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RESEARCH PAPER

Effect of Probiotic *Geotrichum candidum* on Early Rearing of *Labeo rohita* (Hamilton, 1822)

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Abstract

The present study was designed to test whether administration of single strain probiotic during early reaing could improve the survival and growth of rohu, *L.rohita*. Seventy days experiment was conducted in replicate of three and twelve hundred postlarvae (average wet body weight, 0.60 ± 0.05 mg) were equally distributed in two groups , control and treated (n=200 postlarvae /fiberglass tank). Both groups received similar feed except addition of probiotic,*Geotrichum candidum,*QAUGC01 in the rearing water of treated group. Postlarvae raised in the presence of probiotic showed significantly higher (P<0.05) survival rate, improved growth performance (weight gain and specific growth rate), enhanced intestinal protease, amylase and cellulase activity and significantly lower mortality (P = 0.003) after challenge with *Staphylococcus aureus*. Moreover, proximate composition also showed significantly (P<0.05) higher values of crude protein and considerably lower levels of ash content in muscle of fry reared on probiotic supplementation. This study is the first report on the beneficial effects of single locally isolated strainof *G. candidum as* probiotic on early life stages of *L. rohita* and suggests an economically viable way to boost fish production.

Keywords: Postlarvae, probiotic, survival, growth, digestive enzymes.

Introduction

Rohu, Labeo rohia is an important Indian major carp that cultured throughout the world because of its fast growth rate, high economic value and consumer demands. However, during early developmental stages, it shows high mortality (70–80%) and poor growth rate. These factors hamper the availability of quality seed (Mohapatra *et al*., 2012) and expansion of the production of this species (Jha *et al*., 2015), consequently causing an economic loss (Edwards, 2013).

In fish, early juvenile do not possess well-developed digestive system and lack set of necessary digestive enzymes (Ghosh *et al*., 2004). Furthermore, maturation of lymphoid organs and immune-competence also developed lately, generally after certain weeks of hatching (Zapata *et al.*, 2006), while their rearing environment is not free of pathogenic microbes (Wang *et al*., 2008). Hence, low survival and poor growth of early juvenile could be related to week immunity against pathogens and their incompetency to utilize nutrient efficiently.

Survival and growth rate of early juvenile could be enhanced by improving their status of digestive enzymes and immune competency (Liu*et al*., 2010). Some efforts have been made including use of microencapsulated diets (Ghosh *et al*., 2004), dietary exogenous enzymes and multi-strain probiotics (Jha *et al*., 2015), however no one try to rear postlarvae in the presence of single strain probiotic.

Probiotics are live ingested bacteria and yeast, confer health benefits by improving intestinal microbial balance of host (Merrifield *et al*., 2010). They could improve growth performance and feed conversion efficiency of fish by producing short chain fatty acids, digestive enzymes and vitamins (Martínez *et al*., 2012), enhance immune response by effecting T-cell differentiation **(**Merrifield *et al*., 2010), provide resistance to pathogenic organisms (Muñoz-Atienza *et al*., 2013) and increase tolerance under stressful condition (Varela *et al*., 2010). Moreover, also assure the digestion of anti- nutritional factors (Suzer *et al*., 2008), enhance fish appetite, improve carcass and flesh quality (van Nuenen *et al*., 2005).

By viewing the enzymatic potential (amylolytic, proteolytic, lipolytic, cellulolytic and phytase activity) (Abu Bakar, 2014; Eida *et al*., 2013; Boutrou *et al*., 2006) of *G. candidum* and their ability to release antibacterial substances (Dieuleveux *et al*., 1998), the

present study was designed with the assumption that it could improve the early growth and survival of *L. rohita*. To test this hypothesis , the postlarvae (after yolk sac absorption) were reared up to fry stage in the presence locally isolated strain of *G. candidum*, QAUGC01 and its potential benefits on digestive enzymes activity, proximate composition and mortality after challenge to *S. aureus* were evaluated.

