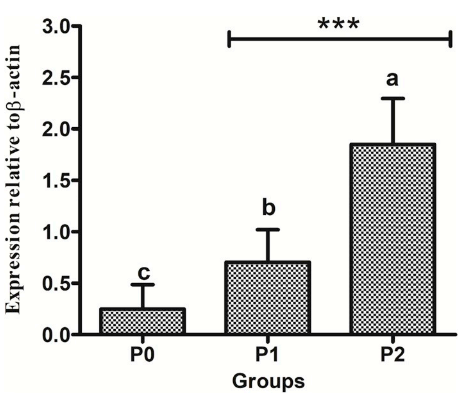

Fig. 5. HSP 70 gene expression in the intestine of *L. rohita* fingerlings after 11 weeks feeding free (un-encapsulated) and encapsulated *G. candidum* supplemented diet. The bar shows the values as average ± SD, n = 9. ANOVA followed by LSD post hoc test represent comparisons between groups. Averages followed by different alphabets on bars are significantly different at $P < 0.05$. P0, fed basal diet devoid of *G. candidum*, while P1 and P2, fed diet supplemented with free (un-encapsulated) and encapsulated *G. candidum*.

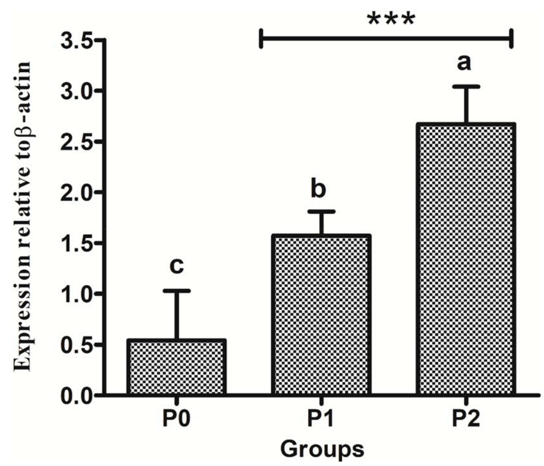

Fig. 6. HSP 70 gene expression in the liver tissue of *L. rohita* fingerlings after 11 weeks feeding free (un-encapsulated) and encapsulated *G. candidum* supplemented diet. The bar shows the values as average ± SD, n = 9. ANOVA followed by LSD post hoc test represent comparisons between groups. Averages followed by different alphabets on bars are significantly different at $P < 0.05$. P0, fed basal diet devoid of *G. candidum*, while P1 and P2, fed diet supplemented with free (un-encapsulated) and encapsulated *G. candidum*.

4. Discussion

Here for evaluating the practical application of local strain of yeast *G. candidum* QAUGC01 as a feed additive, the feeding trial was conducted in earthen ponds under semi-intensive culture conditions. It is well accepted that probiotic work depends on biological and physiochemical factors like, age and size of the cultured fish species [6,7], culture condition and environmental factors [8]. Therefore, feeding trial was conducted in replicate of three under similar conditions, feeding rate, feeding frequency and on the almost similar size of fingerlings. The obtained results clearly demonstrated the beneficial effects of encapsulated *G. candidum* followed by free form (un-encapsulated) on the growth performance and immunity of fish. Our finding is in accord with the observations of many investigators, where dietary probiotic supplementation showed beneficial effect on growth rate [11,21,42–44] immune response [11,45,46] and intestinal enzymes activities [11,35,47] of finfish and shellfish. It seems that *G. candidum* in semi intensive culture conditions, competitively exclude the pathogens and colonize the intestinal mucosal surface of host and exert its beneficial effects on the physiology of fish by secretion of exogenous enzymes and stimulation of immune response [35,36].

Generally, aquatic environment is not free of pathogenic organisms and aquatic organisms are in direct contact with the aquatic environment, thus may at risk of health problems [48]. Normally, remain healthy even in the presence of pathogens, but any stress due to environmental fluctuation and aquaculture practice shift the balance in favor of the disease [49]. Here, somewhat higher survival rate and no sign of disease may be due to improved water quality of culture system [21,22], which reduce the incidence of diseases [50]. In the present study, we did not test pond water for pathogens and simply checked the water quality parameter. Nevertheless, many scientists reported the lower incidence of pathogens and improved water quality of ponds when yeast was provided either directly or indirectly via feed [8,51,52].