Materials and Methods

Two thousands postlarvae (after yolk sac absorption) of *L.rohita*, average wet body weight 0.60 ± 0.05 mg were obtained from Rawal Fish seed Hatchery, Islamabad and were transported live to Fisheries and Aquaculture Research Station, Quaid-i-Azm University, Islamabad.

Preparation of Microbial Inoculum (Probiotic)

Geotrichum candidum QAUGC01,was isolated from indigenously fermented milk product Dahi (yogurt). Pre-culturing was done in1 L tryptone soya broth (TSB; Oxoid, UK) for 48 h at 25°C in a shaker incubator at 120 rpm. For developing a continuous, culture of *G. candidum*, fiberglass tank was filled with stream water and inoculated with stock probiotic preparation (100mlL^{-1}) for 48 h. Daily 9 L water from tank was used and fresh stream water was added in the tank.

Experimental Design And Feeding Strategy

The experiment was conducted in outdoor facility of Fisheries and Aquaculture Research Station in rectangular fiberglass tanks (120 × 60 × 60 cm). Ramli stream water having live feed (Planktons) was used for rearing of fish. Water was first collected in a large concrete open raceway. Filtration was employed by passing water over fine mesh screen before supplying to experimental tanks.

The experiment was performed in triplicate under semi-static conditions.Six rectangular fiberglass tanks were used, three for control group and others three for treatment group. Postlarvae of *L. rohita* (after yolk sac absorption) were stocked at the rate of 200 larvae per tank (stocking density about 9 mg L^{-1}). Aeration in each tank was provided via air stones connected to air pumps. The feeding trials were conducted for 70 days. The control group was provided with control diet (initially juice of 45 % protein starter tilapia feed which was gradually replaced with 34% protein powdered feed) purchased from the local fish feed mill (Oryza Organics Pvt.Limited-Pakistan). However, the treatment group received control feed in addition to *G. candidum* supplement at the rate of 10^9 $CFUL^{-1}$. Feed juice was prepared by dissolving starter feed in small amount of water and passes through gauze by squeezing. The feed juice was provided after every 2 h up to 5 days by using 5 g feed per tank per day, then for the next 15 day juice was replaced with 45 % protein starter tilapia feed and fed thricea day (at 8:00, 12:00 and 16:00) at the rate of 2g per tank. Afterwards, the fry were fed 35% crude protein powdered feed twice a day (at 8:00 and 14:00 h).Every day about 20% water from each tank of experimental group was replaced with a continuous culture of probiotic with the concept to maintain the probiotic concentration (about 10^9 CFU L^{-1} water), while in control group, it was changed with fresh stream water.

Water Quality

Throughout the experiment, water quality parameters like Dissolved oxygen (DO), temperature, pH, and total ammonia were checked by Multi-parameter Hanna HI 9147. Temperature and DO level were noted daily at 7:30 and 14:00 h while pH and total ammonia were monitored weekly.

During the experimental period, water temperature ranged from 23.7 to 26.7°C, dissolved oxygen from 5.5 to 6.6mg L^{-1} pH from 7.5 to 7.9, while total ammonia was less than 0.25 mg L^{-1}. Water quality parameters were found within the range acceptable for rearing of *L. rohita*

Percentage Survival and Growth

At the end of study, total number of fry in each tank were counted and the percentage survival was calculated by using the following formula:

Percentage survival = (Number of Fry Survived / Total Number of Postlarvae stocked)×100.

For calculating average weight, fry from each tank were weighed by using top loading balance (SHIMADZU ELB600) and divided by total number of fry in tank. For evaluating the growth performance, following formulas were used:

Percent weight gain (% WG)= Final liveweight of the fry–Initial live weight of the postlarvae/ Initial body weight of postlarvae×100)

Specific growth rate (SGR%) = *ln* final live weight - *ln initial* live weight / Number of days of feeding trial ×100

Determination of Gut Enzyme Activities

At the end of study, 30 fry were collected from each tank, anesthetized immediately with buffered MS-222 (60 mg L^{-1}), dissected on the ice pad and removed digestive tract. The intestinal content of the fry of each tank was pooled separately, and 1g content with 10 mL phosphate buffer (pH 7.5) was homogenized by using hand held glass homogenizer (Model AHS 200). The homogenate was centrifuged at 15000 rpm (Model Eppendorf centrifuge 5417R) for 15 min at 4°C. The supernatant was collected and stored at -20ºC until analysis.

Protease Activity

For the determination of protease activity, 1ml enzyme solution was mixed with 5mL, 0.65% casein solution. The solution was incubated for 10 min at 37ºC. After that 5 mL of 110 mM trichloroacetic acid solution was added and further incubated for 30 min at the same temperature. Solution was cooled at room temperature and filtered by using Whatsman filter paper. Subsequently, 2 mL of filtratewas mixedwith 1 mL of 0.5 mM Folin–Ciocalteu reagent and 5mL of 500 mM Na_2CO_3 solution and incubated at 37ºC for 30 min. After cooling, absorbance was measured at 660 nm with the help of UV-Visible spectrophotometer

Amylase Activity

Amylase activity was evaluated by 3,5-Dinitrosalicylic acid (DNS) method (Bernfeld, 1955).The method was based on the estimation of reducing sugars at 560 nm using maltose as the standard. Briefly, 0.5 mL enzyme solution was incubated for 3-4 min at room temperature. Then, 500 µL of 1% starch solution was added and kept at ambient temperature for 3 min, followed by the addition of 1 mL of DNS reagent and incubated in a boiling water bath for about 5 min. After cooling at room temperature, 10 mL of reagent grade water was added and absorbance was noted at 540 nm by using spectrophotometer. One amylase unit was defined as the amount of enzyme mL^{-1} filtrate that released one microgram reducing sugar min^{-1}.

Cellulase Activity

Denison & Koehn (1977) method with a few modifications was adopted for measuring cellulase activity. Briefly, the reaction mixture was prepared by mixing 1 mL each of enzyme solution, 1% carboxymethyl cellulose (CMC) solution and 0.1 M citrate buffer and incubated at 50ºC for about half an hr. After incubation, 3 mL DNS (Dinitrosalicylic acid) reagent was added and again boiled in a water bath for 15min. After that 1mL 40% sodium potassium tartrate was added and allowed to cool at room temperature. After cooling, the production of reducing sugar (glucose) from CMC substrate resulted by cellulase activity was measured at 540 nm on UV-Visible spectrophotometer. One unit cellulase activity is defined as the amount of enzyme mL^{-1} filtrate that released 1 mg glucose min^{-1}.

Proximate Analysis

For the determination of muscle composition, 10 samples of each group (each sample had muscles of five fry) were collected and analyzed by adopting standard methods (AOAC, 2000). Ash content was determined by placing 2 g of sample in a muffle furnace and heated at 600 ^{0}C for 24 h, while Crude protein was determined through micro Kjeldahl's method after acid digestion of samples. Moreover, total fat content was determined without acid hydrolysis, through hexane extraction method with the use of soxthlet apparatus.

Challenge Test

After feeding trial, 10 fish from each tank were shifted to glass aquaria well equipped with heaters and aeration system. The water temperature and DO level were maintained at 25± 0.5˚C and 5.5± 0.5 mgL^{-1} respectively. They were acclimatized for about 2 days and challenged with *S.aureus* strain isolated from *L. rohita* by Department of Microbiology, Quaid-i-Azam University, Islamabad. Bacterial suspension was administrated in water at the rate of 2.5×10^7 cfu mL^{-1}. During acclimatization and exposure to pathogen, fish were fed their respective diets up to satiation. Both groups were kept under observation for 15 days and mortalities were record.