Successful colonization of probiotic can regulate the microbial ecology of the gut and improve the digestibility of nutrients by stimulating and secreting various enzymes including digestive enzymes and cellulose degrading enzyme. The strong *in vitro* enzymatic potential of *G. candidum* i.e., higher level of cellulases, α amylases, β-glucanases, xylanases and moderate level of proteases and lipases [31,32] as well as positive impact on intestinal microbial load and digestive enzyme activity of juvenile *L. rohita* is well documented [34–36]. Here the improved secretion of proteases, amylases and cellulases (Table 3) may support the results of better growth and survival of *L. rohita* reared on probiotic supplement. It seems that *G. candidum* due to their higher enzymatic potential, improves the digestibility, absorption and availability of nutrients more efficiently.

The effects of probiotics on health status and immunity of aquatic organism are well documented. Here the decreased serum AST and ALT levels in groups fed *G. candidum* supplemented diets as compared to basal diet also indicated the improved health of fingerlings. Serum AST indicate the liver function and the health condition of fish [53], while ALT more specifically indicate liver cell damage and higher serum cholesterol level [53]. Our results are in agreement with other investigators who reported similar significant decrease in AST level in *Oreochromis niloticus, Cirrhinus mrigala* and *O. niloticus* reared on *Bacillus subtilis*, *B. licheniformis*, and *B. subtilis* + *Saccharomyces cerevisiae* supplementation respectively [11,54–56].

The results of complete blood count (CBC) also indicated the improved health status of *L. rohita* reared on probiotic supplemented diets. In aquaculture, hematological indices are considered as a vital tool for determining the physiological change and health status of fish [11,57]. No comparable study indicates the impact of *G. candidum* on the hematology and immune response of fish. However, like our results Goran et al. [58] reported the positive impact of *S. cerevisiae* on hematological indices of *C. carpio,* while Hassaan et al. [56] observed a significant increase in HB, Hct, RBCs and WBCs of *C. mrigala* in response to a mixture of *B. subtilis* and *Saccharomyces*.

The mucosal surface of aquatic animal, including fish serves as a first-line defense barrier against several bacterial and viral pathogens. It is well accepted that probiotic augment the innate and adaptive immune responses upon binding on mucosal surface [59–62]. Currently, information on the effects of present strain of potential probiotic *G. candidum* QAUGC01 (KTC280407) on the immunity of aquatic animals is limited. Here probiotic in both forms (free and encapsulated) showed a considerable increase in total serum protein, IgM level, phagocytic, respiratory burst and lysozyme activity indicating their role in improving the immune response of *L. rohita*. The increased level of total serum protein in the current study may be attributed to the stimulation of defense molecules like lysozyme, IgM, etc. In agreement with our results, Hassaan et al. [56] observed highest level of total protein in fish when fed the dietary supplement of 1% yeast extract in combination with low level of *B. licheniformis* (0.48×10^6 CFU g^{-1}).

In fish, IgM and lysozymes are the key components of the immune system [1]. Their increased levels in response to both free and encapsulated *G. candidum* dietary supplement indicate the stimulation of defense mechanism of fish i.e., production of antibodies against foreign invaders and stimulation of phagocytosis. It is well documented that successful probiotics have a tendency to stimulate the production of antibodies in fish [1,26] and boost up the activity of lysozyme against bacterial infection [26]. Like our results, a few investigators observed increase IgM level in different fish species, sea bream (*Sparus aurata*) [63], rainbow trout (*Oncorhynchus mykiss*) [64] and leopard grouper (*Mycteroperca rosacea*) [65]. Mohapatra et al. [66] after feeding yeast cells (*S. cerevisiae* and *Debaryomyces hansenii*) as a dietary supplement. Similarly, Guven and Yalcin [67] reported several fold increase in lysozyme activity in rainbow trout and *Catla catla* after administration of *S. cerevisiae* and yeast nucleotides supplement respectively.

Furthermore, the enhanced respiratory burst and phagocytic activity in response to *G. candidum* supplemented diet also indicated the strengthening of bactericidal mechanisms/innate defense of fish [1,23]. Respiratory burst generates reactive oxygen species (ROS) like superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) which are toxic to many microorganisms [23]. Generally, this process occurs in phagocytes which recognize any foreign material/potential pathogens, and degrade them by phagocytosis [68]. Like our results, Ortuno et al. [69] reported improved respiratory burst activity of in sea cucumbers; *S. aurata* after feeding *S. cerevisiae* supplemented diet. It seems β-glucan in the cell wall of yeast/*G. candidum* contribute in improving the health status of fish more efficiently by stimulating the immune system. β-glucan is a potent immune system stimulator [69], have "receptor molecule" on the surface of phagocytes, so, stimulate the process of phagocytosis and also secrete cytokines (signal molecules) for stimulating the formation of new white blood cells.