Statistical Analysis

Data obtained from the experiment were expressed as mean ± SE. The WG %, SGR % and survival % and mortality after challenge test data were analyzed by one way analysis of variance (ANOVA) followed by an LSD test through the SPSS statistical package (Version 20, SPSS Inc, Chicago, IL, USA) and values $P < 0.05$ were considered as statistically significant. Moreover, simple t-test by using Graph Pad Prism software version 5 was used for comparison of the results of proximate composition as well asactivity of digestive enzymes of control and experimental group of fry.

Results

Survival

ANOVA revealed a significant difference ($P < 0.05$) in the % survival of control and probiotic supplemented group of postlarvae of *L. rohita*. The % survival in the probiotic supplemented group was 79%, significantly higher as compared to a control group (60%) (Figure. 1).

Growth Performance

There initial body weights of both control and experimental groups of postlarvae were considerably similar ($P>0.05$). However, at the end of the study, group of fry reared in the presence of probiotic supplement showed significantly higher ($P < 0.05$) weight gain compared to control group. Similarly, the specific growth rate (%) was also significantly higher in a group of postlarvae provided *G. candidum* QAUGC01supplementation (Table 1).

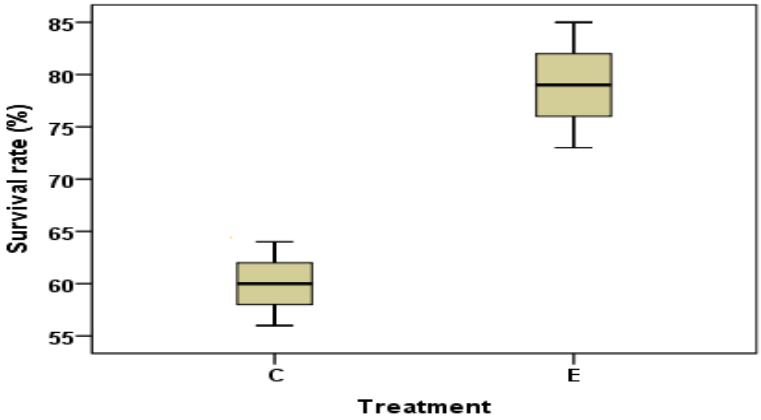

Figure 1. Survival (%) after 70 days rearing of Postlarvae in the presence of *G. candidum* QAUGC01 in Control (C) and probiotic supplemented group. (E).

Table 1. Growth performance of *L. rohita* postlarvae after 70 days rearing in the presence of *G. candidum* QAUGC01

Treatment Parameters	C	E
Mean initial weight Postlarva (mg)	0.61 ± 0.05^{a}	0.59 ± 0.04^{a}
Mean Final weight Fry (g)	1.34 ± 0.02^{b}	1.64 ± 0.03^{a}
Mean weigh gain (%)	21978.57 ± 322.39^{b}	27696.67 ± 411.94^{a}
Initial Biomass (mg)	122 ± 8.32^{a}	118 ± 6.4^{a}
Final Biomass (g)	160.72 ± 5.51^{b}	259.6 ± 16.83^{a}
SGR (%BW/day)	7.80 ± 0.01^{b}	7.96 ± 0.01^{a}

Data are represented as Mean ± SE. (n=3). Means followed by different letter within the row are significantly different (P<0.05). (ANOVA followed by Tukey test). C, Control diet : E, experimental group reared on probiotic supplement.

Digestive Enzymes

Comparative specific activity of digestive enzymes of postlarvae after 70 days of rearing in a control and probiotic supplemented groupis shown in Figure.2. The simple t-test revealed that the activity of digestive enzymes in a group of fry reared on a probiotic supplementation was considerably higher (P<0.05) than observed in a control group of fish. Moreover, amylase and cellulase activities showed high significant difference (P<0.001) between control and experimental group of fry as compared to protease activity (P<0.05).

Nutritional Composition

Comparative proximate composition showed significantly higher (P<0.05) crude protein and fat content in a group of fish provided single strain probiotic in addition to control diet as compared to control group of postlarvae reared in water without any supplement (Figure. 3). However, total ash and carbohydrate content of a control group of postlarvae were significantly higher (P<0.05) as compared to a group of postlarvae provided probiotic *G. candidum* QAUGC01 supplementation through water during experimental trial.

Challenge Test

Figure.4 shows the percentage mortality in both groups of *L. rohita* after challenge with *S. aureus*. Early juvenile reared in the presence of probiotic for 70 days before exposure to pathogen showed significantly (P =0.003) low mortality rate as compared to control group of fish.

Discussions

Early Juvenile of *L. rohita* reared in the presence of single strain probiotic *G. candidum* QAUGC01 supplementation showed about 19% more survival as compared to control group**.** The experiment was conducted near natural conditions, in outdoor facility using rectangular fiberglass tanks well equipped with aerators. Both control and experimental groups were reared under similar condition and get similar feed except the presence of probiotic in the rearing water of experimental group. Thus, higher survival may be due tothe supplementation of probiotic.

In the present study, the survival rate (about 79 %) of postlarvae reared on probiotic supplement was considerably higher as compared to observe at hatcheries of Asian countries (Edwards, 2013) as well as observed by Jha *et al*. (2015) in similar fish species *L. rohita* in response to mixture of probiotics and

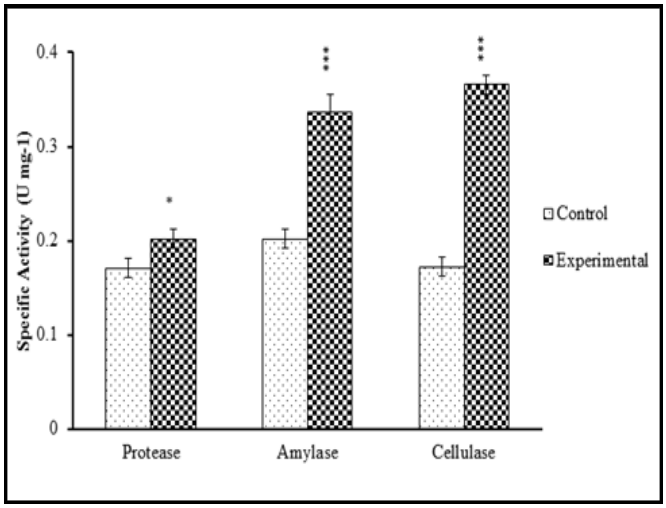

Figure 2. Protease, Cellulase and Amylase activities of *L. rohita* after 70 days rearing in the presence of *G. candidum* QAUGC01. Each bar represents the values as Mean ± SE. Comparison of means byt-test (n=3). * = P< 0.05; ** = P< 0.01, *** = P< 0.001.

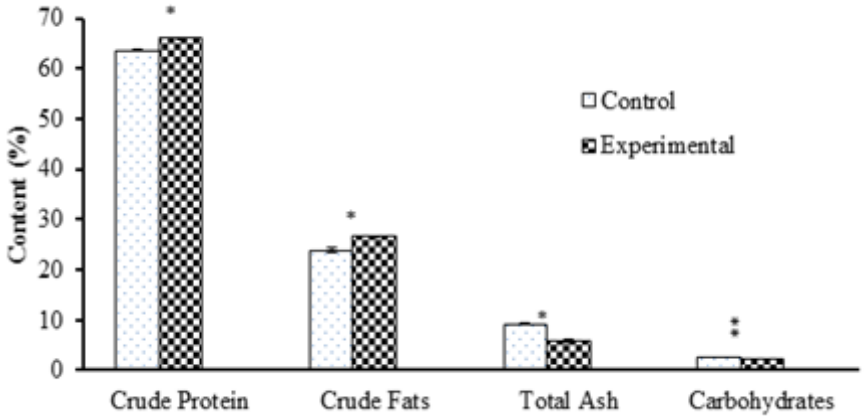

Figure 3. Proximate composition of muscle of L. rohita after 70 days rearing of Postlarvae in the presence of G. candidum QAUGC01. Each bar represents the values as Mean ± SE. Comparison of means byt-test (n=3)(* = P< 0.05;** = P< 0.01; *** = P<0.001).