Beside above mentioned parameters, we also observed a decreasing trend in LDL, total cholesterol and triglyceride levels in groups of *L. rohita* fed *G. candidum* supplemented diet as compared to fish fed diet devoid of probiotic. A similar decrease in cholesterol level was observed in Atlantic salmon (*Salmo sar*) after 89 days feeding diet supplemented with three species of yeasts. i.e., *Candida utilis, Kluyveromyces marxianus* and *S. cerevisiae* [70]. Furthermore, a few investigators reported lower in cholesterol from the culture medium in response to *S. boulardii* and *S. cerevisiae* [71]. Limited literature is available on the effect of probiotics on total cholesterol, LDL and triglycerides levels in fish. However, many investigators reported the LDL and cholesterol lowering effect of *S. boulardii*, *L. casei* and *S. cerevisiae* in mice [53,72] and suggested the involvement of cell wall components of yeast such as β-glucan in lowering plasma cholesterol level [73].

Beside observing a positive effect of G. *candidum* on the growth and immuno-biochemical indices, the positive effect was also observed on the heat shock protein HSP70 gene expression in muscle, intestine and liver. The HSP70 gene was selected because it is involved in the protection of protein structure and also stop cell self-destruction (apoptotic) mechanisms [74]. HSPs are involved in the folding and translocation of newly synthesized proteins as well as in the repair of damaged proteins or bringing them back to their normal conformation [75], hence maintaining their normal function [22] and protein homeostasis [76]. A higher HSP70 level indicates a greater potential to respond to the stressful conditions possibly present in fish farms. The regulatory effect of HSP70 on pro-inflammatory cytokine production in grass carp was observed by Ref. [77], while others reported the implication of HSP70 gene in both innate and adaptive immunity [77,78]. In the present study, the dietary administration of probiotic in both form showed up regulation of HSP70 gene in different organs i.e., muscle, liver and intestine of fish. Moreover, higher HSP70 gene expression was observed in all studied tissues after feeding diet supplemented with encapsulated *G. candidum* as compared to free form. Like our results [79], also reported an up-regulation of HSP70 gene in the intestine, kidney and spleen of tilapia after administration of *Lactobacillus brevis* supplemented diet. Likewise [74], observed a similar trend in fish species after administration of *L. fructivorans* and *L. plantarum*. Moreover, *Lactobacillus* consortium induced HSP70 expression in the intestine of *L. rohita* [80].

The present study also revealed the most significant positive effects of encapsulated *G. candidum* on growth and immune status of *L. rohita* as compared to free *G. candidum.* About 69.2% and 27.3% greater production of the P2 group in comparison to the P0 and P1 groups of fish respectively indicating the suitability of encapsulated *G. candidum* to *L. rohita* reared in semi-intensive culture system. Our results are in agreement with the findings of many investigators, where alginate and alginate-skim milk encapsulated probiotic administration have shown to improve the growth rate and immune responses in fish e.g., Senegalese sole [81], Gilthead seabream [27] and *O. niloticus* [82]. The improved efficiency of encapsulated *G. candidum* as compared to free (un-encapsulated) probiotic may be due to improve stability during processing, storage and passage through the GI tract, higher absorption as well as target release of probiotic. It is well documented that the probiotic microorganisms in transit to the target site, reduce in number of viable cells [83]. Similar to this study [82], reported an improved weight gain and specific growth rate of *O. niloticus* fed alginate encapsulated *L. rhamnosus* as compared to control group.

The present improved survival, growth rate, intestinal enzymes activities, hematology, immune responses and HSP 70 gene expression of *L. rohita* reared on probiotic supplemented diets under semi intensive earthen pond culture conditions indicated practical application of selected probiotic *G. candidum*. Additionally, more pronounced impact of encapsulated *G. candidum* QAUGC01 may be attributed to the improved stability, viability of *probiotic* in the feed and bioavailability in the GI tract of *L. rohita*. Based on these results, encapsulated *G. candidum* QAUGC01 supplemented diet could be recommended as an eco-friendly viable way to supplement the conventional feed for improving production and immune status of *L. rohita*.

Funding

This research did not receive any specific grant from funding agencies in the commercial, public and not-for-profit sectors.

Ethical approval

The research was carried out by following compliance with ethical standards provided by the society for prevention of the cruelty to animals (SPCA) of Pakistan, with ethical approval (BEC-FBS-QAU2017-71) of Bioethical Committee of the Faculty of Biological Sciences, Quaid-i-Azam University.

Declaration of competing interest

Authors have no conflict of interest to declare.

Acknowledgement

We would like to thank Dr. Muhammad Javed Arshad (senior scientific officer) at NVL Islamabad for the facilitation during sample analysis.

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