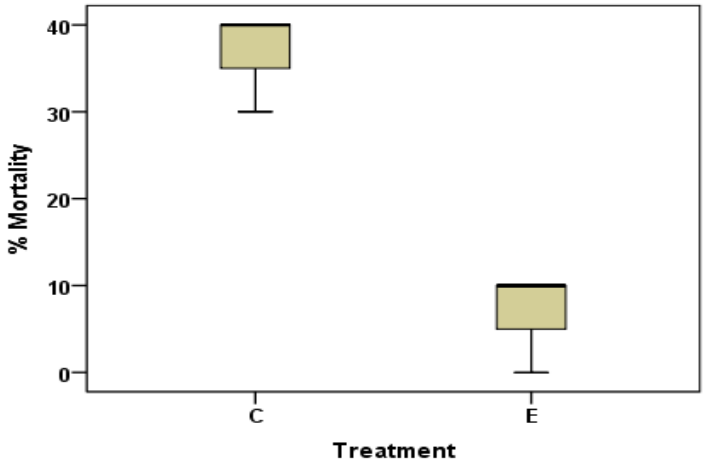

Figure 4. Mortality (%) offry after 70 days rearing in the presence of *G. candidum* QAUGC01 and challenged with *S. aureus*.

exogenous enzyme (four strains of *Lactobacillus* + Sacchromyces + Bifidobacterium + Streptococcus + yeast + Aspergillus + Spirulina + phytase).The higher level of survival in our conditionsin the presence of single strain of probiotic (Figure. 1) might be related to a continuous supply of live natural feed with a steady supply of oxygen via aeration . This fact is strengthened further by the observation that in our experimental conditions, even in the control group, the survival rate was about 60% as compared to 50 % reported previously (Jha*et al*., 2015). But it is quite interesting in our study that supplement of only asingle strain of probiotic *G. candidum* QAUGC01 isolated from local fermented milk product Dahiand administrated via water had shown comparatively higher survival as compared to complex mixture consisting of multiple strain probiotics plus exogenous enzyme (Jha *et al*., 2015).

The very low survival (20-30%) of *L. rohita* during earlier stages at most of hatcheries usually associated with pathogens and unsuitability or indigestibility of the feed (Edwards, 2013). The significantly improved survival of postlarvae of rohu in the present study may be due to the colonization of *G. candidum* QAUGC01 in the gastrointestinal tract and their positive action on the digestibility of nutrients in feed (Mohapatra *et al*., 2012) or might be associated with enhancement of non-specific immune response resulting in improvement of resistance against infection /diseases (Taoka *et al*., 2006; Jha *et al*., 2015). In our conditions, this could also be related with enzymatic potential (Abu Bakar, 2014; Boutrou *et al*., 2006) and secretion of antibacterial substances by *G. candidum* (Dieuleveux *et al*., 1998). These antimicrobial substances have proven to be effective against the proliferation of opportunistic pathogens in culture system and gastrointestinal tract of fry. Like our results, Zhou *et al*.(2009) also reported significant increase survival of shrimp larvae, reared in water having probiotic *Bacillus coagulans*.

Moreover, 22% increase in the body weight of *L. rohita* fry reared on probiotic supplement as compared to control group (Table 1) indicate the growth promoting potentialof *G. candidum* QAUGC01. Like our results, many researchers reported the growth stimulating effects of various probiotics supplementation in different fish species at different life stages (Merrifield *et al*., 2010; Mehrabi *et al*., 2011; Rad *et al*., 2013; Hassaan *et al*., 2014; Jha *et al*., 2015) and pointed out that probiotics improves the feed conversion efficiency by stimulation of digestion due to higher production of digestive enzymes and vitamins (Wang *et al*., 2008). Enzymatic potential of *G. candidum* (amylolytic, proteolytic, lipolytic, cellulolytic and phytase activity) is well documented (Abu Bakar, 2014; Eida *et al*., 2013; Boutrou *et al*., 2006) and also observed in the present study (Figure 2). The intestinal enzymes, amylase, protease and cellulase showed several fold higher activity in a group of fry reared on *G. candidum* QAUGC01 supplement.

Most Cyprinids during early developmental stages have under-developed digestive system and insufficient enzymes for nutrient digestion, thus indicate the need of exogenous enzymes which can be added into larvae diet. The enzymes supplementation (Phytase and amylase) in diet during earlier life stage of *L. rohita* has also been recommended in a recent study along with probiotic strains (Jha *et al*., 2015). However, in the present study, QAUGC01 strain of *G. candidum* without addition of exogenous enzymes, significantly improved the survival and growth rate (Table 1, Figure 1). It appears that after colonizationin the intestinal tract,*G. candidum* release sufficient enzymes and indirectly effect survival and growth rate (WG%, SGR%) by improving the digestion, absorption and availability of nutrients.

Probiotics immune-stimulatory effect, resistance to pathogenic organisms and disease is well documented (Merrifield *et al*., 2010; Muñoz-Atienza *et al*., 2013). The results of the present study also indicated that administration of probiotic in water during early life stages of *L. rohita* increased resistance to *S. aureus* and significantly lower the mortality rate (P= 0.003) as compared to control group (Figure. 4). It seems that *G. candidtum* out compete the proliferation of opportunistic pathogen and colonize in the GI tract of Postlarvae/ fry (Muroga *et al*., 1987). Thus, improve survival of fry after challenge with *S. aureus* may be associated with the activation of innate immune defense or decrease in permeability of epithelium for macromolecules and toxins (Sun & O'Riordan,2013).

Supplementation *of G. candidum* QAUGC01 also showed positive effect on the muscles proximate composition of rohu (Figure. 3). The muscles dry mass of fry reared on probiotic supplement showed 3.7 and 11.27% higher value of crude protein and fats respectively as compared to control group. Our finding of higher values of crude protein and fats agrees with the result of Rad *et al*.(2013), who fed probiotic (*S. cerevisiae)* supplemented diet to Nile tilapia (*Oreochromis niloticus*) fingerlings and obtained significantly higher ($P<0.05$) difference compared to control group. Many other scientists also reported the similar positive effects of probiotics supplemented diet on crude protein and fat contents of fish viz juvenile *C. carpio* (Noveirian &Nasrollahzadeh, 2012),*O. niloticus* (Lara-Flores *et al*., 2003) in response to *Streptococcus faecium*, *Lactobacillus acidophilus* and *Saccharomyces cerevisiae. niloticus* in response to *Bacillus licheniformis* and yeast extract(Hassaan *et al*., 2014) and suggested that change in chemical composition of fish in response to probiotic supplementation may be related to better nutrient digestibility and absorption (Abdel-Tawwab *et al*., 2008) and efficient conversion of ingested food into structural proteins that resulted in more muscles (Mehrabi *et al*., 2011). Thus, improved flesh qualityof rohu (fry)of experimental

group may be due to enhance digestion and absorption of nutrient due to proteolytic and lipolytic activity of *G. candidum*- QAUGC01 (Abu Bakar, 2014).

Conclusion

The results of present study showed that supplement of single strain probiotic *G. candidum* QAUGC01 via water during early rearing can improve the % survival, growth performance, digestive enzymes activities, resistance to disease and muscle composition of *L. rohita*. These positive effects could be attributed to the higher enzymatic potential and immune-stimulatory effect of *G. candidum*. Based on the results, single strain probiotic *G. candidum* QAUGC01 through water can be recommended as an economically viable way as compare to dietary multi-strains probiotics mixture with exogenous enzymes (Jha*et al*., 2015) for improving survival and growth during early rearing of *L. rohita*. However, further pilot studies are needed for optimization of probiotic concentration and evaluation of the molecular basis of growth stimulating factors.

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